厚生労働科学研究費補助金

萌芽的先端医療技術推進研究事業

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18年度　総括研究報告書

主任研究者　水島　徹

平成19（2007）年3月

目次

厚生労働科学研究費補助金（萌芽的先端医療技術推進研究事業）
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　熊本大学大学大学院医学薬学研究部教授

研究要旨

様々な方法で新たに80ほどの新規ヒトストレス遺伝子を発見し、それを既に開発していたヒトストレス遺伝子チップに加え、改良型ヒトストレス遺伝子チップを開発した。

これまでの研究（NSAIDsにより誘導される遺伝子をストレス遺伝子チップで解析した結果）を基に我々は、膜傷害性のないNSAIDsは胃潰瘍副作用のないNSAIDsになることを発見している。本年度我々は、複数の膜傷害性のないNSAIDsを発見しそれらが胃潰瘍を起こさないこと、及び既存薬と変わらない抗炎症作用を示すことを見いだした。以上の結果はこのNSAIDsが有用な医薬品になること、すなわちストレス遺伝子チップを用いた解析（トキシコゲノミックス）が副作用のない医薬品の開発にも有用であることを示している。

我々はトキシコゲノミックスから得られた情報を基に、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し副作用感受性の予測システムを確立できると考え、NSAIDs潰瘍を例としてその証明を行いたいと考えている。本年度我々は、NSAIDsにより誘導される遺伝子の解析、微生物をNSAIDs耐性化する遺伝子の解析から、NSAIDs潰瘍感受性の個人差を規定している遺伝子の候補を十数個選定した。またSNPのデータベースからいくつかの遺伝子に関してはそのSNPにより蛋白質の活性が変化すること（即ち、NSAIDs 潰瘍感受性が変化する可能性）を明らかにした。

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（あるいは視覚的に判断できる）毒性しか分からないという問題もある。そこで新しい毒性試験法の確立が求められている。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。本研究提案の目標の一つは、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既存の医薬品（消化管・肝・腎毒性が臨床で問題になっている抗菌薬、抗ウィルス薬、免疫抑制薬など）の細胞傷害機構を調べることによりその副作用メカニズムを解明し、副作用のない新しい医薬品の開発戦略を確立する研究も行う。我々はこの方法で、胃潰瘍を起こさない安全なNSAIDsの開発法を確立した。そこで本研究で、この開発戦略に従い新しいNSAIDsを合成し、実際にそのNSAIDsに胃潰瘍副作用がないことを示し、トキシコゲノミックスが副作用のない医薬品の開発に貢献することを実証する。同時にこの研究は、NSAIDs潰瘍に苦しんでいる多くの患者さんを救う、及び医療費の削減にもつながる（米国では年間16500人がNSAIDs潰瘍で亡くなっており、これはエイズ死者数よりも多い、また胃潰瘍副作用のため臨床現場では、NSAIDsと同時に胃薬が処方されている）。

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。細胞はストレスに対し、適切な遺伝子を発現することによって、自らをストレス耐性化する。そこで我々は、まず比較的短時間で遺伝子解析が出来る微生物を用いて、特定の医薬品に対して細胞を耐性化する遺伝子を検索し、次にその遺伝子のヒトホモログを取り、その遺伝子多型と副作用感受性の個人差との相関性を調べる。

B. 研究方法

新規ストレス遺伝子の検索

ストレスとしては、NSAIDs、アルコール、活性酸素を使用した。細胞に各ス

トレスを与えた時に誘導される遺伝子を、既存のDNAチップ（ゲノム情報からランダムに遺伝子をチップ化したもの）を使って検索した。また我々が既に作成しているストレス遺伝子チップも用いた。一方未知の遺伝子の発見を目指して、ディファレンシャルディスプレイ法でも検索を行った。同定された遺伝子に関しては、RT-PCR法で確認するともに、DNAチップに用いるための配列をコンピューターを使って検索した。

副作用のないNSAIDsの発見

我々が見いだしたNSAIDsの膜傷害性に関する構造活性相関を基に、新たに30種のNSAIDsを合成した。また大正製薬、及び三共から1000種以上のNSAIDsを入手した。まずこれらの膜傷害性を我々が特許化している方法で調べ（一次スクリーニング）、次に細胞傷害性をモルモット胃粘膜初代培養細胞で調べた（二次スクリーニング）。さらにCOX阻害活性を確認しCOX-2選択性を持たないものを選択した後（三次スクリーニング）、動物実験で胃潰瘍副作用と抗炎症作用を調べた。

C.研究結果

NSAIDs により誘導される遺伝子

junction plakoglobin

hypothetical protein similar to mouse Fbw5

KIAA0013 gene product

small optic lobes (Drosophila) homolog

glucosidase, beta; acid (includes glucosylceramidase)

zinc finger protein homologous to Zfp103 in mouse

solute carrier family 1 (neutral amino acid transporter), member 5

interferon induced transmembrane protein 1

upstream transcription factor 1

protease, serine, 8 (prostasin)

seven transmembrane domain protein

PHD finger protein 3

fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, Bombay

phenotype included)

PTD008 protein

phosphoenolpyruvate carboxykinase 2 (mitochondrial)

Sequence 5 from Patent WO9954461.

