厚生労働科学研究費補助金

創薬基盤推進研究事業

ストレス遺伝子チップを用いた医薬品の副作用機構の解明と、副作用のない新規医薬品開発戦略の確立

平成19年度　総括研究報告書

主任研究者　水島　徹

平成20（2008）年　3月

目次

厚生労働科学研究費補助金（創薬基盤推進研究事業）
総括研究報告書

ストレス遺伝子チップを用いた医薬品の副作用機構の解明と、副作用のない新規医薬品開発戦略の確立

主任研究者　水島　徹　熊本大学大学院医学薬学研究部教授

研究要旨

我々は、薬剤性間質性肺炎副作用が問題になっている抗リウマチ薬等に関しても同様のトランスクリプトソーム解析を行い、これらの医薬品が抗炎症作用を持つタンパク質の発現を強く抑えることがこの副作用の原因であることを示唆すると共に、これまで成功していなかった薬剤性間質性肺炎の実験動物モデルの確立に成功した。

A. 研究目的

製薬企業を始め、新しい物質を商品化する企業にとって、毒性試験は必須である。現在、動物実験で毒性試験を行っているため、莫大な費用と時間がかかるという問題に加え、生死に関する毒性しか分からないという問題もある。そこで動物実験に代わる方法として、新規物質を細胞に作用させ、誘導される遺伝子を網羅的に解析することによって、その物質の毒性を予想する方法が考えられている。そのためには、ストレス遺伝子（種々のストレスによって誘導される遺伝子）を網羅したDNAチップ（ストレス遺伝子チップ）が有効である。本研究の目標の一つは、ヒトストレス遺伝子チップを開発したという実績を基に、更なるストレス遺伝子の網羅的同定を行い、改良型ヒトストレス遺伝子チップを開発することである。即ち本研究は、本プロジェクトの指定研究を支える研究と位置づけることができる。また、このストレス遺伝子チップを使って、臨床現場でその副作用が問題になっている医薬品を解析し、その副作用メカニズムを解明し、副作用の少ない新しい医薬品の開発戦略を確立する研究も行う（トキシコゲノミックスが副作用の少ない医薬品の開発に貢献することを実証する）。我々はこの方法で、胃潰瘍を起こしにくい安全なNSAIDsの開発法を確立し、実際この方法を用いて新しいNSAIDsを合成しその胃潰瘍副作用がほとんどないことを示した（この研究は、NSAIDs潰瘍に苦しんでいる多くの患者さん（米国では年間16500人がNSAIDs潰瘍で亡くなっている）の救済、及び医療費の削減（胃潰瘍副作用のため、NSAIDsと同時に胃薬が処方されている）にもつながる。同様の方法で、臨床現場でその間質性肺炎副作用が問題になっている医薬品（抗癌剤、抗リウマチ薬、漢方薬）をストレス遺伝子チップで解析しその副作用メカニズムを解明し、間質性肺炎副作用の少ない新しい医薬品の開発戦略を確立する研究もおこなっている。平成18年度、ストレス遺伝子チップを用いた解析から、これら医薬品による間質性肺炎副作用に関与する新たなメカニズムを発見したので、平成19年度はその証明と間質性肺炎副作用の少ない新しい医薬品開発法の確立を目指す。一方本研究で我々は、微生物を利用して医薬品の細胞毒性（副作用）に関する新しいヒト遺伝子を同定し、副作用感受性の個人差を規定している遺伝子多型を同定する研究も行っている。過去2年間の解析からNSAIDs潰瘍副作用感受性の個人差を規定している可能性がある、

COX、HSP、TETRAN遺伝子に関する多型（SNP）を発見したので、平成19年度はその更なる解析を行い、患者の NSAIDs潰瘍感受性を予測する方法の確立を目指す。

B. 研究方法

ストレス遺伝子チップを用いた、医薬品副作用による間質性肺炎発症機構メカニズムの解明

抗癌剤（ゲフィチニブ（イレッサ））、抗リウマチ薬（レフルノミド、エタネルセプト、インフリキシマブ）、漢方薬（小柴胡湯、牛車腎気丸）による間質性肺炎副作用が臨床現場で大きな問題になっている（多数の死者が報告されている）。しかし、その発症メカニズムはほとんど分かっていない。さらにこれらの医薬品は欧米でもよく使用されているが、我が国ほど間質性肺炎副作用は問題になっていない。そこでこれらの医薬品による間質性肺炎副作用発症機構を解明し、副作用の少ない新薬を開発することは大変重要である。平成 18 年度我々は、これらの医薬品が SOD、HO-1、Nrf2、HSP など抗炎症作用を持つタンパク質の発現を強く抑えることを見出した。この結果は、これらの医薬品が抗炎症タンパク質を低下させることにより、間質性肺炎を引き起こしている可能性を示すものであるので更なる解析を行う。まず siRNA 法や各種阻害剤などを用いて、これらの医薬品によるこれら遺伝子の抑制機構を解明する。そしてその抑制がこれらの医薬品の主作用（抗癌や抗リウマチなど）と関連があるのかを調べる（我々は関連がないと予想している）。またこれらの遺伝子を恒常的に発現しているトランスジェニックマウス、及びこれらの遺伝子のノックアウトマウスを入手し、間質性肺炎誘導性を調べ、これらの遺伝子が間質性肺炎に関与しているかを調べる。また有機化学合成によりこれらの医薬品（漢方薬を除く）の誘導体を合成し、それが間質性肺炎を起こすのか、またこれらの遺伝子（SOD、HO-1、Nrf2、HSP）を抑制するのかを調べる。以上の研究から、これらの医薬品による間質性肺炎発症機構を解明し、副作用のない新規医薬品の開発戦略を確立することが本研究の最終目標である。

