transcription of ER stress response-related genes, such as *GRP78* [10,15–20]. Three types of *cis*-acting sequences, ER stress response element (ERSE), ERSE-II and ATF6-binding element, have been reported as consensus sequences for the binding of p50-ATF6 [21–23]. Recently, post-translational modifications of ATF6 other than protein degradation, such as intermolecular disulfide bridge formation and *N*-linked glycosylation, were reported to be involved in ER stressor-induced activation and translocation of ATF6 [24,25]. Thus, it is believed that ATF6 activity is mainly regulated at the post-translational level.

We recently reported that NSAIDs up-regulate the expression of *ATF6* mRNA [7]. Furthermore, some ER stressors (hypoxia and tunicamycin) were also reported to up-regulate *ATF6* mRNA expression [26,27]. In the current study, we have shown that all of the ER stressors tested (celecoxib, tunicamycin and thapsigargin) up-regulate *ATF6* mRNA expression. Furthermore, results in this study suggest that this up-regulation is due to the ER stressor-induced generation of p50-ATF6 and contributes to enhance the ER stress response.

Materials and methods

Chemicals and plasmids. Antibodies against ATF6, GRP78, lamin and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The RNeasy kit, small interfering RNAs (siRNAs) and HiPerFect transfection reagent were from Qiagen (Valencia, CA). Lipofectamine (TM2000) and pcDNA3.1 plasmid were obtained from Invitrogen (Carlsbad, CA). HilyMax was from Dojindo Laboratories (Kumamoto, Japan).

Cell culture, transfection, and real-time RT-PCR analysis. AGS, HCT-15, HeLa and Kato III are human carcinoma cell lines derived from stomach (AGS and Kato III), colon (HCT-15) or uterine cervix (HeLa) tissue. Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO_2 at 37 C.

Transfection of AGS cells with plasmids was carried out using Lipofectamine (TM2000) or HilyMax according to the manufacturer's protocol. On the other hand, cells were transfected with siRNA using HiPerFect transfection reagent according to the manufacturer's instructions.

Real-time RT-PCR was done as descrived previously [7].

Immuno-blotting analysis. The protein concentration of samples was determined by the Bradford method. Samples were applied to polyacrylamide SDS gels, subjected to electrophoresis, and the resultant proteins then immuno-blotted with their respective antibodies.

Statistical analysis. All values are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Sche e's multiple comparison test was used for evaluation of di erences between groups. The Student's *t*-test for unpaired results was used for the evaluation of di erences between two groups. Di erences were considered to be significant for values of $P < 0.05$.

Results and discussion

Up-regulation of ATF6 mRNA expression by various ER stressors

We recently reported that treatment of AGS cells with 80 μM celecoxib for 6 h caused a five-fold increase in the amount of *ATF6* mRNA [7]. In the current study, we have used real-time RT-PCR to examine the e ect of various ER stressors, including celecoxib, on *ATF6* mRNA expression. As shown in Fig. 1A, thapsigargin (2 μM) up-regulated *ATF6* mRNA expression in AGS cells in an incubation period-dependent manner. This is the first demonstration that thapsigargin up-regulates the level of *ATF6* mRNA. This time-course profile was similar to that of thapsigargin-induced up-regulation of *GRP78* mRNA expression, although the extent of up-regulation was more marked for *GRP78* than *ATF6* (Fig. 1B). We also examined the e ect of other ER stressors (5 μg/ml tunicamycin and 100 μM celecoxib) on *ATF6* mRNA expression and found that both of these ER stressors up-regulate expression of *ATF6* mRNA (Fig. 1C and D). We also confirmed that these concentrations of chemicals up-regulate expression

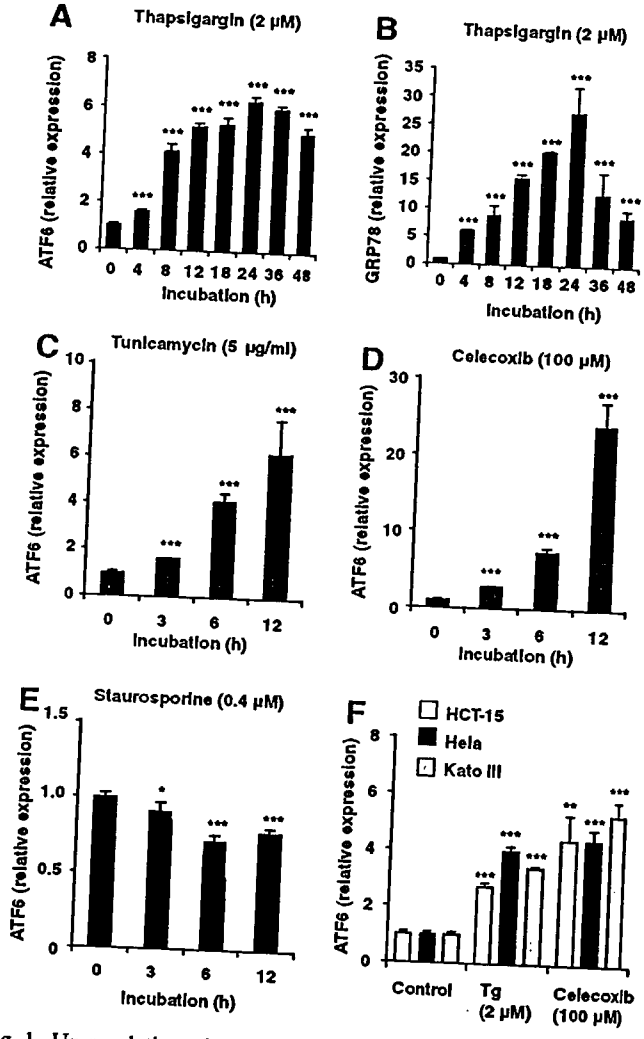

Fig. 1. Up-regulation of *ATF6* mRNA expression by ER stressors. AGS cells (A–E) or HCT-15, HeLa and Kato III cells (F) were incubated with the indicated concentrations of thapsigargin (Tg) (A,B,F), tunicamycin (C), celecoxib (D,F) or staurosporine (E) for the specified time periods (A–E) or 12 h (F). Total RNA was extracted and subjected to real-time RT-PCR using primers specific for *ATF6* or *GRP78*. Values were normalized to actin gene expression and expressed relative to the control sample (time 0 or without drugs). Values are given as means ± SD ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

of *GRP78* mRNA (data not shown). In contrast, staurosporine, which is known to lack ER stress response-inducing activity [7], did not up-regulate (but rather slightly down-regulated) expression of *ATF6* mRNA (Fig. 1E).

As shown in Fig. 1F, both thapsigargin and celecoxib up-regulated the level of *ATF6* mRNA in all of the cell lines tested (HCT-15, HeLa and Kato III). Overall, the results in Fig. 1 show that ER stressors generally up-regulate the levels of *ATF6* mRNA in various human cells, suggesting that this up-regulation is mediated by the ER stress response.

Mechanism for up-regulation of ATF6 mRNA levels by ER stressors

In general, up-regulation of the levels of distinct mRNAs is due to either transcriptional activation of the gene or stabilization of the mRNA. We examined the e ect of ER stressors on the stability of *ATF6* mRNA. After treatment with thapsigargin or celecoxib, cells were further incubated in the presence of actinomycin D, an inhibitor of RNA synthesis, and the amount of *ATF6* mRNA was determined. As shown in Fig. 2A, the amount of *ATF6* mRNA was indistinguishable between thapsigargin-treated and non-treated cells. This demonstrates that thapsigargin does not a ect the stability of *ATF6* mRNA. On the other hand, the results in Fig. 2B show that celecoxib slightly stabilizes *ATF6* mRNA; however, this stabilization was not as distinct as that recently reported for *CHOP* mRNA (Fig. 2C) [28]. In conclusion, the results in Fig. 2A–C suggest that the up-regulation of *ATF6* mRNA levels by ER stressors is generally due to transcriptional activation rather than mRNA stabilization; however, it is possible that celecoxib-dependent stabilization of *ATF6* mRNA contributes to the up-regulation of *ATF6* mRNA levels in celecoxib-treated cells.

Next, we examined the contribution of the ER stress response to the up-regulation of *ATF6* mRNA levels by ER stressors. All of the three ER transmembrane proteins, IRE1, PERK and ATF6, are involved in ER stressor-induced gene expression directly (ATF6) or indirectly (IRE1 and PERK). IRE1 splices *XBP-1* mRNA, resulting in translation of the active form of this protein and PERK phosphorylates eukaryotic initiation factor-2α (eIF-2α) leading to activation of ATF4 expression [29,30]. Both XBP-1 and ATF4 are involved in ER stressor-induced gene expression [13]. Thus, three transcription factors, p50-ATF6, XBP-1 and ATF4, were candidates for mediating ER stressor-induced up-regulation of *ATF6* mRNA levels. At first, we examined the contribution of XBP-1 and ATF4 to the ER stressor-induced up-regulation of *ATF6* mRNA levels by use of siRNA techniques. As shown in Fig. 2D and E, transfection with a given siRNA clearly suppressed the thapsigargin- or celecoxib-induced mRNA expression of its target gene. On the other hand, transfection with siRNA for XBP-1 or ATF4 did not drastically a ect the up-regulation of *ATF6* mRNA, although slight inhibition

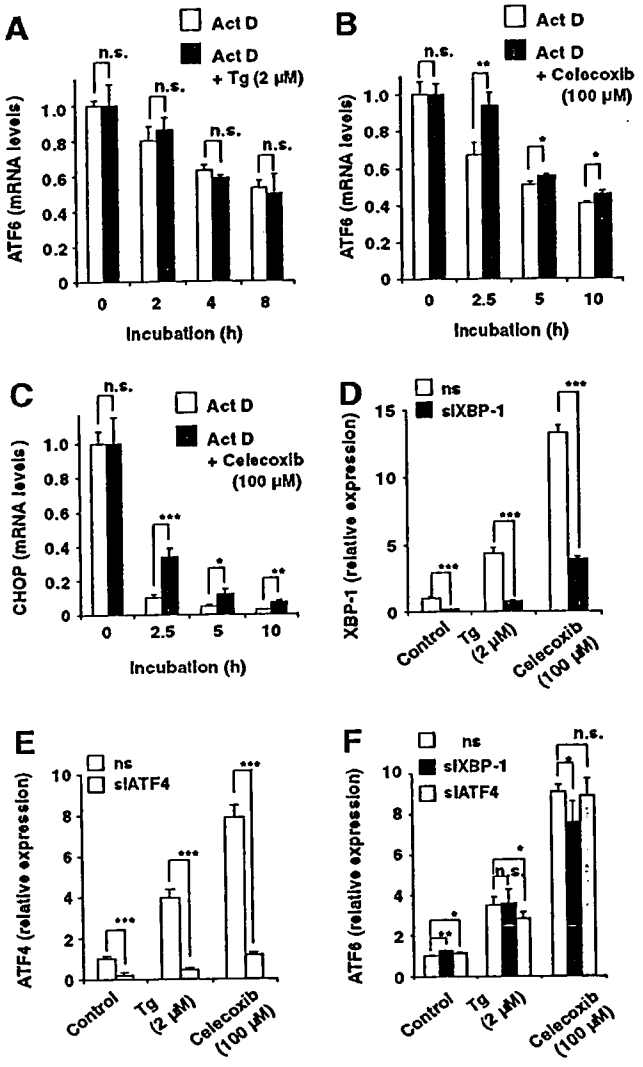

Fig. 2. Stability of *ATF6* mRNA in the presence of ER stressors and e ect of siRNA for XBP-1 or ATF4 on the ER stressor-induced up-regulation of *ATF6* mRNA expression. AGS cells were pre-incubated with or without thapsigargin (Tg) (A) or celecoxib (B,C), at the indicated concentrations, for 6 h and further incubated with 1 μg/ml actinomycin D (Act D) for the specified time periods under the same conditions as the pre-incubation step (A–C). AGS cells were transfected with siRNA for XBP-1 (siXBP-1) or ATF4 (siATF4) or with non-silencing (ns) siRNA. After 24 h, cells were incubated with or without the indicated concentration of thapsigargin (Tg) or celecoxib for 6 h (D–F). The levels of *ATF6* (A,B,F), *CHOP* (C), *XBP-1* (D) and *ATF4* (E) mRNA were estimated by real-time RT-PCR experiments as described in the legend of Fig. 1. Values are given as means ± SD ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. n.s., Not significant.

was observed in thapsigargin-treated cells transfected with siRNA for ATF4 and in celecoxib-treated cells transfected with siRNA for XBP-1 (Fig. 2F). Based on the results in Fig. 2F and the results with AEBSF shown in Fig. 3 (see below), it may be concluded that neither XBP-1 nor ATF4 is a main transcription factor responsible for ER stressor-induced up-regulation of *ATF6* mRNA expression.