KIAA0842 protein

BCL2/adenovirus E1B 19kD-interacting protein 1

vascular endothelial growth factor

stratifin

RAD9 (S. pombe) homolog

fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, Bombay

phenotype included)

copine I

myo-inositol 1-phosphate synthase A1

S100 calcium-binding protein P

four and a half LIM domains 3

KIAA0339 gene product
poly(A)-binding protein, nuclear 1
ubiquitin-conjugating enzyme E2M (homologous to yeast UBC12)
ferritin, light polypeptide
nuclear receptor subfamily 1, group H, member 2
Sequence 1 from Patent WO9966039.
inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
poly(rC)-binding protein 4
RAP1, GTPase activating protein 1
seven transmembrane domain protein
lymphocyte adaptor protein
Incyte EST

エタノールにより誘導される遺伝子

Rhesus monkey p53 mRNA, complete cds.
major histocompatibility complex, class II, DQ alpha 1
actinin, alpha 4
ATPase, Ca++ transporting, plasma membrane 2
Human bone sialoprotein (BNSP) gene, exons 6 and 7.
ribosomal protein S6 kinase, 70kD, polypeptide 2
KIAA1484 protein
G protein-coupled receptor kinase-interactor 1
ryanodine receptor 3
hypothetical protein FLJ20277
glycoprotein, synaptic 2
procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3
heme oxygenase (decycling) 1
activating transcription factor 3
ribosomal protein, large, P1
phosphoinositide-3-kinase, catalytic, gamma polypeptide
translocase of inner mitochondrial membrane 17 (yeast) homolog B
Rab geranylgeranyltransferase, alpha subunit
interleukin 1 receptor-like 2
glutathione S-transferase M2 (muscle)
cysteinyl-tRNA synthetase
ring finger protein 15
ATPase, Class II, type 9A
dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)
CG10153 gene product
STIP1 homology and U-Box containing protein 1
hypothetical protein FLJ12628

活性酸素により誘導される遺伝子

endothelin converting enzyme 1
microsomal triglyceride transfer protein (large polypeptide, 88kD)
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 1
hypothetical protein
glutathione synthetase
Homo sapiens cDNA: FLJ23529 fis, clone

LNG06042
Fc fragment of IgG, receptor, transporter, alpha
protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1
KIAA0664 protein
cytokine-inducible kinase
cysteine-rich protein 2
Homo sapiens mRNA for FLJ00067 protein, partial cds
smooth muscle myosin light chain kinase; smMLCK
linker for activation of T cells
cholinergic receptor, nicotinic, beta polypeptide 3

これらの新規ストレス遺伝子を加えた、改良型ストレス遺伝子チップを開発し、それが DNA チップとして機能することを確認した。

副作用のないNSAIDsの発見

自ら合成した NSAIDs、及び協力企業から得た NSAIDs を出発材料とし、スクリーニングを行った。まずこれらの膜傷害性を我々が特許化している方法で調べ（一次スクリーニング）、対照医薬品であるイブプロフェンより膜傷害性の少ない 60 種を選択した。次に細胞傷害性をモルモット胃粘膜初代培養細胞で調べた（二次スクリーニング）。その結果、選択した 60 種のほとんどが、イブプロフェンより弱い細胞傷害性を示した。この結果は、NSAIDs の細胞傷害性の原因がその膜傷害性にあるという我々の考えを支持している。さらに COX 阻害活性を確認し COX-2 選択性を持たないものを選択した後（８種）、それらの NSAIDs に関して、動物実験で胃潰瘍副作用を調べた。その結果、全ての NSAIDs はイブプロフェンより弱い胃潰瘍副作用を示した。この結果は、NSAIDs 潰瘍の原因が、その細胞傷害性（膜傷害性）にあるという我々の考えを支持している。さらに特に胃潰瘍副作用の少なかった４種に関して抗炎症作用を調べところ、イブプロフェンより弱い抗炎症作用を示すものが２種、同程度の抗炎症作用を示すものが２種存在した。この結果は、NSAIDs の細胞傷害性（膜傷害性）は、NSAIDs の抗炎症作用には関係がないという我々の考えを支持している。以上のスクリーニングにより、胃潰瘍副作用の少ない NSAIDs を発見できた。