C. 研究結果

ゲフィチニブ、レフルノミド、エタネルセプト、インフリキシマブを用いて検討したところ、これらの薬剤が HSF1 のリン酸化を抑制することを見出した。HSF1 はリン酸化され活性化させ、HSP などの遺伝子発現を促進することが知られている。そこでまず HSF1 を過剰発現させた細胞を用いて解析したところ、これら薬剤による SOD、HO-1、Nrf2、HSP の発現抑制が見られなくなった。この結果は、これら薬剤は HSF1 のリン酸化を

抑制し、HSF1 を不活性化することにより、これら遺伝子の発現を抑制していることを示唆している。HSF1 が、HO-1, Nrf2, SOD の発現制御に寄与していることが示唆されたのはこれが初めてである。

またこれらの遺伝子の過剰発現細胞を用いて、これら薬剤の作用（抗癌作用や抗リウマチ作用）をコントロール細胞と比較したところ、大きな差は見られなかった。従って、これら遺伝子の発現抑制はこれらの医薬品の主作用とは関連がないと考えられる。

一方、Nrf2 ノックアウトマウス、HSP70 トランスジェニックマウスにおいて、ブレオマイシン誘導性肺炎（間質性肺炎のモデル）を野生型マウスと比較したとこと、Nrf2 ノックアウトマウスではより肺炎を起こしやすいこと、逆に HSP70 トランスジェニックマウスでは肺炎を起こしにくいことを見出した。この結果は、これら薬剤（ゲフィチニブ、レフルノミド、エタネルセプト、インフリキシマブ）による Nrf2、HSP70 の発現抑制が、間質性肺炎副作用に寄与していることを示唆している。

一方、レフルノミドの誘導体を合成しているが、まだ全ての合成が完了していないので、副作用を調べるには至っていない。

NSAID 感受性に関する遺伝子に関しては、遺伝子多型のデータベースから候補遺伝子（COX-1、COX-2、PGES、HSP70、GRP78、HO-1、TETRAN）に関する情報を得て、その中から胃粘膜細胞の NSAID 感受性を規定する遺伝子多型 22 種同定した。

薬剤性間質性肺炎研究が遅れていたのは、その動物モデルが確立されていなかったためである。我々は、TNF-a（薬剤性間質性肺炎において重要な役割を果たしている）、及び低用量ブレオマイシン（高用量ブレオマイシン単独で、間質性肺炎症状が現れる）をあらかじめ投与したマウスに、レフルノミドやエタネルセプトを投与すると、間質性肺炎症状が現れることを見出し、薬剤性間質性肺炎モデルを確立したと考えている。このモデルにおいて、PC-SOD（SOD を修飾し安定性を高めた製剤で、現在、間質性肺炎治療薬としての臨床試験中）、及び HO-1 の誘導剤により、この間質性肺炎様症状が改善することを見出した。以上の結果は、これらの医薬品が抗炎症タンパク質を低下させることにより、間質性肺炎を引き起こしている可能性を示している。

D.考察

結果の欄に記載した

E.結論

以上の結果から、これら薬剤（ゲフィチニブ、レフルノミド、エタネルセプト、インフリキシマブ）による SOD、HO-1、Nrf2、HSP70 の発現抑制が、間質性肺炎

副作用に寄与していること、及び我々が構築したモデルが、薬剤性間質性肺炎のモデルとして使用出来ることを示唆している。

F.健康危険情報

該当なし

G.研究発表

1. 論文発表

1. Mima, S., Ushijima, H., Hwang, H-J., Tsutsumi, S., Makise, M., Yamaguchi, Y., Tsuchiya, T., Mizushima, H. and Mizushima, T. Identification of the *TPO1* gene in yeast, and its human orthologue TETRAN, which cause resistance to NSAIDs. *FEBS Lett.* 581,1457-1463. (2007)
2. Ishihara, T., Hoshino, T. Namba, T., Tanaka, K. and Mizushima, T. Involvement of up-regulation of PUMA in non-steroidal anti-inflammatory drug-induced apoptosis. *Biochem. Biophys. Res. Commun.* 356,711-717. (2007)
3. Katsu, T., Imamura, T., Komagoe, K., Masuda, K. and Mizushima, T. Simultaneous measurements of K+ and calcein release from liposomes and the determination of pore size formed in the membrane. *Anal. Sci.* 23,517-522. (2007)
4. Tanaka, K. Namba, T., Arai, Y., Fujimoto M., Adachi, H., Sobue, G., Takeuchi, K., Nakai, A. and Mizushima, T. Genetic evidence for a protective role for heat shock factor 1 and heat shock protein 70 against colitis. *J. Biol. Chem.* 282,23240-23252. (2007)
5. Hoshino, T., Nakaya, T., Homan, T.,Tanaka, K., Sugimoto, Y., Araki, W., Narita, M., Narumiya, S., Suzuki, T. and Mizushima, T. Involvement of prostaglandin E2 in production of amyloid-b peptides both in vitro and in vivo. *J. Biol. Chem.* 282,32676-32688. (2007)

2. 学会発表（招待講演のみ）

1. Tohru Mizushima Direct genetic evidence for a protective role of heat shock proteins against irritant-induced gastric lesion.2.Tohru Mizushima Molecular mechanism for chemopreventive effect of non-steroidal anti-inflammatory drugs. Meeting of the pacific basin group on gastroduedental disorders. Digestive Disease Week (2007) (Washington)
2. Tohru Mizushima Molecular mechanism for chemopreventive effect of non-steroidal anti-

inflammatory drugs. 3rd International Congress on Gastrointestinal Oncology. 3rd INTERNATIONAL CONGRESS ON GASTROINTESTINAL ONCOLOGY (2007) (Greece)