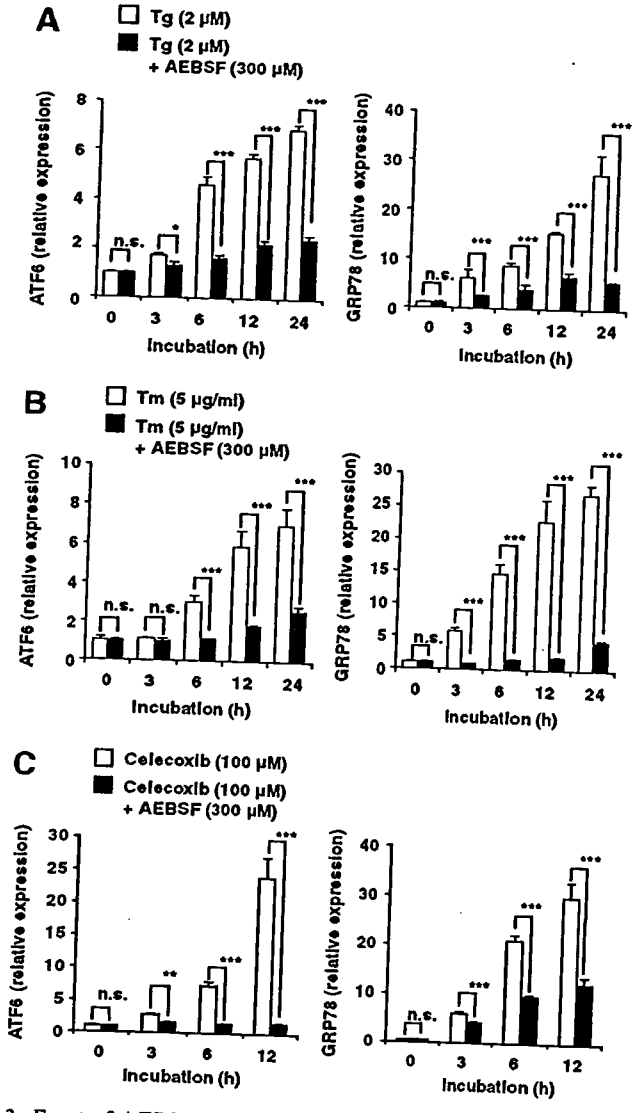

Fig. 3. E ect of AEBSF on ER stressor-induced up-regulation of *ATF6* mRNA expression. AGS cells were pre-incubated with or without 300 μM AEBSF for 1 h and then, still in the presence or absence of AEBSF, further incubated for the time periods indicated with thapsigargin (Tg) (A), tunicamycin (Tm) (B) or celecoxib (C). The levels of *ATF6* and *GRP78* mRNA were estimated by real-time RT-PCR experiments as described in the legend of Fig. 1. Values shown are means ± SD ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. n.s. Not significant.

In order to examine the contribution of ATF6 to up-regulation of *ATF6* mRNA by ER stressors, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), an inhibitor of S1P, that was reported to inhibit the ER stressor-induced activation of ATF6 (degradation of p90-ATF6 into p50-ATF6) was used [16]. As shown in Fig. 3A, treatment of cells with AEBSF (300 μM) clearly inhibits the thapsigargin-dependent up-regulation of *ATF6* mRNA expression. We confirmed that thapsigargin-dependent up-regulation of *GRP78* mRNA expression (Fig. 3A) and the appearance of p50-ATF6 (data not shown) were inhibited by 300 μM AEBSF. Furthermore, this concentration of AEBSF did not decrease (but rather slightly increases) the amount of *ATF6* mRNA in the absence of thapsigargin (data not shown). Similar results were obtained with tunicamycin and celecoxib (Fig. 3B and C). AEBSF almost completely inhibited the celecoxib-dependent up-regulation of *ATF6* mRNA expression but only partially inhibited that of *GRP78* mRNA expression (Fig. 3C). This reflects the observation that ATF4 is involved in celecoxib-dependent up-regulation of *GRP78* mRNA expression [31] but not *ATF6* mRNA expression (Fig. 2F). In order to examine the specificity of this e ect of AEBSF, we examined its e ect on the celecoxib-induced up-regulation of *claudin 4* mRNA expression that was reported in our previous paper [5]. AEBSF did not inhibit (but rather slightly stimulated) celecoxib-induced up-regulation of *claudin 4* mRNA expression (data not shown), suggesting that the e ect of AEBSF is specific for *ATF6*. Overall, the results in Fig. 3 suggest that up-regulation of *ATF6* mRNA by ER stressors is mediated by activation of ATF6, in other words, by the S1P-dependent degradation of p90-ATF6 into p50-ATF6.

For confirmation of this idea, we examined the e ect of over-expression of the active form of ATF6 on the expression of ATF6. Transfection of cells with the plasmid pATF6(373), containing DNA sequences corresponding to amino acid residues 1–373 of ATF6, was reported to induce the ER stress response, suggesting that the translated fragment of ATF6 (ATF6(373)) acts as an active form of ATF6 similar to p50-ATF6 [10]. We confirmed that transfection with pATF6(373) caused up-regulation of not only ATF6(373) (data not shown) but also of *GRP78* mRNA and GRP78 protein (Fig. 4A and B). As shown in Fig. 4A, transfection of cells with pATF6(373) caused up-regulation of *ATF6* mRNA levels. Because the primers used in obtaining the results in Fig. 4A (for *ATF6*) do not recognize the mRNA derived from pATF6(373), the results in Fig. 4A show that over-expression of ATF6(373) caused up-regulation of expression of mRNA derived from the endogenous *ATF6*. Transfection with pATF6(373) also caused up-regulation of p90-ATF6 (Fig. 4B). The results in Fig. 4A and B suggest that the active form of ATF6 positively regulates the transcription of *ATF6*.

We searched for three types of ATF6-binding consensus sequences in the promoter of *ATF6* and found two ATF6-binding elements (TGACGT) (from 2525 to 2520 and from 987 to 982), based on consensus sequences described in a previous study [23]. Thus, it is possible that p50-ATF6 binds to these elements to induce the transcription of *ATF6* and that this induction is responsible for the up-regulation of *ATF6* mRNA expression by ER stressors.

A role for up-regulation of ATF6 mRNA expression in ER stress response

We assumed that the up-regulation of *ATF6* mRNA by ER stressors contributes to enhancement of the ER stress response; in other words, to enhancement of ER stressor-induced up-regulation of various genes such as *GRP78*. To test this idea, we examined the e ect of over-expression

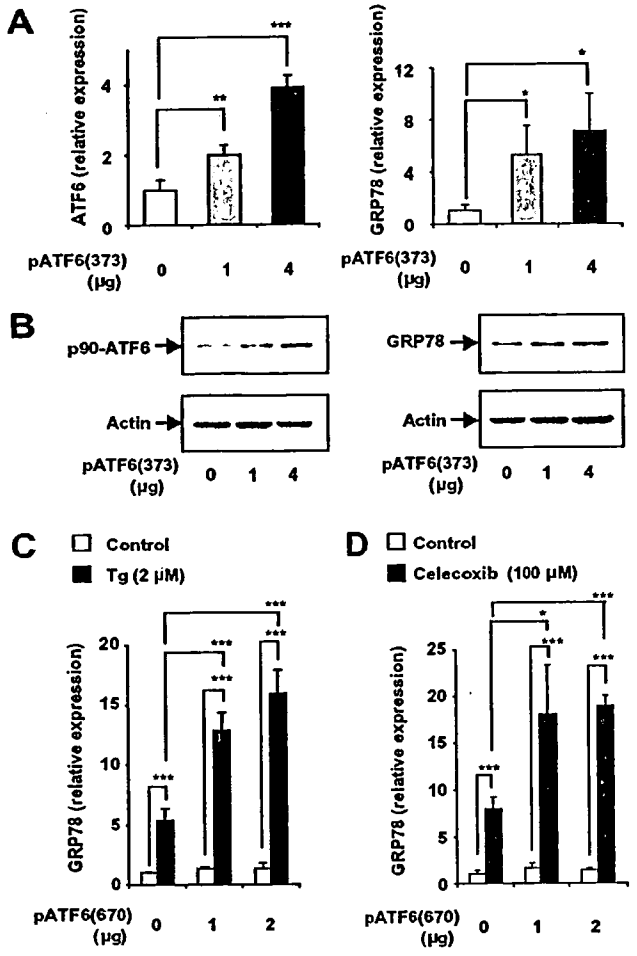

Fig. 4. Up-regulation of ATF6 and *ATF6* mRNA by the active form of ATF6 and e ect of over-expression of p90-ATF6 on the ER stress response. AGS cells were transiently transfected with the indicated amount of expression plasmid for ATF6(373) (pATF6(373)) and/or control vector (total DNA amounts were fixed at 4 μg) and cultured for 6 h (B) or 12 h (A) (A,B). AGS cells were transiently transfected with the indicated amount of expression plasmid for p90-ATF6 (pATF6(670)) and/or control vector (total DNA amounts were fixed at 2 μg) and cultured for 24 h. Cells were further incubated with or without the indicated concentration of thapsigargin (Tg) (C) or celecoxib (D) for 6 h (C,D). Whole cell extracts were analyzed by immuno-blotting with antibodies specific for p90-ATF6, GRP78 or actin (B). The levels of *ATF6* and *GRP78* mRNA were estimated by real-time RT-PCR experiments as described in the legend of Fig. 1. Values shown are means ± SD ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ (A,C,D).

of p90-ATF6 on ER stressor-induced up-regulation of *GRP78* mRNA expression. Transfection of AGS cells with pATF6(670) (the expression plasmid for p90-ATF6) caused over-expression of p90-ATF6 and *ATF6* mRNA (data not shown). As shown in Fig. 4C and D, transfection with pATF6(670) stimulated the thapsigargin- or celecoxib-induced up-regulation of *GRP78* mRNA expression. For thapsigargin, similar results have previously been reported [21]. On the other hand, transfection with pATF6(670) did not increase the amount of *GRP78* mRNA in the absence of ER stressors (Fig. 4C and D). These results show that over-expression of p90-ATF6 cannot induce the ER stress response by itself; however, it can stimulate the ER stress response induced by ER stressors and suggests that up-regulation of *ATF6* mRNA expression by ER stressors contributes to enhancement of the ER stress response.

From the results of this study, we propose that various ER stressors induce the transcription of *ATF6* and that the active form of ATF6 (p50-ATF6) positively regulates the transcription of *ATF6*. As for the physiological role of this up-regulation, we have considered two possibilities. As described above, one possibility is that the up-regulation is involved in the stimulation of the ER stressor-induced ER stress response. The other possibility is that it is involved in the maintenance of p90-ATF6 in cells and in preparation for the next induction of the ER stress response. The amount of p90-ATF6 rapidly decreases upon exposure to ER stressors due to cleavage by S1P and S2P or proteasome-dependent degradation [16,32], however, it returns to the original level within 12–24 h [21]. This compensation may be necessary to ensure rapid induction of the next ER stress response and we assume that ER stressor-induced up-regulation of *ATF6* mRNA expression contributes to this compensation. It is also interesting to consider that maintenance of p90-ATF6 in cells may not only be important because it is the precursor for p50-ATF6 but because it may play other, as yet unknown, cellular roles.