NSAIDs潰瘍感受性の個人差を規定している遺伝子多型を同定

我々はトキシコゲノミックスから得られた情報を基に、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し副作用感受性の予測システムを確立できると考え、NSAIDs潰瘍を例としてその証明を行いたいと考えている。本年度我々は、NSAIDsにより誘導される遺伝子の解析

からS100p, clausin-1, clausin-4, clausin-12、HO-1,GRP78, ORP150, COX-2, GRP94, CHOP を、微生物をNSAIDs耐性化する遺伝子の解析からHSP72, HSP90, HSP104, HSP60, HSF1, TETRANを、 NSAIDs潰瘍感受性の個人差を規定している遺伝子の候補として選定した。次にSNPのデータベースからこれらの遺伝子のSNPを検索した。その中で我々はTETRANとHSP72に注目した。

TETRANの主なSNP（TETRAN-SNP-1、TETRAN-SNP-2）に注目し、その遺伝子を培養細胞で発現し、TETRAN活性（NSAIDs排出活性）を測定した。TETRAN-SNP-1では野生型と変わらないNSAIDs排出活性を示したのに対し、TETRAN-SNP-2では野生型の30％程度の排出活性しか示さなかった。一方、HSP72の主なSNP（HSP72-SNP-1、HSP72-SNP-2、HSP72-SNP-3）に注目し、その遺伝子を培養細胞で発現し、HSP72活性（NSAIDs耐性化活性）を測定したところ、SNP-3では野生型と変わらないNSAIDs排出活性を示したのに対し、SNP-1, 2では野生型より弱い活性を示した。

D.考察

本研究で開発した改良型ストレス遺伝子チップは、トキシコゲノミックスの研究に有用であると考えられる。実際我々はこの DNA チップを用いて NSAIDs で誘導されるストレス遺伝子の解析を行い、NSAIDs 潰瘍感受性の個人差を規定している遺伝子多型の候補遺伝子の同定に成功した。

これまでの我々の研究から、COX-2 に対する選択性がなく、かつ膜傷害性のない NSAIDs は、胃潰瘍誘発副作用、及び心筋梗塞誘発副作用のない真に安全な NSAIDs になることが示唆されていた。今年度我々はこのアイデアに従い、実際に COX-2 に対する選択性がなく、かつ膜傷害性のない NSAIDs のスクリーニングを行い、そのような NSAIDs が胃潰瘍誘発副作用、及び心筋梗塞誘発副作用のない真に安全な NSAIDs であることを示した。この結果は我々のアイデアが正しいことを示すだけでなく、新しい医薬品開発への道を開いたという点でも評価できる。

臨床現場で問題になっているのが、NSAIDs 感受性に関する個人差である。即ち、同じ量の NSAIDs を投与しても、胃潰瘍を発症する患者としない患者がおり、NSAIDs 感受性に関する個人差を予測することが出来れば、画期的である。今回我々は、NSAIDs 感受性に関与する遺伝子の SNP 解析を行い、複数の興味深い SNP を発見した。その中には、細胞の NSAIDs 感受性を変化させるものもあり、NSAIDs 感受性に関する個人差を予測する方法論の確立に貢献すると思われる。

E.結論

本研究により、トキシコゲノミックスの有用性が示された。

F.健康危険情報

該当なし

G.研究発表

1. 論文発表

1. Tsutsumi, S., Namba, T., Tanaka, K., Arai, Y., Ishihara, T., Aburaya, M., Mima, S., Hoshino, T., and Mizushima, T. Celecoxib up-regulate endoplasmic reticulum chaperones that inhibit celecoxib-induced apoptosis in human gastric cells. *Oncogene* 25, 1018-1029. (2006)
2. Yamada. Y., Shiota, S., Mizushima, T., Kuroda, T. and Tsuchiya T. Functional gene cloning and characterization of MdeA, a multidrug efflux pump from *Staphylococcus aureus*. *Biol Pharm Bull.* 29, 801-804. (2006)
3. Aburaya, M., Tanaka, K., Hoshino, T. Tsutsumi, S., Suzuki, K., Makise M., Akagi, R. and Mizushima, T. Heme oxygenase-1 protects gastric mucosal cells against non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* 281,33422-33432. (2006)
4. Makise, M., Takahashi, N., Matsuda, K., Yamairi, F., Suzuki, K., Tsuchiya, T., and Mizushima, T. Mechanism for the degradation of origin recognition complex containing Orc5p with a defective walker A motif and its suppression by over-production of Orc4p in yeast cells. *Biochem. J.* in press. (2007)
5. Hoshino, T., Nakaya, T., Araki, W., Suzuki, K., Suzuki, T. and Mizushima, T. Endoplasmic reticulum chaperones inhibit the production of amyloid-b peptides. *Biochem. J.* in press. (2007)
6. Namba, T., Hoshino, T. Tanaka, K., Tsutsumi, S., Ishihara, T., Mima S., Suzuki, K., Ogawa, S. and Mizushima, T. Up-regulation of 150-kDa oxygen-regulated protein (ORP150) by celecoxib in human gastric carcinoma cells. *Mol. Pharmacol.* in press. (2007)
7. Tanaka, K., Tsutsumi, S., Arai, Y., Hoshino, T. Suzuki, K., Takai, E., Itoh, T., Takeuchi, K., Nakai, A. and Mizushima, T. Genetic evidence for a protective role of heat shock factor 1 against irritant-induced gastric lesions. *Mol. Pharmacol.* in press. (2007)
8. Mizushima, T. Various stress proteins protect gastric mucosal cells against non-steroidal anti-inflammatory drugs.