3. 水島徹　胃潰瘍、炎症性腸疾患に対するHSPの保護効果　第29回日本フリーラジカル学会学術集会ランチョンセミナー（2007）（名古屋）
4. 水島徹　分子標的創薬とTranslational研究　第28回日本炎症・再生医学会（2007）（東京）
5. 水島徹　胃潰瘍、炎症星腸疾患に対するHSPの保護効果　第35回日本潰瘍学会イブニングセミナー（2007）（鳥取）
6. 水島徹　熱ショック蛋白質の炎症性腸疾患における役割　第26回サイトプロテクション研究会（2007）（京都）

H.知的財産権の出願・登録状況

1.特許取得

該当なし

2.実用新案登録

該当なし

3.その他

該当なし

研究成果に刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Mima, S., Ushijima, H., Hwang, H-J., Tsutsumi, S., Makise, M., Yamaguchi, Y., Tsuchiya, T., Mizushima, H. and Mizushima, T.	Identification of the *TPO1* gene in yeast, and its human orthologue TETRAN, which cause resistance to NSAIDs.	*FEBS Lett.*	581	1457-1463	2007
Ishihara, T., Hoshino, T. Namba, T., Tanaka, K. and Mizushima, T.	Involvement of up-regulation of PUMA in non-steroidal anti-inflammatory drug-induced apoptosis.	*Biochem. Biophys. Res. Commun.*	356	711-717	2007
Katsu, T., Imamura, T., Komagoe, K., Masuda, K. and Mizushima, T.	Simultaneous measurements of K+ and calcein release from liposomes and the determination of pore size formed in the membrane.	*Anal. Sci.*	23	517-522	2007
Tanaka, K. Namba, T., Arai, Y., Fujimoto M., Adachi, H., Sobue, G., Takeuchi, K., Nakai, A. and Mizushima, T.	Genetic evidence for a protective role for heat shock factor 1 and heat shock protein 70 against colitis.	*J. Biol. Chem.*	282	23240-23252	2007
Hoshino, T., Nakaya, T., Homan, T.,Tanaka, K., Sugimoto, Y., Araki, W., Narita, M., Narumiya, S., Suzuki, T. and Mizushima, T.	Involvement of prostaglandin E2 in production of amyloid-b peptides both in vitro and in vivo.	*J. Biol. Chem.*	282	32676-32688	2007

FEBS Letters 581 (2007) 1457–1463

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

Shinji Mima[a,1], Hironori Ushijima[a,1], Hyun-Jung Hwang[b,1], Shinji Tsutsumi[a], Masaki Makise[a], Yoshihiro Yamaguchi[b], Tomofusa Tsuchiya[b], Hiroshi Mizushima[c], Tohru Mizushima[a,*]

[a] *Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan*
[b] *Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan*
[c] *Center for Medical Genomics, National Cancer Center Research, Tokyo 104-0045, Japan*

Received 19 February 2007; accepted 1 March 2007

Available online 8 March 2007

Edited by Horst Feldmann

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%

*Corresponding author. Fax: +81 96 371 4323.
E-mail address: mizu@gpo.kumamoto-u.ac.jp (T. Mizushima).

[1]These three authors equally contribute to this work.

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

0014-5793/$32.00
doi:10.1016/j.febslet.2007.03.001

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 $P < 0.05$.