Acknowledgments

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Identification of the *TPO1* gene in yeast, and its human orthologue TETRAN, which cause resistance to NSAIDs

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Abstract **Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, have serious gastrointestinal side effects. Since their direct cytotoxicity was suggested to be involved in this side effect, we here tried to identify NSAID-resistant genes. We screened for *Saccharomyces cerevisiae* genes whose overexpression causes indomethacin resistance and identified the *TPO1* gene, which encodes a major facilitator superfamily transporter. Its overexpression or deletion made yeast cells resistant or sensitive, respectively, to some NSAIDs. A BLAST search identified the possible human orthologue of Tpo1p, tetracycline transporter-like protein (TETRAN), whose overexpression in cultured human cells caused resistance to some NSAIDs, suggesting that TETRAN is an efflux pump for some NSAIDs.**

Keywords: Non-steroidal anti-inflammatory drugs; TPO1; Tetracycline transporter-like protein

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most frequently used medicines in the world and account for nearly 5% of all prescribed medications [1]. However, NSAIDs frequently have side effects, including gastrointestinal ulcers and bleeding [2]. Individual variation of sensitivity is a serious clinical problem, but the genetic factors determining this variation are unknown.

Inhibition of cyclooxygenase (COX) by NSAIDs, which is responsible for their anti-inflammatory activity, was previously thought to be fully responsible for their gastrointestinal side effects [3]. However, the increased incidence of gastrointestinal ulcers and the decrease in prostaglandin (PG) levels induced by NSAIDs are not always linked, suggesting that additional mechanisms could be implicated [4]. We recently showed that these involve the direct cytotoxic activity of NSAIDs that is independent on their COX-inhibition activity [5]. Therefore, genes that affect this cytotoxicity may determine risk of gastric lesions, but such genes have not been identified.

For identifying genes that affect the cytotoxicity, we screened *Saccharomyces cerevisiae* genes whose overexpression confers resistance to indomethacin, and we thus identified *TPO1*, which belongs to the major facilitator superfamily (MFS) of transporters. By a BLAST search, we identified a possible human orthologue, tetracycline transporter-like protein (TETRAN), which is predicted to be a drug transporter because of its strong amino acid sequence similarity to a tetracycline transporter in *E. coli* [6]. When TETRAN was overexpressed in cultured human cells, cells became resistant to some NSAIDs. We therefore consider that TETRAN is probably responsible for the efflux of NSAIDs from cells.

2. Materials and methods

2.1. Plasmids and yeast strains

The *tpo* mutants and the parent strain (YPH499) were kindly donated by Dr. K. Igarashi (Chiba University) [7].

A plasmid containing cDNA for the human *TETRAN* gene was obtained from Invitrogen. After digestion by *Eco*RI and *Xho*I, the resultant DNA fragment was inserted into the *Eco*RI–*Xho*I site of pcDNA3.1(+) to obtain the expression plasmid for TETRAN. Transfection of the plasmid was carried out using Lipofectamine (TM2000) or HilyMax according to the manufacturer's protocol. Transfection of siRNA was performed by HiPerFect transfection reagent according to the manufacturer's instructions. Real-time RT-PCR analysis was done as described [8].

2.2. Preparation of yeast genomic library and screening of indomethacin-resistant transformants

Total chromosomal DNA from yeast W303-1 cells was partially digested by *Sau*3AI. DNA fragments (4–10 kb) were purified by ultra-centrifugation in the presence of CsCl, and ligated into the *Bam*HI site of pYES2 (Invitrogen). The resultant yeast genomic library was introduced into W303-1 cells and indomethacin-resistant transformants were selected on synthetic complete (SC) agar plates containing 0.6 mM indomethacin.

2.3. Northern blotting

Total RNA was extracted from yeast or human cells 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%

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Abbreviations: ABC, ATP binding cassette; COX, cyclooxygenase; Drtet, *D. melanogaster* tetracycline resistance protein; FBS, fetal bovine serum; MFS, major facilitator superfamily; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NSAID, non-steroidal anti-inflammatory drug; OD, optical density; PG, prostaglandin; SC, synthetic complete; SNP, single nucleotide polymorphism; TETRAN, tetracycline transporter-like protein

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formaldehyde, and blotted onto nylon membranes (Amersham Bioscience). Partial DNA fragments of *TPO1* were amplified by PCR as described [9] and partial DNA fragments of *TETRAN* were produced by digesting the full-length cDNA with *Nde*I and *Aor*51HI. These were radioactively labeled and used as probes.

2.4. Assay for sensitivity of cells to NSAIDs

Cells were cultured in RPMI1640 medium containing 10% fetal bovine serum. Cell viability as determined by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method [10]. Apoptotic chromatin condensation was observed as described [10].

2.5. Statistical analysis

Values are expressed as a means ± standard error (S.E.M.). One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison was used to evaluate differences between groups. Results were considered to be significant for values of $P < 0.05$.

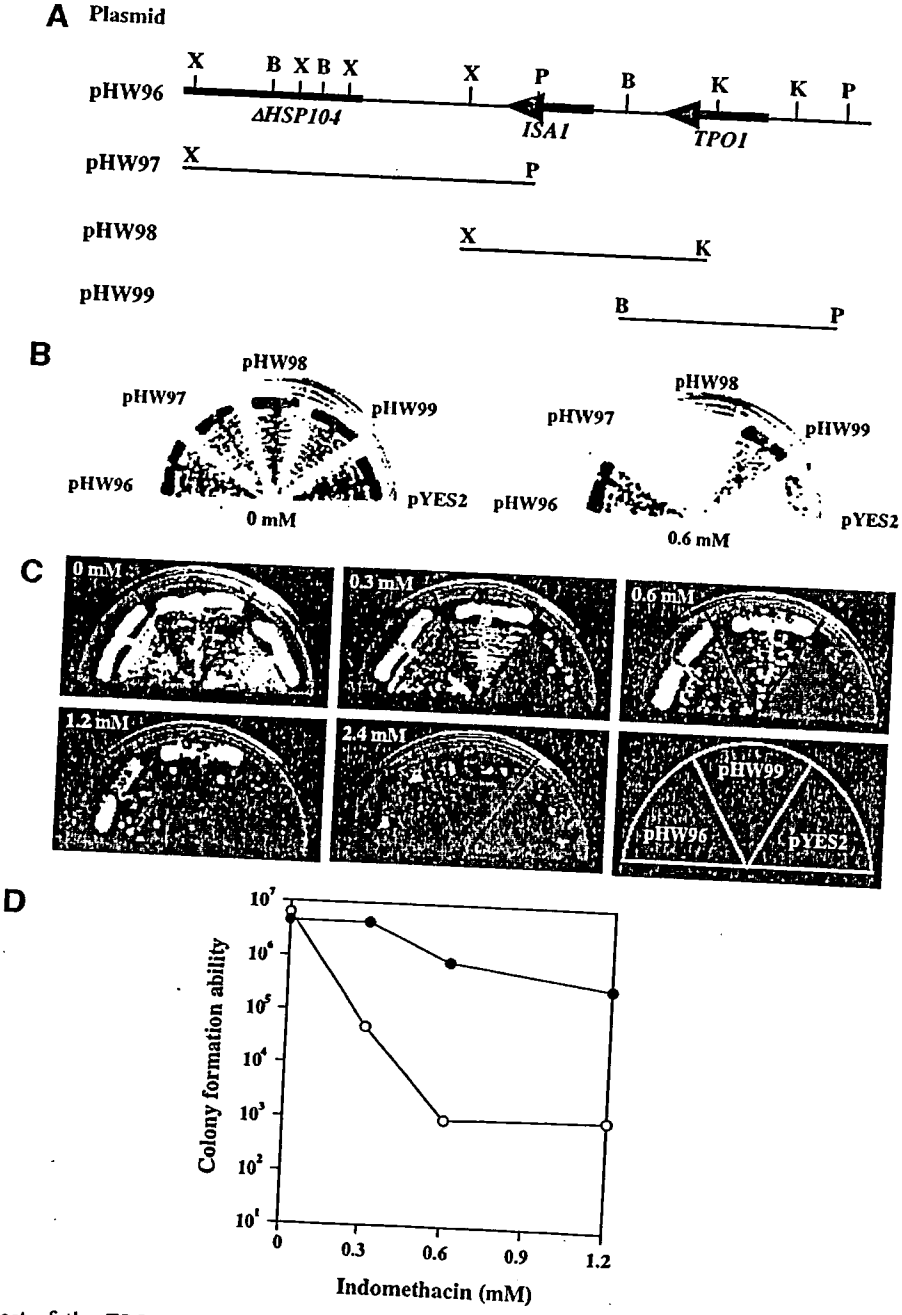

Fig. 1. Identification in yeast of the *TPO1* gene, which caused resistance to indomethacin. (A) Restriction enzyme maps for pHW96 and its derivatives (X, *Xba*I; B, *Bal*I; P, *Pvu*II; K, *Kpn*I). The *GAL* promoter of pYES2 is located on the right side of each insert. (B) W303-1 cells harboring each plasmid were streaked on SC agar plates containing 0 or 0.6 mM indomethacin and plates were incubated at 30 °C for 4 days. (C) W303-1 cells harboring pHW96, pHW99 or the vector (pYES2) were streaked on SC agar plates containing the indicated concentrations of indomethacin, and plates were incubated for 4 days. (D) A full growth suspension of W303-1 harboring pHW99 (closed circle) or pYES2 (open circle) was diluted appropriately and streaked on SC agar plates containing the indicated concentrations of indomethacin. Plates were incubated for 4 days. Colonies were counted and colony-forming units (colonies formed per 1 ml full growth suspension) were determined.

3. Results

3.1. Screening for indomethacin-resistance genes in yeast

DNA fragments prepared from chromosomes of *S. cerevisiae* W303-1 cells were introduced into pYES2, under the control of the *GAL* promoter. After transformation of W303-1 cells with this DNA library, we identified 18 independent indomethacin resistant colonies from about 20000 transformants on SC agar plates containing 0.6 mM indomethacin. A plasmid from one of clones that were positive after re-transformation experiments (17 clones) was subjected to direct DNA sequencing. The plasmid (pHW96) has two complete genes, *TPO1* and *ISA1*, and a fragment of *HSP104* (Fig. 1A). Subcloning revealed that *TPO1* is responsible for resistance; a plasmid, which contained only *TPO1* (pHW99) made cells resistant to indomethacin and deletion of *TPO1* from pHW96 (pHW97 and pHW98) diminished the resistance (Fig. 1B). Plasmid from other 16 positive clones also contained *TPO1*.

Further analysis was preformed using cells overexpressing *TPO1*. While W303-1/pYES2 did not significantly grow on SC agar plates containing 0.3 mM indomethacin, W303-1/pHW96 and W303-1/pHW99 could grow in the presence of 1.2 mM indomethacin (Fig. 1C). We also found that transformation of W303-1 with pHW99 dramatically increased its ability to form colonies in the presence of 0.3 – 1.2 mM indomethacin (Fig. 1D). Furthermore, we draw growth curve in the presence of various concentrations of indomethacin. While W303-1/pYES2 could not grow in liquid medium containing 0.3 mM indomethacin, W303-1/pHW99 could grow in the presence of 1.2 mM indomethacin (Fig. 2). These results indicate that cells harboring pHW99 are resistant to indomethacin.