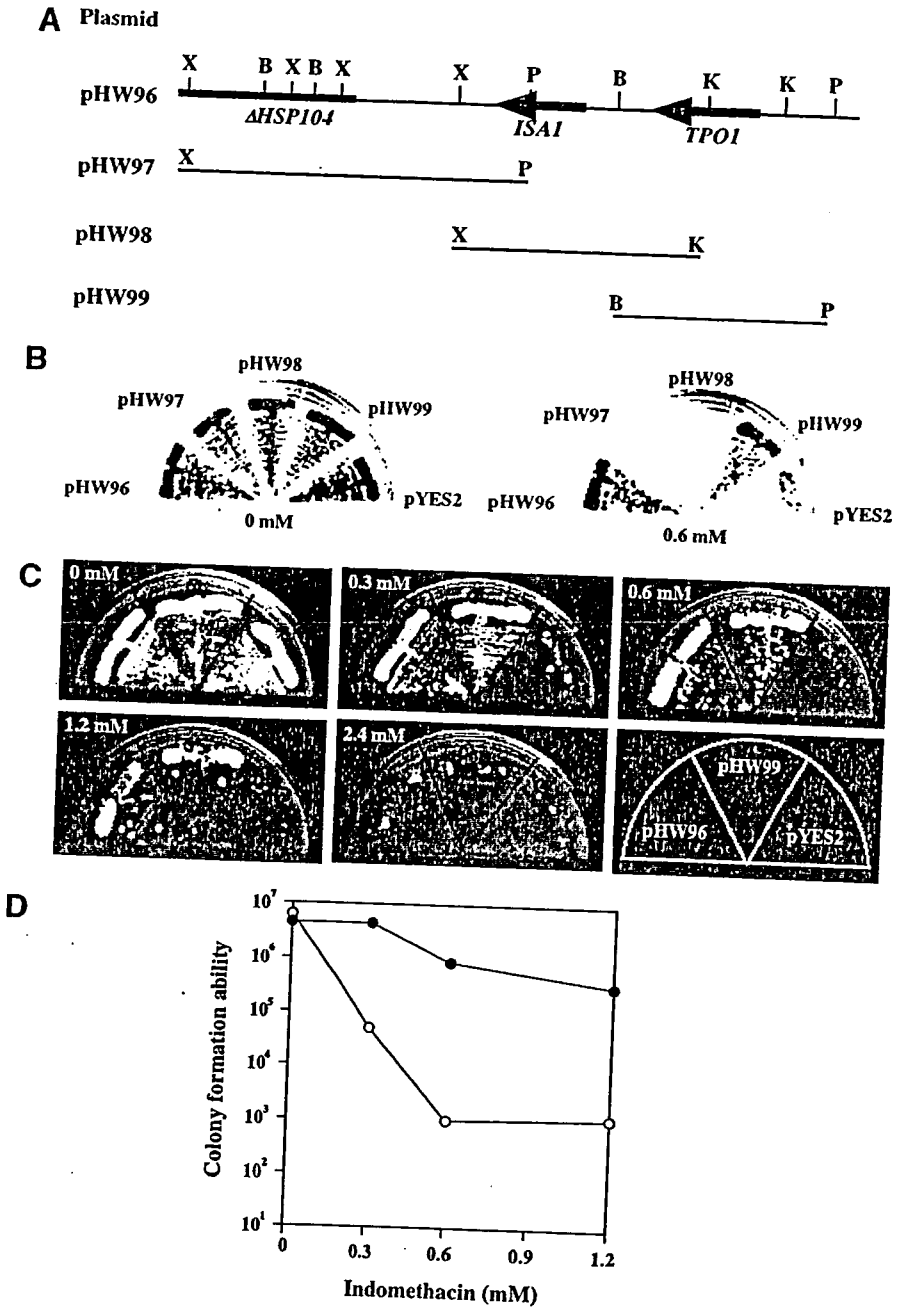

Fig. 1. Identification in yeast of the *TPO1* gene, which caused resistance to indomethacin. (A) Restriction enzyme maps for pHW96 and its derivatives (X, *Xba*I; B, *Bal*I; P, *Pvu*II; K, *Kpn*I). The *GAL* promoter of pYES2 is located on the right side of each insert. (B) W303-1 cells harboring each 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,

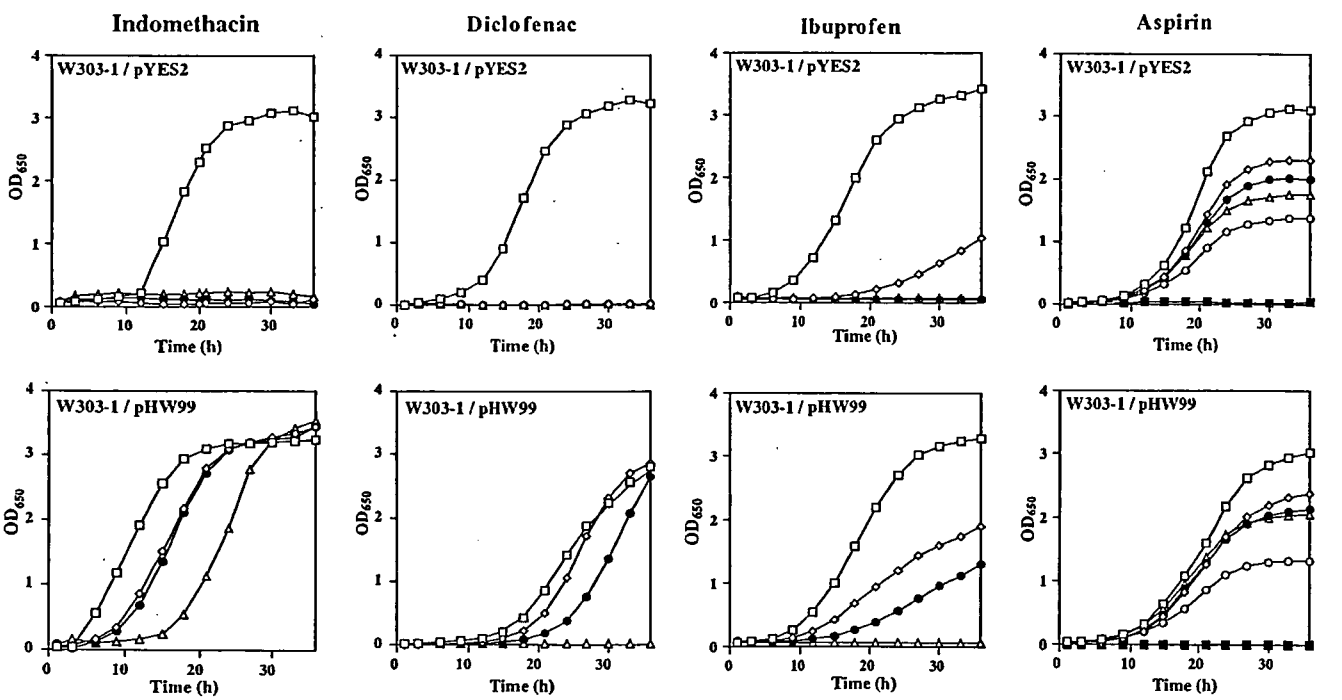

Fig. 2. Growth of yeast cells overexpressing *TPO1* in the presence of NSAIDs. Full growth suspensions of W303-1/pHW99 or W303-1/pYES2 were 1/50 diluted and cultured at 30 °C in the presence of 0 (open square), 0.3 (open diamond), 0.6 (closed circle), and 1.2 (open triangle) mM indomethacin; 0 (open 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.

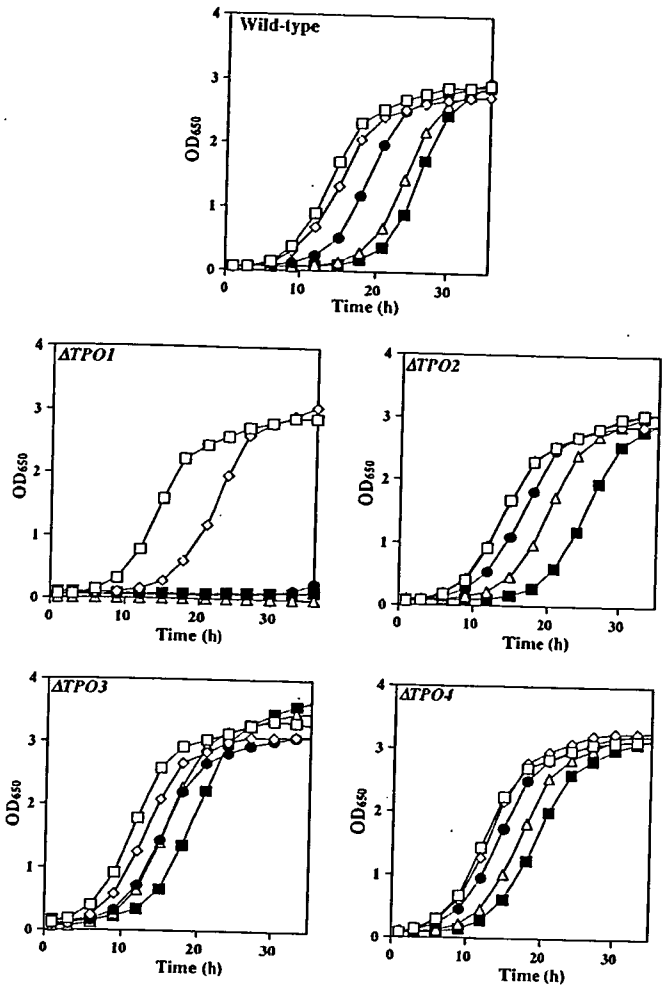

Fig. 3. Growth of yeast *tpo* mutants in the presence of indomethacin. Full growth suspensions of Δ*TPO1*, Δ*TPO2*, Δ*TPO3*, Δ*TPO4* and the wild-type strain, YPH499, were 1/50 diluted and cultured at 30 °C in the presence of 0 (open square), 0.05 (open diamond), 0.1 (closed circle), 0.15 (open triangle)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

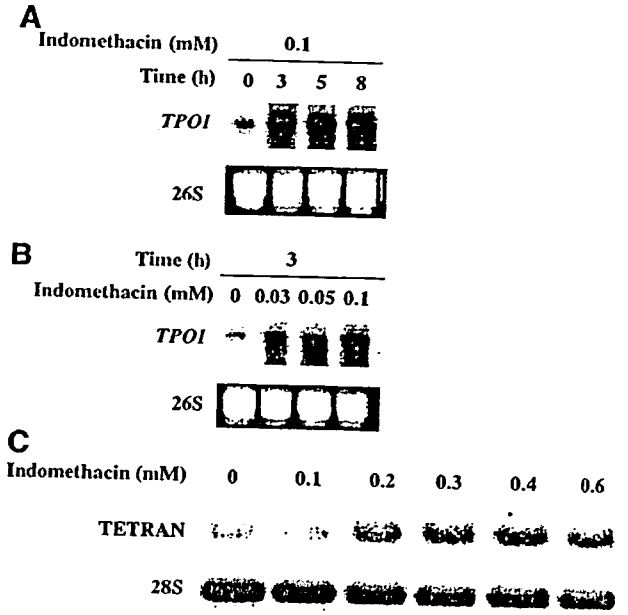

Fig. 4. Induction of *TPO1* mRNA by indomethacin. Exponentially growing W303-1 cells were (A) treated with 0.1 mM indomethacin for the indicated periods or (B) with the indicated concentrations of indomethacin for 3 h. MKN45 ells were exposed to various concentrations of indomethacin for 24 h (C). The 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
Tpol        1 MSDHSPISNKENHLLPSDSSRSSSSDMHSTGTTGTTGVEPVDFTGEGAKYTTATEGNGGA 60
Homology

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

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

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

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

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

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

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

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

TETRAN    407 ARAAG--PLVAASVYWLAGAQACFTTWSGLFLLPFFLLQKLSYPAQTLKAE-          455
Drtet     437 ARALG--PVVGCIAFWCVGSRITYIAGGLLLIYPAMALQRARI---------          477
Tpol      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

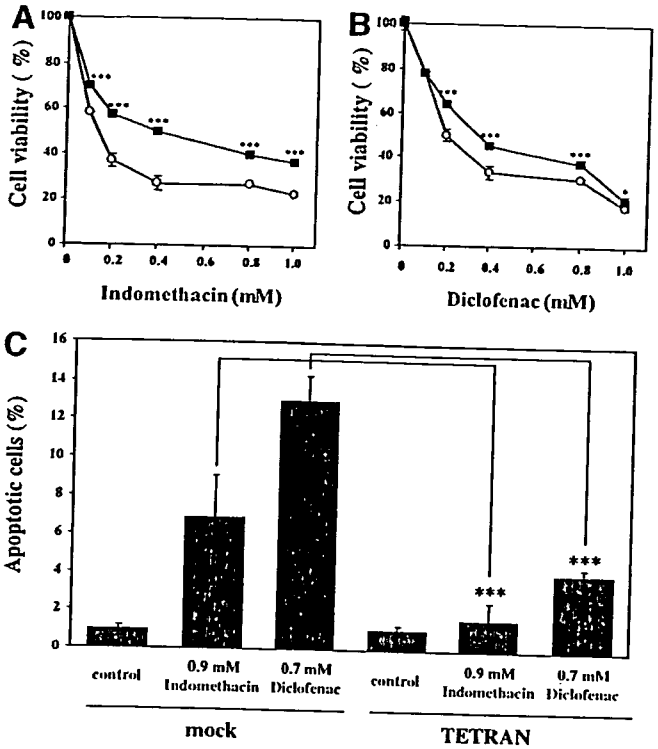

Fig. 6. Sensitivity to NSAIDs of human cells overexpressing TETRAN. A stable transfectant of gastric carcinoma line MKN45 with the expression vector for TETRAN (closed square) and with the vector only (open circle) were exposed for 38 h to the indicated concentrations of indomethacin or diclofenac. Cell 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.