W303-1/pYES2 did not grow in liquid medium containing 0.1 mM diclofenac, but W303-1/pHW99 could grow in liquid medium containing 0.2 mM diclofenac. In the presence of ibuprofen, a little difference in growth was also seen, suggesting that the resistance is not specific for indomethacin. On the other hand, in the presence of aspirin, there was no clear difference in growth, showing that W303-1/pHW99 is not resistant to all NSAIDs (Fig. 2).

3.2. Sensitivity of tpo mutants to indomethacin

Results described above suggest that Tpo1p protects yeast cells from NSAIDs. To further test this hypothesis, we examined a *tpo1* disruption mutant, Δ*TPO1*. As shown in Fig. 3, the wild-type strain could grow in liquid medium containing 0.2 mM indomethacin, but Δ*TPO1* mutant did not grow in liquid medium containing 0.1 mM indomethacin, again showing that Tpo1p is involved in indomethacin-resistance in wild-type yeast cells.

Recently, homologues of *TPO1* (*TPO2*, *TPO3* and *TPO4*) were identified in *S. cerevisiae*, all involved in polyamine transport and resistance to polyamines [7]. We examined their contribution to indomethacin resistance, by use of disruption mutants (Δ*TPO2*, Δ*TPO3* and Δ*TPO4*). As shown in Fig. 3, all mutants (Δ*TPO2*, Δ*TPO3* and Δ*TPO4*) showed indomethacin-sensitivity similar to that of the wild-type strain. This suggests that indomethacin resistance specifically involves Tpo1p, whereas polyamine resistance involves several genes.

3.3. Induction of TPO1 mRNA by indomethacin

Since herbicides induce the transcription of *TPO1* [9], we tested by Northern blotting analysis whether indomethacin also induces the transcription of *TPO1*. As shown in Fig. 4,

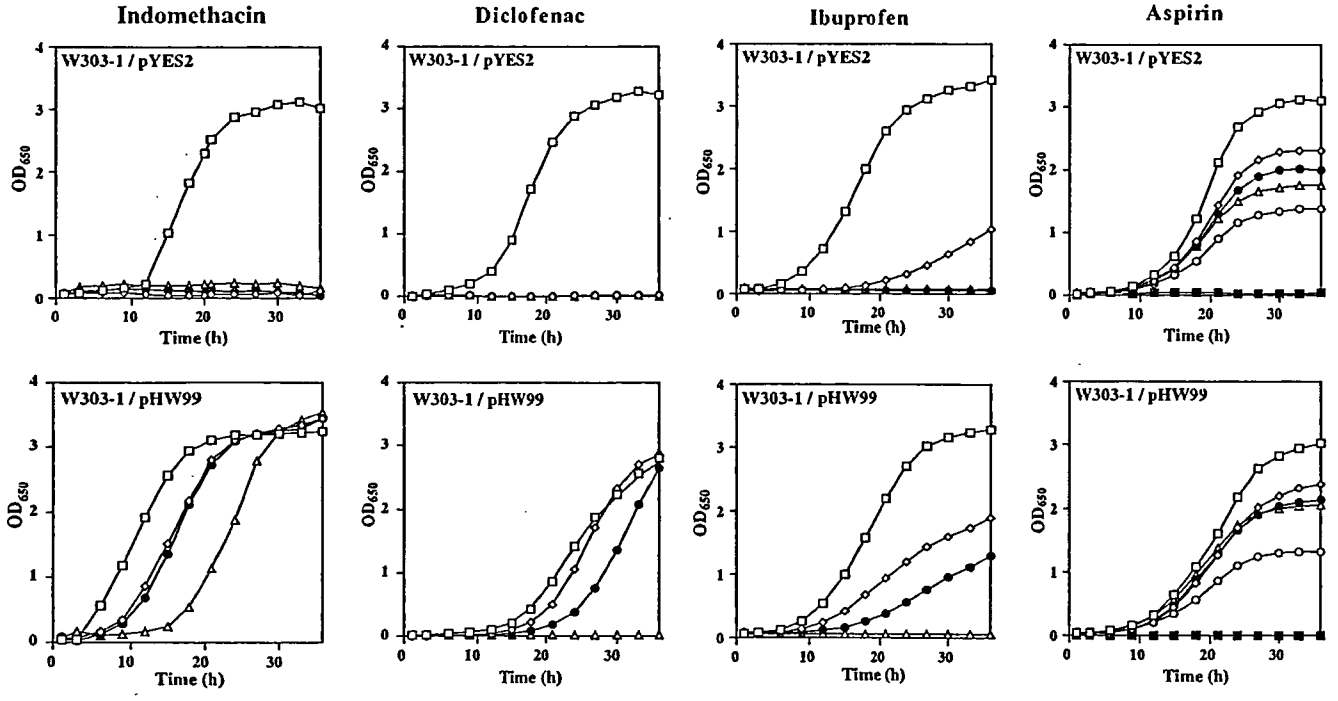

Fig. 2. Growth of yeast cells overexpressing *TPO1* in the presence of NSAIDs. Full growth suspensions of W303-1/pHW99 or W303-1/pYES2 were 1/50 diluted and cultured at 30 °C in the presence of 0 (open square), 0.3 (open diamond), 0.6 (closed circle), and 1.2 (open triangle) mM indomethacin; 0 (open square), 0.1 (open diamond), 0.2 (closed circle), and 0.4 (open triangle) mM diclofenac; 0 (open square), 0.1 (open diamond), 0.2 (closed circle), and 0.4 (open triangle) mM ibuprofen; 0 (open square), 1 (open diamond), 2 (closed circle), 4 (open triangle), 8 (open circle), and 10 (closed square) mM aspirin. The optical density (OD) at 650 nm was monitored.

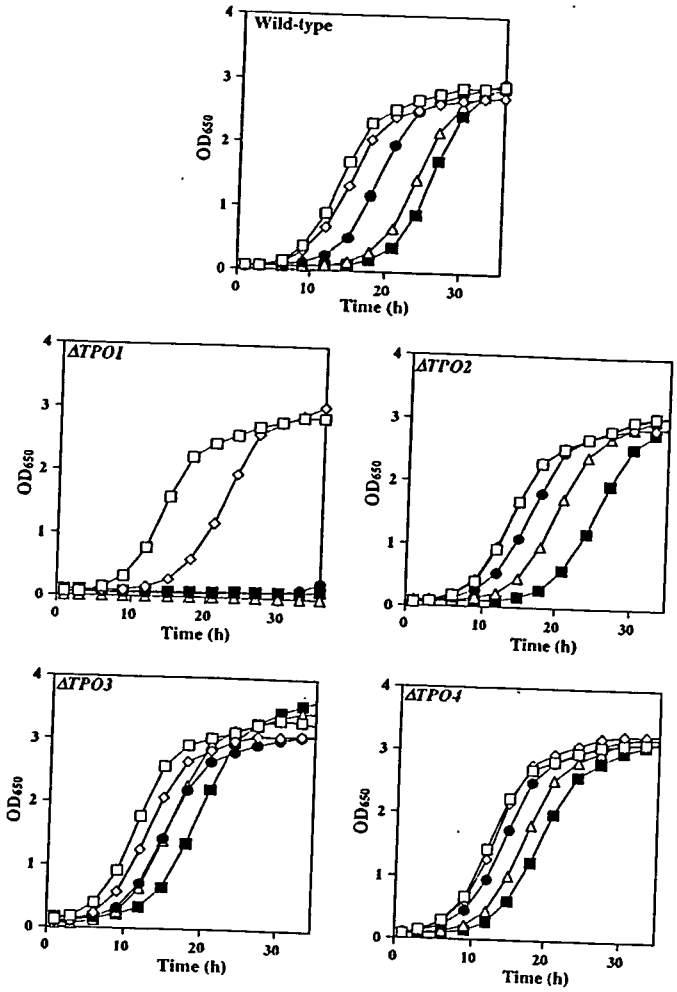

Fig. 3. Growth of yeast *tpo* mutants in the presence of indomethacin. Full growth suspensions of Δ*TPO1*, Δ*TPO2*, Δ*TPO3*, Δ*TPO4* and the wild-type strain, YPH499, were 1/50 diluted and cultured at 30 °C in the presence of 0 (open square), 0.05 (open diamond), 0.1 (closed circle), 0.15 (open triangle) and 0.2 (closed square) mM indomethacin. The OD at 650 nm was monitored.

TPO1 mRNA was induced by indomethacin. The induction of *TPO1* probably contributed to the protection of yeast cells from indomethacin.

3.4. Identification of the human orthologue of TPO1

A direct BLAST search of the human genome, using the amino acid sequence of yeast Tpo1p as a query, did not find any matching genes. We therefore searched for the *D. melanogaster* orthologue of Tpo1p in a similar way and identified the tetracycline resistance protein (Drtet; AE003733 [GenBank]), which is predicted to be tetracycline efflux pump, based on its amino acid sequence (information from GenBank homepage). Drtet shows 8% identity and 25% similarity to yeast Tpo1p in total. Finally, we identified a human orthologue of Drtet, TETRAN (L11669 [GenBank]) [6]. TETRAN shows 40% identity and 74% similarity to Drtet in total. TETRAN has putative 12-membrane-spanning domains, belongs to the MFS class of proteins, and has significant similarity to the *E. coli* tetracycline transporter (TetA) [6], which has also 12-membrane-spanning domains and also belong to MFS [11]. As shown in Fig. 5, some amino acid residues were conserved

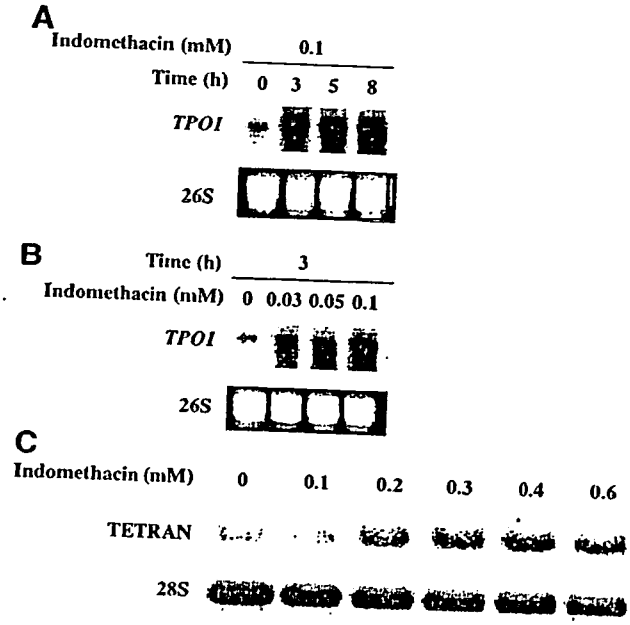

Fig. 4. Induction of *TPO1* mRNA by indomethacin. Exponentially growing W303-1 cells were (A) treated with 0.1 mM indomethacin for the indicated periods or (B) with the indicated concentrations of indomethacin for 3 h. MKN45 cells were exposed to various concentrations of indomethacin for 24 h (C). The level of *TPO1* mRNA (A, B) or *TETRAN* mRNA (C) was monitored by northern blotting analysis. Lower panel shows ribosomal RNA (26S or 28S) stained with ethidium bromide.

between TETRAN, Drtet and Tpo1p. These data suggest that TETRAN is the human orthologue of Tpo1p.

3.5. Effect of TETRAN on the sensitivity of cultured human cells to NSAIDs

No biochemical and biological activities have been reported for TETRAN. To characterize TETRAN, we obtained stable transfectants of MKN45 (a human adenocarcinoma gastric cell line) with an expression plasmid containing *TETRAN* gene. This clone expressed about 2 times more TETRAN mRNA than the control strains. This TETRAN-overexpressing clone and control clone were incubated with various concentrations of indomethacin and the cell viability was determined. As shown in Fig. 6A, the TETRAN-overexpressing clone was more resistant to indomethacin than vector-transfected cells. Furthermore, the TETRAN-overexpressing clone was also resistant to diclofenac (Fig. 6B), suggesting that overexpression of TETRAN in MKN45 cells makes cells resistant to some NSAIDs.