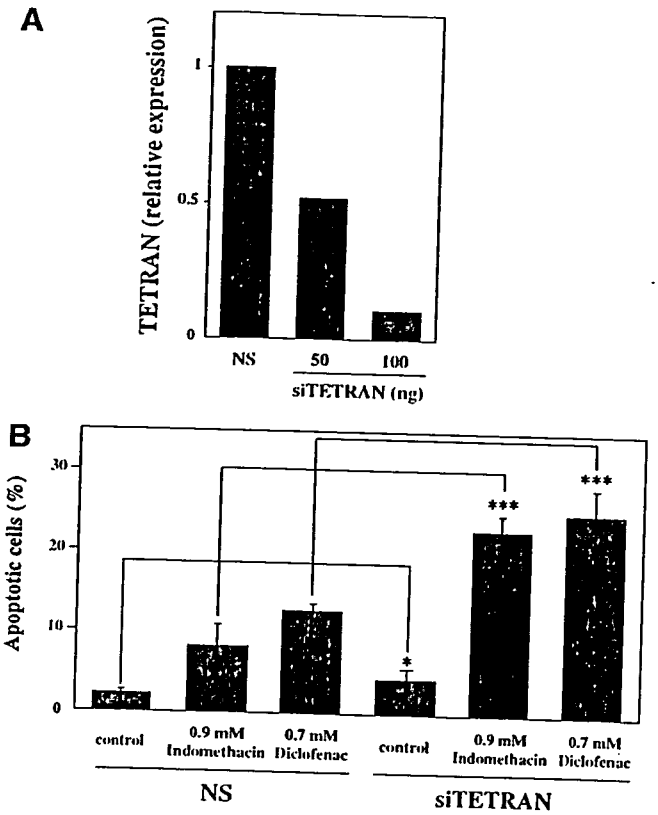

Fig. 7. Effect of siRNA for TETRAN on NSAIDs-induced apoptosis. MKN45 cells were transfected with indicated amounts (A) or 100 ng (B) of siRNA for TETRAN (siTETRAN) or non-silencing siRNA (NS) and cultured for 24 h. Cells were further incubated with or without indicated concentration of indomethacin 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.

Acknowledgments: We thank Drs. K. Igarashi and K. Kashiwagi (Chiba University) for giving us the *tpo* mutants and for helpful discussion.

References

[1] Smalley, W.E., Ray, W.A., Daugherty, J.R. and Griffin, M.R. (1995) Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. Am. J. Epidemiol. 141, 539–545.

[2] Hawkey, C.J. (2000) Nonsteroidal anti-inflammatory drug gastropathy. Gastroenterology 119, 521–535.

[3] Vane, J.R. and Botting, R.M. (1996) Mechanism of action of anti-inflammatory drugs. Scand. J. Rheumatol. Suppl. 102, 9–21.

[4] Lichtenberger, L.M. (2001) Where is the evidence that cyclooxygenase inhibition is the primary cause of nonsteroidal anti-inflammatory drug (NSAID)-induced gastrointestinal injury? Topical injury revisited. Biochem. Pharmacol. 61, 631–637.

[5] Tomisato, W., Tsutsumi, S., Hoshino, T., Hwang, H.J., Mio, M., Tsuchiya, T. and Mizushima, T. (2004) Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. Biochem. Pharmacol. 67, 575–585.

[6] Duyao, M.P. et al. (1993) A gene from chromosome 4p16.3 with similarity to a superfamily of transporter proteins. Hum. Mol. Genet. 2, 673–676.

[7] Tomitori, H., Kashiwagi, K., Asakawa, T., Kakinuma, Y., Michael, A.J. and Igarashi, K. (2001) Multiple polyamine transport systems on the vacuolar membrane in yeast. Biochem. J. 353, 681–688.

[8] Mima, S. et al. (2005) Induction of claudin-4 by nonsteroidal anti-inflammatory drugs and its contribution to their chemopreventive effect. Cancer Res. 65, 1868–1876.

[9] Teixeira, M.C. and Sa-Correia, I. (2002) *Saccharomyces cerevisiae* resistance to chlorinated phenoxyacetic acid herbicides involves Pdr1p-mediated transcriptional activation of TPO1 and PDR5 genes. Biochem. Biophys. Res. Commun. 292, 530–537.

[10] Tomisato, W., Tsutsumi, S., Tsuchiya, T. and Mizushima, T. (2001) Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced necrosis by induction of heat-shock proteins. Biol. Pharm. Bull. 24, 887–891.

[11] Levy, S.B. (1992) Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. 36, 695–703.

[12] Tomisato, W., Tsutsumi, S., Rokutan, K., Tsuchiya, T. and Mizushima, T. (2001) NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. Am. J. Physiol. Gastrointest Liver Physiol. 281, G1092–G1100.

[13] Tomitori, H., Kashiwagi, K., Sakata, K., Kakinuma, Y. and Igarashi, K. (1999) Identification of a gene for a polyamine transport protein in yeast. J. Biol. Chem. 274, 3265–3267.