Based on our previous report [12], we considered that cell death seen in Fig. 6A and B is mediated by apoptosis, in other words, overexpression of TETRAN makes cells resistant to NSAID-induced apoptosis. For confirming this point, we examined the effect of overexpression of TETRAN in MKN45 cells on NSAID-induced apoptosis by counting cells with apoptotic chromatin condensation. As shown in Fig. 6C, treatment with indomethacin caused less apoptotic cells in TETRAN-overexpressing clone than in control clone (mock). Similar results were obtained with diclofenac (Fig. 6C), suggesting that overexpression of TETRAN makes cells resistant to NSAID-induced apoptosis. For further confirmation of this point, we used the siRNA for TETRAN.

```
TETRAN        1 ----------------------------------------------------MGWGGG 6
Drtet         1 ----------------------------------------------------MADLRSRHN 9
Tpo1          1 MSDHSPISNKENHLLPSDSSRSSSSDMHSTGTTGTTGVEPVDFTGEGAKYTTATEGNGGA 60
Homology

TETRAN        7 GGCTPRPPIHQQP--------------------PERR--VVIVVFLGLLLD--------- 35
Drtet        10 GTAAMEKQSHSQTGSHHHNNNKALDKEASENGKPEKSDPMIYIIFVSLLFD--------- 60
Tpo1         61 DLAIQRTTTMNSAAESEVNITRRLTKILTGSVNEPDRVEVDYTNCAPMGGDRPYPPSLPS 120
Homology        . .  .    ...                              :       :  *

TETRAN       36 --LLAFTLLLP---LLPGLLESHGRAHDP-LYGSWQGGVDWFATAIGMPV---EKRYNSV 86
Drtet        61 --LLAFTIILP---LLPSLLEHYRQNDSSGLYAVLTDRVRWFQQLLGAP-----DRYISV 110
Tpo1        121 RDLYEVTFDGPNDPLHPFNWPMKKKVLLCLVLCLDSIAIAMCSSIFASAVPQICEIYHVI 180
Homology          *  .*:  *   * *       :     :       :      :. .     . *  :

TETRAN       87 LFGGLIGSAFSVLQFLCAPLTGA-TSDCLGRRPVMLLCLMGVATSYAVWATSRSFAAFLA 145
Drtet       111 LFGGFLGSMFSFLQFVASPIVGG-LSDYYGRKPVLLACASGIALSYLIWACSSNFALFVL 169
Tpo1        181 EVVAILGITLFVLGFAASPVIYAPLSELYGRKGVLVLSAFGFALFQFAVATAENLQTIFI 240
Homology          . .::*  : .* * .:*:  .  *:  **: *:: .  *.*      * : .:  :.

TETRAN      146 SRLIGGIS-KGNVSLSTAIVADLGSPLARSQGMAVIGVAFSLGFTLGPMLGASLPLEMAP 204
Drtet       170 ARFVGGIS-KGNISLCMSVITDVSSVKTRGRGMALVGVAFSLGFIVGPMIGALFAIFSDK 228
Tpo1        241 CRFFGGFIGAAPMAVVPAAFADMFDTNVRGKAIALFSLGVFVGPILSPVMGSYIAQRTTW 300
Homology        .*:.**:   . :::  : .:*: .  .*.:.:*:..:.. :*  :.*::*: :.

TETRAN      205 ----------WFALLFAASDLLFIFCFLPETLP--------LEKR-------APSIALGF 239
Drtet       229 SGSTWFVLPSLLAFGLAVGDLVVLACCLRETLP--------KEKR-------VKEISSAL 273
Tpo1        301 R------WLEYVVGCFASAVFVAIVLFFEETHHPTILVNKAKQMRKQSNNWGIHAAHEDV 354
Homology                  ..  :* . :: :   : **           : *              .

TETRAN      240 RDAADLLSPLALLRFSAVARGQDPPSGDRLSSLRRLGLVYFLYLFLFSGLEYTLSFLTHQ 299
Drtet       274 SYGLQLLNFSAIFRFAAIKNVPK----KDIAALRSIGLVYFLYLFLYSGLEFTVTFLMYH 329
Tpo1        355 ELSIKDIVQKTVTRPIIMLFVEPLLLFVTIYNSFVYGILYLLLEAYPLVFVEGYGFTENG 414
Homology          . . :   :: *   :            :      *::*:*       :     *

TETRAN      300 RFQFSSLQQGKMFFLIGLTMATIQGAYARRIHPGGEVAAVKRALLLLVPAFLLIGWGRSL 359
Drtet       330 KFGYTSMDQAKMFLTTGVIMTLLQGSVVRRLPEAKIKGYAIFSLYLIVPAFVVVGLAEGS 389
Tpo1        415 ELPYIALIIGMMVCAAFIWYMDNDYLKRCRAKGKLVPEARLYAMVIAGTVFPIGILWFCW 474
Homology        .: : ::  . *.    :     :     *            :: :  ..* :

TETRAN      360 PVLG-----------LGLLLYSFAAAVVVPCLSSVVAGYGSPGQKGTVMGTLR--SLGAL 406
Drtet       390 RMLY-----------AGMTLFAISTAFAVTCLTTLVSKYGNDDQKGSVLGIFR--SLGAL 436
Tpo1        475 TGYYPHKIHWMVPTVGGAFIGFGLMGIFLPCLNYIIESYLLLAASAVAANTFMRSAFGAC 534
Homology                      *  :     .. :.**. ::  *     .. . . :   ::**

TETRAN      407 ARAAG--PLVAASVYWLAGAQACFTTWSGLFLLPFFLLQKLSYPAQTLKAE-          455
Drtet       437 ARALG--PVVGCIAFWCVGSRITYIAGGLLLIYPAMALQRARI---------          477
Tpo1        535 FPLFAGYMFRGMGIGWAGLLLGLFAAAMIPVPLLFLKYGESIRKKSKYAYAA          586
Homology              .   . .    *       : :     .    :   .
```

Fig. 5. Amino acid sequences of human TETRAN, *D. melanogaster* Drtet, and *S. cerevisiae* TPO1. The alignment was performed by CLUSTAL W from the Pole Bio-Informatique Lyonnais web server (* identical residues, : strongly similar residues, · weakly similar residues).

As shown in Fig. 7A, transfection of siRNA for TETRAN into MKN45 cells caused suppression of the mRNA expression of TETRAN in its dose-dependent manner. Transfection of this siRNA stimulated the apoptosis induced by indomethacin or diclofenac (Fig. 7B), confirming that expression of TETRAN makes cells resistant to NSAID-induced apoptosis. The trasnfection of this siRNA also elevated the background level of apoptosis (without NSAIDs), suggesting that expression of TETRAN also suppresses the spontaneous apoptosis under the conditions.

We also examined the effect of indomethacin on the expression of *TETRAN* mRNA in MKN45 cells. As shown in

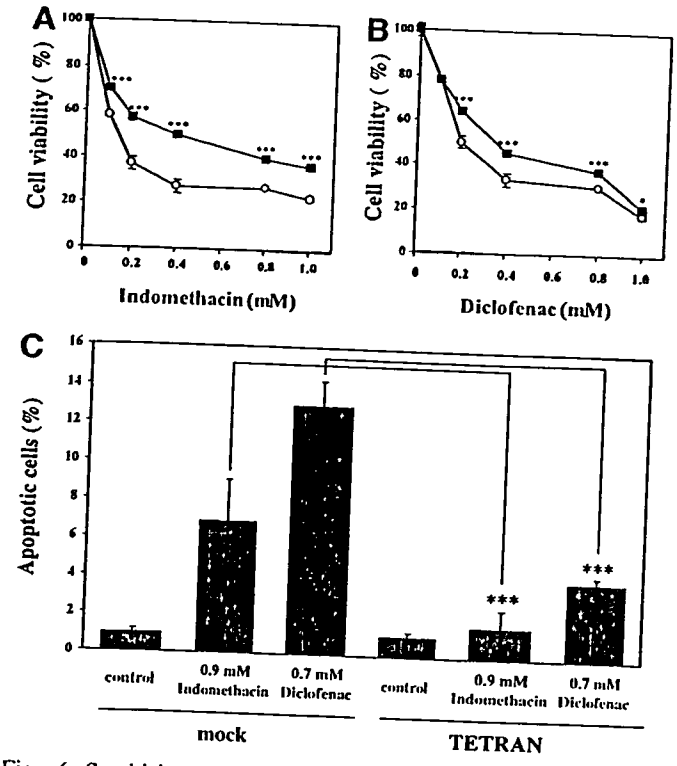

Fig. 6. Sensitivity to NSAIDs of human cells overexpressing TETRAN. A stable transfectant of gastric carcinoma line MKN45 with the expression vector for TETRAN (closed square) and with the vector only (open circle) were exposed for 38 h to the indicated concentrations of indomethacin or diclofenac. Cell viability was determined by the MTT method (A, B). Apoptotic cells with condensed chromatins were counted and expressed relative to total cells (C). Values are means ± S.E.M. ($n = 6$). $^{*}P < 0.05$, $^{***}P < 0.001$.

Fig. 4C, indomethacin induced the expression of *TETRAN* mRNA in a dose-dependent manner. This induction probably contributes to the protection of gastric cells from indomethacin.

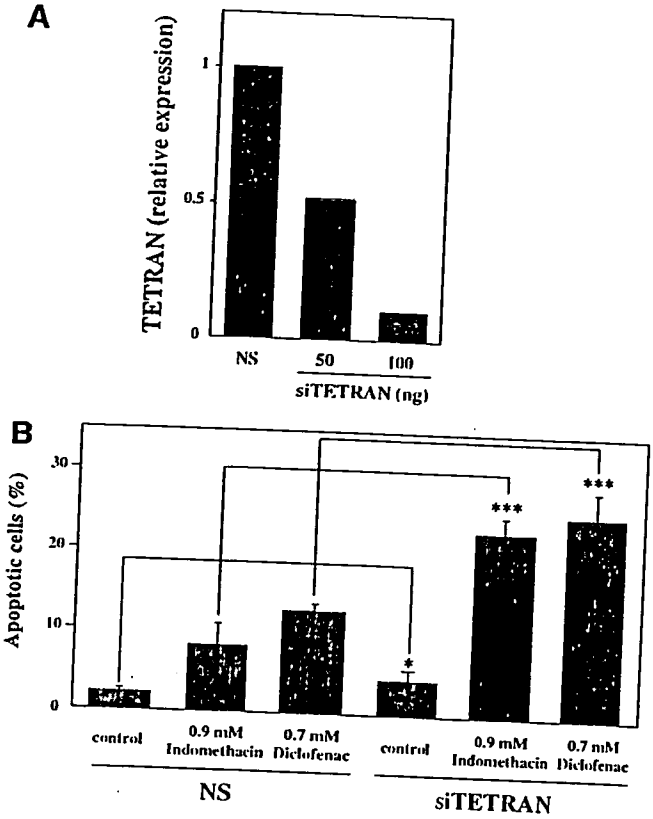

Fig. 7. Effect of siRNA for TETRAN on NSAIDs-induced apoptosis. MKN45 cells were transfected with indicated amounts (A) or 100 ng (B) of siRNA for TETRAN (siTETRAN) or non-silencing siRNA (NS) and cultured for 24 h. Cells were further incubated with or without indicated concentration of indomethacin or diclofenac for 24 h (B). The levels of *TETRAN* mRNA were estimated by real-time RT-PCR experiments using a specific primer for each gene. Values were normalized to actin gene expression and expressed relative to the control sample (A). Apoptosis was monitored as described in the legend of Fig. 6 (B). Values are means ± S.E.M. ($n = 3$). $^{*}P < 0.05$, $^{***}P < 0.001$.