[14] Albertsen, M., Bellahn, I., Kramer, R. and Waffenschmidt, S. (2003) Localization and function of the yeast multidrug transporter Tpo1p. J. Biol. Chem. 278, 12820–12825.

[15] do Valle Matta, M.A., Jonniaux, J.L., Balzi, E., Goffeau, A. and van den Hazel, B. (2001) Novel target genes of the yeast regulator Pdr1p: a contribution of the TPO1 gene in resistance to quinidine and other drugs. Gene 272, 111–119.

[16] Desmoucelles, C., Pinson, B., Saint-Marc, C. and Daignan-Fornier, B. (2002) Screening the yeast "disruptome" for mutants affecting resistance to the immunosuppressive drug, mycophenolic acid. J. Biol. Chem. 277, 27036–27044.

[17] Delling, U., Raymond, M. and Schurr, E. (1998) Identification of *Saccharomyces cerevisiae* genes conferring resistance to quinoline ring-containing antimalarial drugs. Antimicrob. Agents Chemother. 42, 1034–1041.

[18] Van Bambeke, F., Balzi, E. and Tulkens, P.M. (2000) Antibiotic efflux pumps. Biochem. Pharmacol. 60, 457–470.

[19] Paulsen, I.T., Sliwinski, M.K. and Saier Jr., M.H. (1998) Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. J. Mol. Biol. 277, 573–592.

[20] Sa-Correia, I. and Tenreiro, S. (2002) The multidrug resistance transporters of the major facilitator superfamily, 6 years after disclosure of *Saccharomyces cerevisiae* genome sequence. J. Biotechnol. 98, 215–226.

Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Biochemical and Biophysical Research Communications 356 (2007) 711–717
BBRC
www.elsevier.com/locate/ybbrc

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

Tomoaki Ishihara, Tatsuya Hoshino, Takushi Namba, Ken-ichiro Tanaka, Tohru Mizushima *

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan

Received 5 March 2007
Available online 13 March 2007

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.

* Corresponding author. Fax: +81 96 371 4323.
E-mail address: mizu@gpo.kumamoto-u.ac.jp (T. Mizushima).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.03.034

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

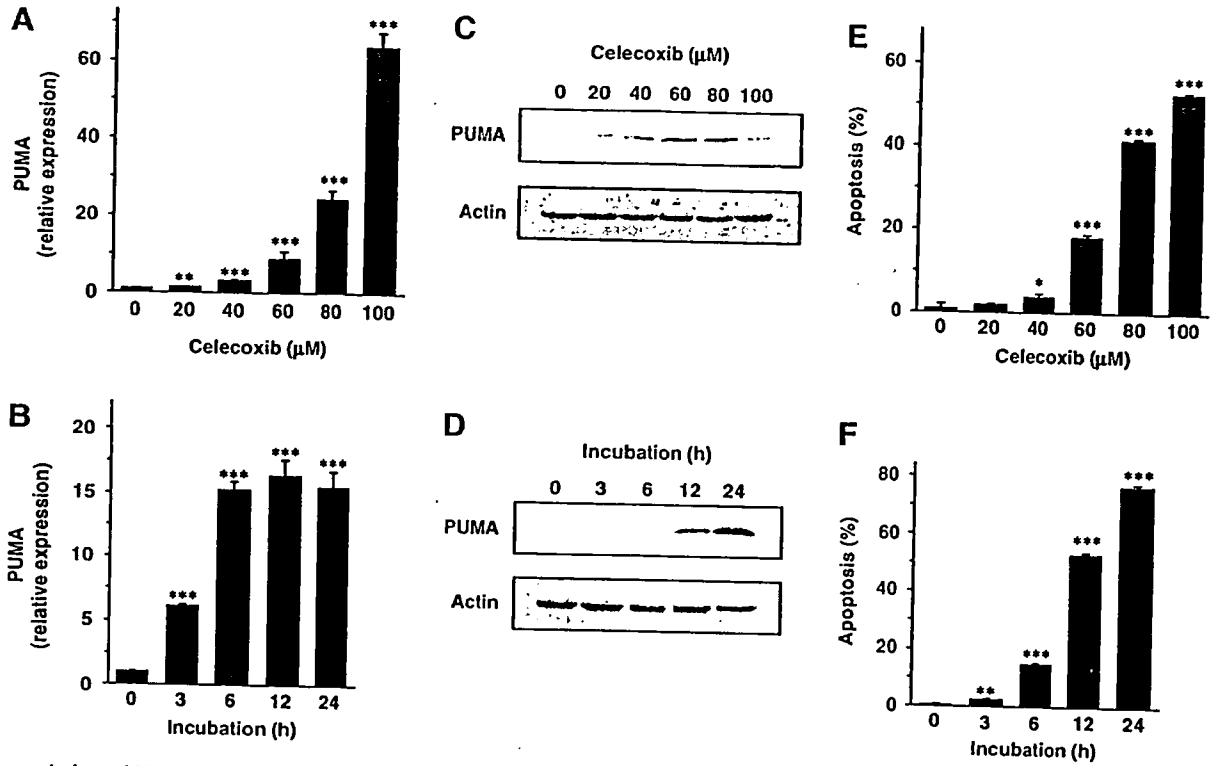

Fig. 1. Up-regulation of PUMA by NSAIDs. AGS cells were incubated with either the indicated concentrations (A, C, and E) or 80 μM of celecoxib (B, D, and F) for 12 h (A, C, and E) or the time periods indicated (B, D, and F). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer 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 ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