4. Discussion

Patients vary in sensitivity to the gastrointestinal side effects of NSAIDs, making clinical use difficult. As the first step to understand this variation, we tried to identify genes that affect the direct cytotoxicity of NSAIDs, which may be at least partly responsible for causing gastric ulcers [5]. In yeast, the *TPO1* gene was shown to be involved in NSAID-resistance. It is the first such gene to be identified in yeast. We also identified a possible human orthologue, TETRAN. Overexpression of TETRAN or suppression of its expression by siRNA technique made cultured human cells resistant or sensitive, respectively, to some NSAIDs. TETRAN is the first protein shown to affect indomethacin resistance in human cells. In future, single nucleotide polymorphism (SNP) analysis of TETRAN may be important to understand the mechanism of variation of patients for sensitivity to the gastrointestinal side effects of NSAIDs.

Tpo1p has putative12 membrane-spanning domains, belongs to the MFS class of proteins, and is located on plasma membranes [13,14]. It was first identified as a transporter for polyamines [7,13], however, at present, it is thought to be a multidrug efflux pump, because its expression also caused resistance to herbicides, cycloheximide, quinidine and immunosuppressive drugs [9,15–17]. Therefore, Tpo1p is probably involved in indomethacin export from yeast cells.

Based on the sequence similarity between TETRAN and other drug efflux pumps, including Tpo1p, we consider that TETRAN is probably an efflux pump for NSAIDs in human cells. TETRAN's amino acid sequence strongly suggests that it also belongs to the MFS class of proteins [6]. Drug efflux pumps can be separated into two groups based on the mode of transport and energy source: primary and secondary active transporters. Primary active transporters are also referred to as ATP binding cassette (ABC) proteins, and use the energy of ATP hydrolysis. Secondary active transporters, e.g. MFS class proteins, act as anti-ports coupled with ion transport [18]. In bacteria, secondary active transporters are predominant for drug efflux [19], but in eukaryotic cells, primary active transporters are predominant [20]. TETRAN is the first MFS protein identified that is involved in drug resistance in human cells, and further studies of such proteins will be important to understand drug efflux mechanisms in human cells.

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Involvement of up-regulation of PUMA in non-steroidal anti-inflammatory drug-induced apoptosis

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Abstract

NSAIDs such as celecoxib induce apoptosis in cancer cells. Although this apoptotic effect is involved in the anti-tumor activity associated with such drugs, the mechanism by which this occurs is not fully understood. We report here that various NSAIDs, including celecoxib, up-regulate PUMA, a Bcl-2 family protein with potent apoptosis-inducing activity, in human gastric carcinoma cell line, accompanying the induction of apoptosis. Experiments using siRNA and an intracellular Ca^{2+} chelator revealed that Ca^{2+}-dependent up-regulation of ATF4 and CHOP is involved in this up-regulation of PUMA. The siRNA for PUMA inhibited the celecoxib-induced activation and translocation of Bax, release of cytochrome *c* into the cytosol and induction of apoptosis, suggesting that PUMA plays an important role in celecoxib-induced mitochondrial dysfunction and the resulting apoptosis.

Keywords: PUMA; Endoplasmic reticulum; ATF4; CHOP; Apoptosis

A range of epidemiological studies have revealed that prolonged non-steroidal anti-inflammatory drugs (NSAIDs) use reduces the risk of cancer (such as colonic, rectal, and stomach cancer), while preclinical and clinical studies have indicated that some NSAIDs are effective in the treatment and prevention of cancer [1]. NSAID-induced apoptosis in cancer cells is thought to play an important role in this anti-tumor action [2]. NSAID-induced apoptosis was thought to be mediated only through the inhibition of cyclooxygenase (COX). However, since a derivative of the NSAID sulindac (sulindac sulfone), which has no COX-inhibitory activity, was shown to induce apoptosis in tumor cells, NSAID-induced apoptosis also involves a COX-independent mechanism.

We revealed that NSAIDs induced various endoplasmic reticulum (ER) stress response-related genes, including C/EBP homologous transcription factor (CHOP, a transcription factor with apoptosis-inducing activity) in a COX-independent manner and this up-regulation of CHOP is involved in NSAID-induced apoptosis [3,4]. However, an NSAID-induced apoptosis pathway downstream of CHOP has not been identified.

Both activating transcription factor (ATF)4-pathway and ATF6-pathway are involved in the ER stress response; ER stressors phosphorylate protein kinase R-like ER kinase (PERK), which in turn phosphorylates eukaryotic initiation factor-2α (eIF-2α, leading to an increase in ATF4 (ATF4-pathway) and cause cleavage of ATF6 (p90-ATF6) into p50-ATF6, the active form that acts as a transcription factor (ATF6-pathway) [5,6]. We recently showed that both ATF4- and ATF6-pathways are induced by NSAIDs [7,8]. We also showed that NSAIDs increase the intracellular Ca^{2+} level and this Ca^{2+}-increase is involved in NSAID-induced apoptosis through activation of calpain, a Ca^{2+}-dependent cysteine protease with apoptosis-inducing ability, and the up-regulation of CHOP [9,10]. However, as with CHOP, the NSAID-induced apoptosis pathway downstream of calpain has remained unknown.

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Mitochondrial outer membrane permeabilization and the resulting release of mitochondrial proteins (such as cytochrome *c*) into the cytosol play a major role in stressor-induced apoptosis, and the Bcl-2 family proteins play an important part in this process [11]. Bcl-2 family proteins can be divided into two groups, the multidomain protein group and the Bcl-2 homology 3 (BH3) only protein group. Multidomain proteins directly regulate mitochondrial outer membrane permeability and both positive (such as Bax and Bak) and negative (such as Bcl-2 and Bcl-xl) permeabilization factors are included in this group [12]. For example, in response to apoptotic stimuli, Bax undergoes a conformational change (activation) and translocates from the cytosol to the mitochondria to form multimers that permeabilize the mitochondrial outer membrane. On the other hand, BH3 only domain proteins, such as Bid and Bad, regulate the mitochondrial permeability by controlling the activity of multidomain proteins [12]. A number of previous studies have shown that various NSAIDs cause mitochondrial outer membrane permeabilization and stimulated the translocation of Bax from the cytosol to the mitochondria [9,13,14]. These results suggest that Bcl-2 family proteins, especially Bax, play an important role in NSAID-induced apoptosis; however, the mechanism which links the ER stress response to Bax has not been identified.

PUMA (p53 up-regulated modulator of apoptosis) is a BH3 only domain protein with potent apoptosis-inducing activity [15,16]. Furthermore, exogenous overexpression of PUMA stimulated conformational change, translocation and multimerization of Bax, and the pro-apoptotic function of PUMA was almost completely abolished in Bax-null cells, suggesting a strong connection between PUMA and Bax [17–19]. Although PUMA was originally reported to be a factor regulated by p53, it is now believed that some stressors up-regulate PUMA in a p53-independent manner [20]. Furthermore, ER stressors (such as tunicamycin and thapsigargin) were reported to up-regulate PUMA [21,22]. Therefore, PUMA could link the ER stress response to Bax in the NSAID-induced apoptosis pathway. In this study we found that various NSAIDs, including celecoxib, up-regulate PUMA in a p53- and COX-independent manner. Experiments using a small interfering RNA (siRNA) specific for PUMA showed that PUMA up-regulation was involved in celecoxib-induced activation and translocation of Bax, mitochondrial outer membrane permeabilization, and induction of apoptosis. Furthermore, the results suggest that NSAID-induced up-regulation of PUMA is mediated through an increase in the intracellular Ca^{2+} level, up-regulation of ATF4 and up-regulation of CHOP.

Materials and methods

Cell culture, treatment with NSAIDs, real-time RT-PCR analysis, and fluorescence activated cell sorting (FACS) analysis. AGS and Kato III are human carcinoma cell lines derived from stomach. Cells were cultured in RPMI 1640 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. Cells (9×10^5 cells in 100 mm diameter plates) were cultured for 24 h prior to use in experiments. Cells were exposed to NSAIDs by replacing the initial medium with fresh medium containing an appropriate concentration of the desired NSAID. Real-time RT-PCR analysis and FACS analysis were performed as described [7].

Immuno-blotting analysis. Whole cell extracts were prepared as described previously [23]. The protein concentration of the samples was determined by the Bradford method. The samples were electrophoresed on polyacrylamide gels containing SDS and the proteins then transferred to membranes and detected using each antibody.

Staining of cells. Cells were cultured on 4-well Lab-Tek II glass slides (Nunc). After fixation with 4% formaldehyde for 20 min and permeabilization with 0.5% Triton X-100 for 5 min, non-specific binding sites were blocked with 3% BSA for 30 min. Immuno-staining to detect the active form of Bax [24] was performed with a polyclonal antibody against the N-terminal region of Bax (Bax N20). Immuno-detection was carried out using Alexa Fluor 488 goat anti-rabbit immuno-globulin G. Cells were simultaneously stained with DAPI (5 μg/ml). Cells were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus BX51).

Statistical analysis. All values are expressed as the means ± standard deviation (SD). Two-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison was used for evaluation of differences between the groups. A Student's *t*-test for unpaired results was performed to evaluate differences between two groups. Differences were considered to be significant for values of $P < 0.05$.

Results and discussion

NSAIDs up-regulate PUMA in a COX- and p53-independent manner

Fig. 1A and B shows dose–response and time–course profiles of celecoxib-dependent up-regulation of *PUMA* mRNA in AGS cells, respectively, as monitored by real-time RT-PCR; celecoxib up-regulated *PUMA* mRNA in both a dose- and time-dependent manner. Similar results were obtained at the protein level as monitored by immuno-blotting (Fig. 1C and D). We also monitored the celecoxib-dependent induction of apoptosis by FACS analysis (Fig. 1E and F). Dose–response and time–course profiles of celecoxib-induced apoptosis (Fig. 1E and F) correlated well with celecoxib-induced up-regulation of PUMA (Fig. 1A–D), suggesting that this up-regulation of PUMA is involved in celecoxib-induced apoptosis.

We also examined the effect of NSAIDs other than celecoxib on PUMA expression. All of the NSAIDs tested (indomethacin, diclofenac and meloxicam) up-regulated PUMA in a dose-dependent manner (data not shown). The concentration of each NSAID required for this up-regulation was much the same as that required for induction of apoptosis (data not shown).

COX exists as two subtypes, COX-1 and COX-2, for which celecoxib and meloxicam, but not indomethacin and diclofenac, are COX-2-selective [25]. We examined the celecoxib-dependent up-regulation of PUMA in Kato III cells, in which *COX-1* but not *COX-2* mRNA is expressed [26]. As shown in Fig. 2A, celecoxib up-regulated PUMA even in Kato III cells, suggesting that up-regulation

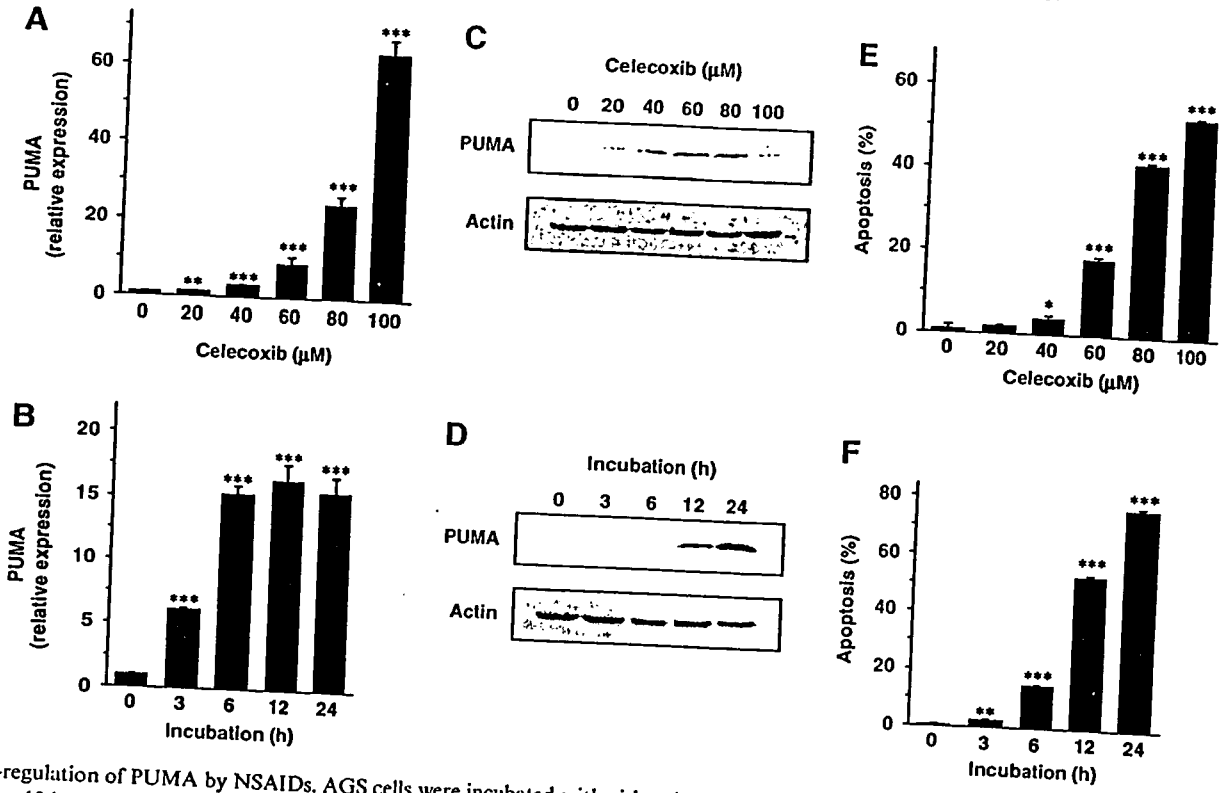

Fig. 1. Up-regulation of PUMA by NSAIDs. AGS cells were incubated with either the indicated concentrations (A, C, and E) or 80 μM of celecoxib (B, D, and F) for 12 h (A, C, and E) or the time periods indicated (B, D, and F). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer set for *PUMA*. Values were normalized to actin gene expression and expressed relative to the control sample (i.e., without celecoxib or time 0) (A,B). Whole cell extracts were analyzed by immuno-blotting with an antibody against PUMA or actin (C,D). Apoptotic cell numbers were determined by FACS (E,F). Values are given as means ± SD ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

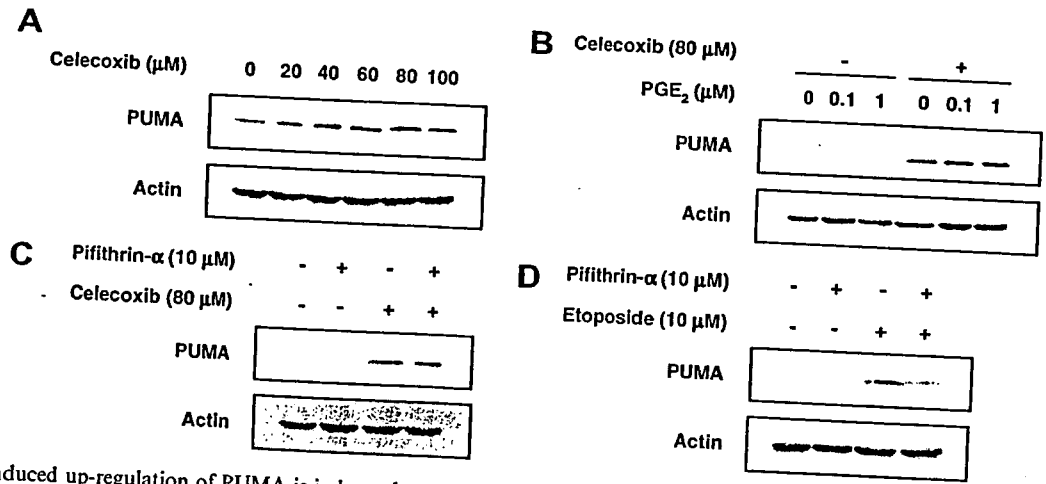

Fig. 2. Celecoxib-induced up-regulation of PUMA is independent of COX and p53. Kato III cells were incubated with the indicated concentrations of celecoxib for 24 h (A). AGS cells were incubated with or without 80 μM celecoxib (B,C) or 10 μM etoposide (D) in the presence or absence of indicated concentrations of PGE_2 (B) or pifithrin-α (C,D) for 12 h (B,C) or 24 h (D). The expression of PUMA was monitored by immuno-blotting as described in the legend of Fig. 1A–D.

of PUMA by NSAIDs is independent of COX-inhibition. For further confirmation of this point, we examined the effect of exogenously added PGE_2 on the celecoxib-induced up-regulation of PUMA and found that PGE_2 (0.1 or 1 μM) did not affect the expression of PUMA in the presence or absence of celecoxib (Fig. 2B).

As described in Introduction, some stressors (such as etoposide) up-regulate PUMA in a p53-dependent manner and others in a p53-independent manner. We tested the case of celecoxib using an inhibitor of p53, pifithrin-α. As shown in Fig. 2C, treatment of cells with 10 μM pifithrin-α did not affect the celecoxib-dependent up-regulation of

PUMA. This concentration of pifithrin-α did not affect cell viability (data not shown). We confirmed that etoposide-dependent up-regulation of PUMA is suppressed by 10 μM pifithrin-α (Fig. 2D). These findings suggest that celecoxib up-regulates PUMA in a p53-independent manner.

Molecular mechanism governing celecoxib-dependent up-regulation of PUMA

Next, we tested the contribution of the ER stress response (ATF4-pathway and ATF6-pathway) to celecoxib-dependent up-regulation of PUMA using siRNA for ATF4 and ATF6. We confirmed previous results [8] that transfection of a given siRNA decreased mRNA expression of its target gene, but had no effect on the expression of the other gene in both absence and presence of celecoxib (data not shown). Transfection of a siRNA for ATF4, but not that for ATF6, inhibited the celecoxib-dependent up-regulation of *PUMA* mRNA (Fig. 3A), suggesting that the ATF4-pathway but not the ATF6-pathway is involved in this up-regulation.

The contribution of CHOP to the celecoxib-dependent up-regulation of PUMA was also tested using the siRNA technique. Transfection of a siRNA for CHOP partially suppressed celecoxib-dependent up-regulation of not only *CHOP* mRNA but also *PUMA* mRNA (Fig. 3A and B), suggesting that CHOP is also involved in celecoxib-dependent up-regulation of PUMA. The siRNA for ATF4 suppressed celecoxib-dependent up-regulation of *CHOP* mRNA (Fig. 3B), suggesting that ATF4 positively, but indirectly, regulates the expression of the *PUMA* gene via up-regulation of *CHOP*. On the other hand, double-transfection of siRNA for both ATF4 and CHOP clearly exerted a stronger suppressive effect on up-regulation of *PUMA* mRNA than transfection of CHOP siRNA alone (Fig. 3A), suggesting that ATF4 also plays a direct role in the positive regulation of *PUMA* gene expression.

As described in Introduction we previously reported that NSAIDs increase the intracellular Ca^{2+} level. In this study we used BAPTA-AM, an intracellular Ca^{2+} chelator that is permeable to the cytoplasmic membrane to test the contribution of this Ca^{2+}-increase to celecoxib-dependent up-regulation of PUMA. BAPTA-AM inhibited celecoxib-induced up-regulation of PUMA at both the mRNA (Fig. 3C) and protein (data not shown) level, suggesting that celecoxib-induced up-regulation of PUMA is mediated by the increase in the intracellular Ca^{2+} level. BAPTA-AM also inhibited celecoxib-induced up-regulation of both *ATF4* and *CHOP* mRNAs (Fig. 3D and E) but did not

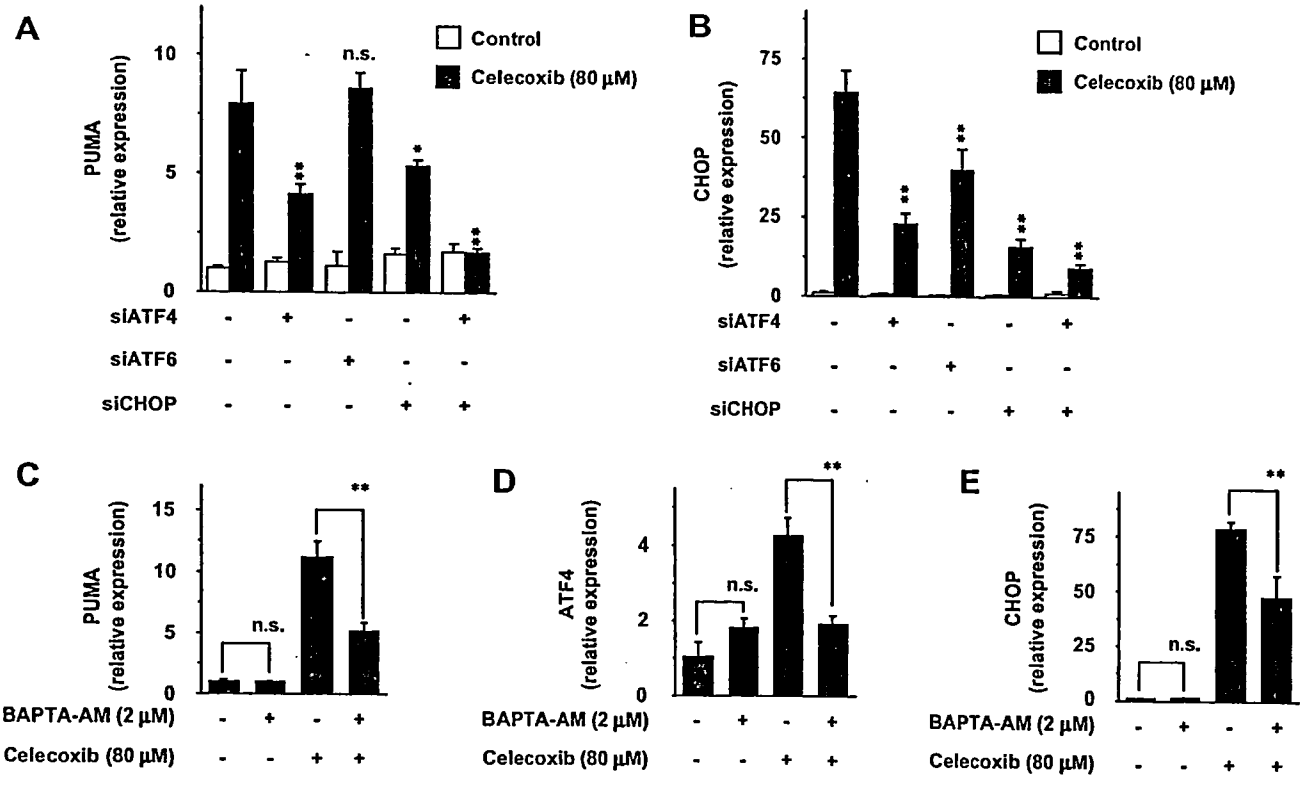

Fig. 3. Contribution of the increase in the intracellular Ca^{2+} level and up-regulation of ATF4 and CHOP to celecoxib-induced up-regulation of PUMA. AGS cells were transfected with siRNA for ATF4 (siATF4), ATF6 (siATF6), CHOP (siCHOP), or non-silencing siRNA. After 48 h cells were incubated with or without 80 μM celecoxib for 6 h (A,B). AGS cells were pre-incubated with or without BAPTA-AM (C–E) for 1 h and further incubated with or without 80 μM celecoxib under the same conditions as in the pre-incubation step for 6 h (C–E). The levels of *PUMA* mRNA (A,C), *ATF4* mRNA (D) and *CHOP* mRNA (B,E) were estimated by real-time RT-PCR as described in the legend of Fig. 1. Values shown are means ± SD ($n = 3$). $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant.

inhibit the overall mRNA synthesis (data not shown), suggesting that celecoxib-dependent Ca^{2+}-increase induces PUMA through up-regulation of both ATF4 and CHOP.