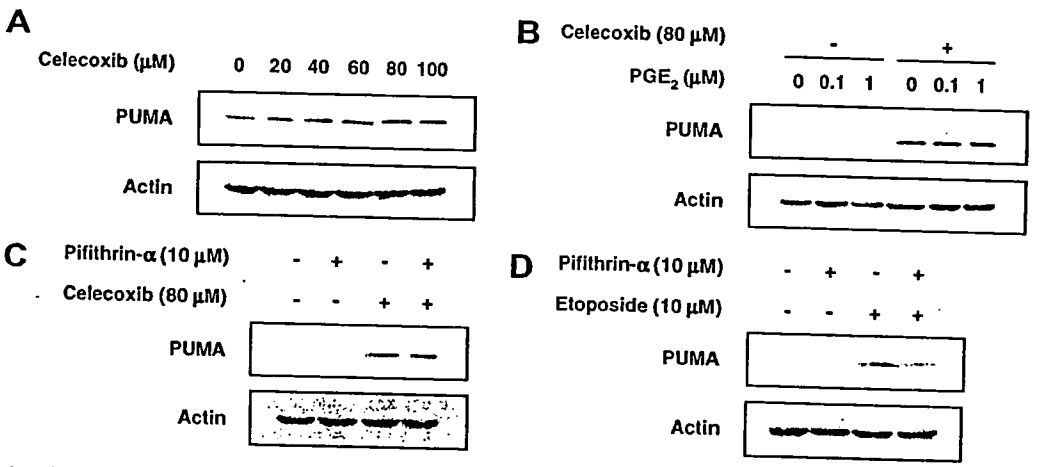

Fig. 2. Celecoxib-induced up-regulation of PUMA is independent of COX and p53. Kato III cells were incubated with the indicated concentrations of celecoxib for 24 h (A). AGS cells were incubated with or without 80 μM celecoxib (B,C) or 10 μM etoposide (D) in the presence or absence of indicated concentrations 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

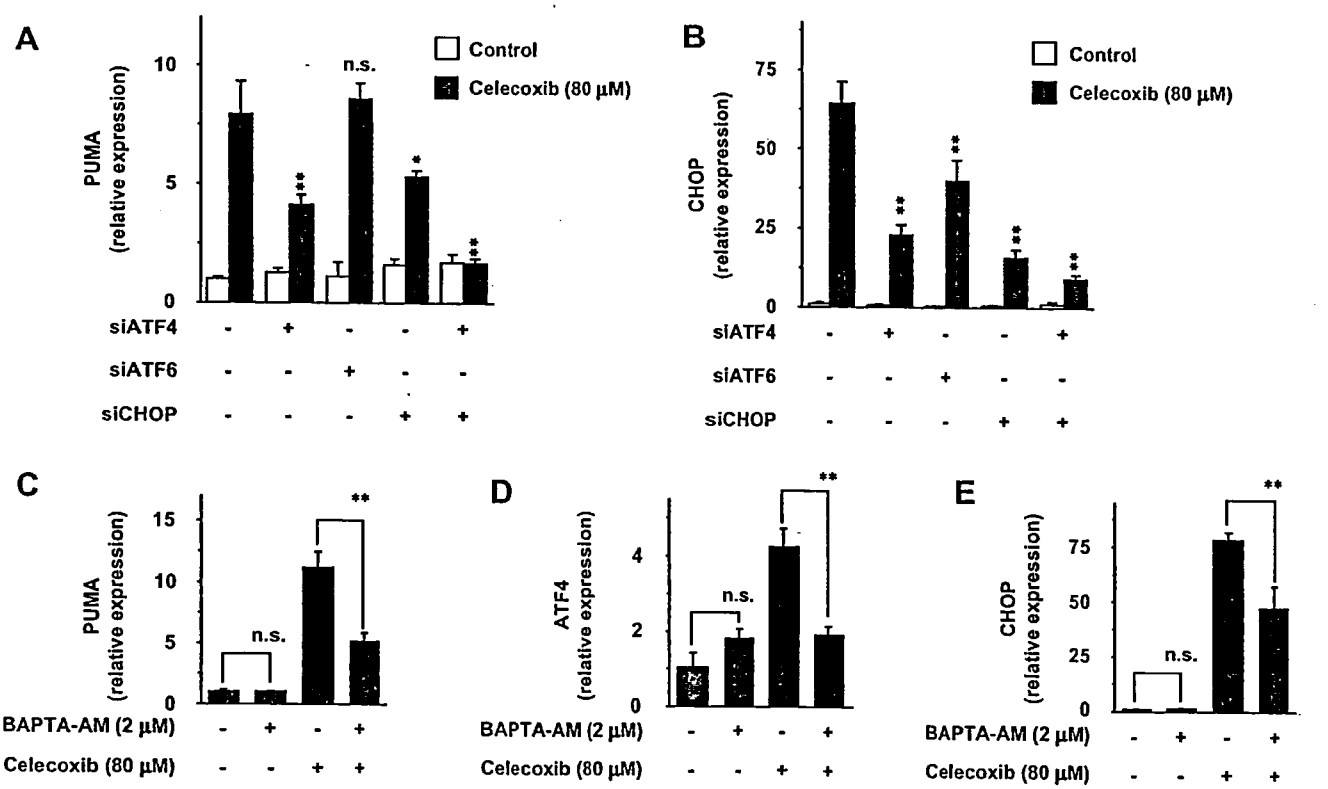

Fig. 3. Contribution of the increase in the intracellular Ca^{2+} level and up-regulation of ATF4 and CHOP to celecoxib-induced up-regulation of PUMA. AGS cells were transfected with siRNA for ATF4 (siATF4), ATF6 (siATF6), CHOP (siCHOP), or non-silencing siRNA. After 48 h cells were incubated with or 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.