We also examined the participation of calpain in celecoxib-induced up-regulation of PUMA using an inhibitor of calpain, Z-Leu-Leu-H. We reported that Z-Leu-Leu-H inhibited the celecoxib-dependent activation of calpain and apoptosis [9]. Addition of Z-Leu-Leu-H did not affect the celecoxib-dependent up-regulation of PUMA (data not shown), suggesting that calpain is not involved in celecoxib-induced up-regulation of PUMA.

Role of up-regulation of PUMA in celecoxib-induced apoptosis

We then used the siRNA technique to examine the contribution of celecoxib-induced up-regulation of PUMA in celecoxib-induced Bax activation and translocation, mitochondrial outer membrane permeabilization (release of cytochrome *c* into the cytosol) and induction of apoptosis. We confirmed that transfection of a siRNA for PUMA inhibited the celecoxib-dependent up-regulation of *PUMA* mRNA (Fig. 4A) and PUMA protein (Fig. 4B). FACS analysis showed that this transfection partially suppressed celecoxib-induced apoptosis (Fig. 4C). We also found that this transfection partially suppressed celecoxib-dependent activation of caspase 3 (data not shown), suggesting that the up-regulation of PUMA is involved in celecoxib induced apoptosis.

As shown in Fig. 4D, the amount of cytochrome *c* in the cytosol fraction increased in the presence of celecoxib and this increase was partially suppressed by the transfection of siRNA for PUMA, suggesting that celecoxib-dependent mitochondrial outer membrane permeabilization is partially mediated by up-regulation of PUMA. Results shown in Fig. 4D also indicated that the amount of Bax in the cytosol fraction decreased in the presence of celecoxib but almost completely recovered to the original level by the transfection of siRNA for PUMA. These findings suggest that the celecoxib-dependent translocation of Bax from cytosol to mitochondria is mediated by the up-regulation of PUMA.

The conformational change (activation) of Bax occurs prior to Bax translocation, and seems to be required for this translocation [24]. We here monitored the conformational change of Bax by immuno-staining analysis with an antibody that specifically recognizes the active form of Bax [24]. As shown in Fig. 4E, the number of Bax (active form)-positive cells drastically increased in the presence of celecoxib, and the transfection of siRNA for PUMA returned this number to the background level, suggesting that the celecoxib-dependent activation of Bax is mediated by up-regulation of PUMA. It was recently reported that PUMA can directly bind to Bax and cause the

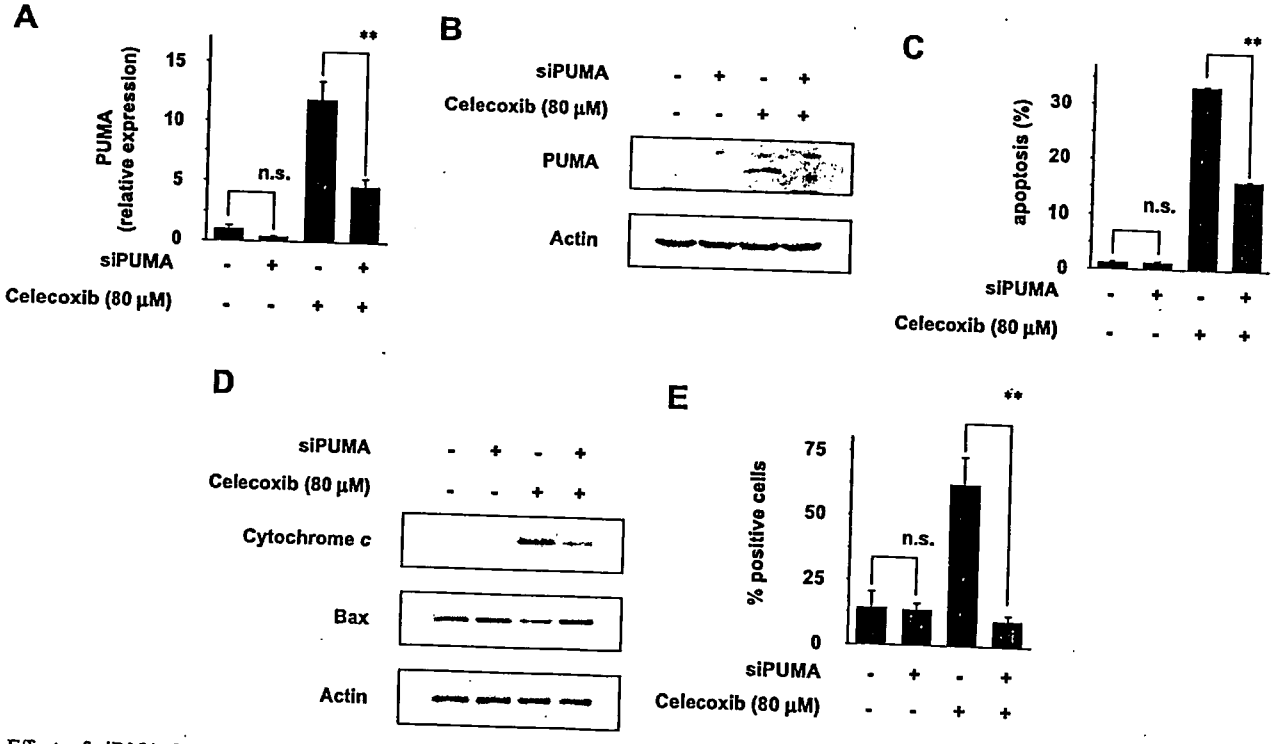

Fig. 4. Effect of siRNA for PUMA on celecoxib-induced apoptosis. AGS cells were transfected with siRNA for PUMA (siPUMA) or non-silencing siRNA. After 48 h cells were incubated with or without 80 μM celecoxib for 6 h (A), 12 h (B, D, and E) or 18 h (C). Cytosol fractions were prepared (D). The levels of *PUMA* mRNA (A), PUMA protein (B), cytochrome *c* and Bax proteins (D) were estimated by immuno-blotting or real-time RT-PCR experiments as described in the legend of Fig. 1. Apoptosis was monitored as described in the legend of Fig. 1C. Immuno-staining with the antibody against the N-terminal region of Bax (Bax N20) and DAPI-staining were performed as described in the experimental procedures. Approximately 500–700 cells were randomly counted for staining with Bax N20 (E). Values shown are means ± SD ($n = 3$–5). $^{**}P < 0.01$; n.s., not significant.

conformational change [27]. Therefore, it seems that celecoxib-induced mitochondrial dysfunction is mediated by the direct binding of PUMA to Bax, and the resulting activation and translocation of Bax. PUMA-mediated dissociation of Bax from Bcl-2 and Bcl-xl [15,17] may also be involved in the activation of Bax by PUMA, because Bcl-2 and Bcl-xl were reported to inhibit the Bax conformational change [28,29].

NSAID-induced apoptosis plays an important role not only in the anti-tumor activity of these drugs, but also in the pathology of NSAID-induced gastric ulcers, which is a major side-effect of NSAIDs in their clinical use; we recently suggested that both COX-inhibition and NSAID-induced cell death (such as apoptosis) at the gastric mucosa are necessary factors leading to the production of NSAID-induced gastric ulcers *in vivo* [30]. On this basis, we examined the molecular mechanism governing NSAID-induced apoptosis in several of our papers and found that the ER stress response (up-regulation of CHOP) and mitochondrial outer membrane permeabilization play an important role in this apoptosis [4,9,30]. In the present study, we have identified PUMA as a factor that could provide a link between the ER stress response and mitochondrial outer membrane permeabilization. In summary, the results from this study suggest that NSAID-induced apoptosis is mediated by various mechanisms, one of which is a pathway involving an increase in the intracellular Ca^{2+} level, the ER stress response, up-regulation of ATF4, up-regulation of CHOP, up-regulation of PUMA, activation and translocation of Bax and mitochondrial outer membrane permeabilization.

Acknowledgment

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Simultaneous Measurements of K^+ and Calcein Release from Liposomes and the Determination of Pore Size Formed in a Membrane

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The changes induced by biologically active substances in the permeability to K^+ and calcein of liposomes composed of egg phosphatidylcholine and cholesterol were measured simultaneously in order to rapidly screen the sizes of pores formed in a membrane, using different sized markers. The substances examined in the present study were classified into three types based on differences in the rates at which K^+ and calcein were released. The first type released only K^+, and included gramicidin A. The second type predominantly released K^+, preceding the release of calcein, and included amphotericin B and nystatin. The third type, including antimicrobial peptides, such as gramicidin S, alamethicin, and melittin, and several membrane-active drugs, like celecoxib (non-steroidal anti-inflammatory drug), 1-dodecylazacycloheptan-2-one (named azone; skin permeation enhancer), and chlorpromazine (tranquilizer), caused the release of K^+ and calcein simultaneously. Thus, the sizes of pores formed in a liposomal membrane increased in the following order: types one, two, and three. We determined the size more precisely by conducting an osmotic protection experiment, measuring the release of calcein in the presence of osmotic protectants of different sizes. The radii of pores formed by the second type, amphotericin B and nystatin, were 0.36 - 0.46 nm, while the radii of pores formed by the third type were much larger, 0.63 - 0.67 nm or more. The permeability changes induced by substances of the third type are discussed in connection with a transient pore formed in a lipid packing mismatch taking place during the phase transition of dipalmitoylphosphatidylcholine liposomes.

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Introduction

Assays of the permeability of liposomes are fundamental for investigations of the interactions between biologically active substances and membranes.[1,2] Rapid changes in the permeability of liposomal membranes can be brought about by interactions with drugs, calcium-mediated fusion, or antibody/complement-mediated lysis of antigen-bearing liposomes. Among various markers so far developed, calcein has most widely been used for such measurements.[1,3-7] Calcein fluoresces very weakly at high concentrations because of self-quenching, but its fluorescence increases at lower concentrations as quenching is reduced. Thus, the calcein entrapped inside liposomes at high concentrations is weakly fluorescent, while the calcein that has leaked out of the liposomes is highly fluorescent. Calcein-loaded liposomes have been widely used for permeability assays, because liposomal leakage can be measured *in situ* without any separation of liposomes in an assay medium.[1,3-7]

In the present study, we were particularly interested in the combination of calcein and K^+ for permeability assays and attempted to monitor simultaneously the release of calcein and K^+ from liposomes to obtain information about the sizes of pores formed in membranes. Because K^+ leakage is the first event in the permeabilization of a membrane, measurements of differences in the rates of release of K^+ and calcein, which is larger than K^+, can provide significant information about the sizes of pores made by membrane-active substances. We tested various membrane-active substances; the channel-forming peptide gramicidin A,[8,9] polyene antibiotics such as amphotericin B, nystatin, and filipin,[8,10,11] antimicrobial peptides such as gramicidin S, alamethicin, and melittin,[8,9,12-14] and membrane-disrupting drugs such as celecoxib (non-steroidal anti-inflammatory drug),[6] 1-dodecylazacycloheptan-2-one (named azone; skin permeation enhancer),[15] and chlorpromazine (tranquilizer).[16,17] Although simultaneous monitoring of the different rates of K^+ and calcein release from liposomes provided significant information about the sizes of pores generated by these substances, we further determined the sizes more precisely by conducting an osmotic protection experiment.[3,18] This experiment is based on the fact that, if the solute added to the outer medium does not pass through pores formed in the liposomal membrane, the release of calcein will not be induced because the osmotic pressure of the inner calcein is balanced with that of the solute. Thus, the sizes of pores in the membrane can be evaluated by examining whether the solutes can protect against the release of calcein. We used a series of sugars as

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