Inflammopharmacology in press. (2007)

9. Namba, T., Ishihara, T., Tanaka, K., Hoshino, T. and Mizushima, T. Transcriptional activation of ATF6 by endoplasmic reticulum stressors. *Biochem. Biophys. Res. Commun.* in press. (2007)

学会発表（招待講演のみ）

1. 水島徹　創薬研究センターの紹介、及び副作用のない NSAIDs の開発　熊本県製薬協会年会での特別講演 (2006)（熊本）
2. 水島徹　HSP 誘導による胃粘膜保護　名古屋 GGA 研究会での特別講演 (2006)（名古屋）
3. 水島徹　熊本大学附属創薬研究センターについて　創薬研究センター開所記念式典　(2006)（熊本）
4. Tohru Mizushima HSP and Skin. AISET (Anti-aging International Symposium) (2006) (Tokyo)
5. Tohru Mizushima Heme oxygenase-1 protects gastric mucosal cells against non-steroidal anti-inflammatory drugs. 12th International Conference on Ulcer Research (2006) (Osaka)
6. 水島徹　PLGA ナノ粒子を用いた DDS 戦略　日本 DDS 学会シンポジウム　(2006)（東京）
7. 水島徹　レシチン化 SOD による潰瘍性大腸炎治療　日本炎症・再生学会シンポジウム　(2006)（東京）
8. 水島徹　非ステロイド系抗炎症薬（NSAIDs）の抗癌作用に関する分子機構の解明　癌研セミナー (2006)（東京）
9. 水島徹　基礎研究と創薬研究　鹿児島大学での招待講演　(2006)（鹿児島）
10. Tohru Mizushima Molecular mechanism for chemopreventive effect of non-steroidal anti-inflammatory drugs. The Fifth Sino-Japan Joint Conference for Cancer Research Molecular Targets for Cancer Therapeutics (2006) (Shanghai)
11. 水島徹　薬の作り方、使い方　市民講演会　(2006)（熊本）
12. 水島徹　NSAIDs 潰瘍発症の分子機構と HSP 誘導による胃粘膜保護　臨床ストレス応答学会特別講演 (2006)（京都）
13. 水島徹　NSAIDs 潰瘍発症の分子機構と HSP 誘導による胃粘膜保護　つくば GI リサーチセミナー特別講演　(2006)（つくば）
14. 水島徹　薬学部へ行って薬を作ろう　九州薬科学研究教育連合主催講演会　招待講演　(2006)（福岡）
15. 水島徹　DDS スペシャリスト養成

プログラムについて　教育研究拠点FD セミナー　招待講演　(2006) (熊本)

C. 知的財産権の出願・登録状況

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
Tsutsumi, S., Namba, T., Tanaka, K., Arai, Y., Ishihara, T., Aburaya, M., Mima, S., Hoshino, T., and Mizushima, T.	Celecoxib up-regulate endoplasmic reticulum chaperones that inhibit celecoxib-induced apoptosis in human gastric cells.	*Oncogene*	25	1018-1029	2006
Yamada. Y., Shiota, S., Mizushima, T., Kuroda, T. and Tsuchiya T.	Functional gene cloning and characterization of MdeA, a multidrug efflux pump from *Staphylococcus aureus.*	*Biol Pharm Bull*	29	801-804	2006
Aburaya, M., Tanaka, K., Hoshino, T. Tsutsumi, S., Suzuki, K., Makise M., Akagi, R. and Mizushima, T.	Heme oxygenase-1 protects gastric mucosal cells against non-steroidal anti-inflammatory drugs.	*J. Biol. Chem.*	281	33422-33432	2006
Makise, M., Takahashi, N., Matsuda, K., Yamairi, F., Suzuki, K., Tsuchiya, T., and Mizushima, T.	Mechanism for the degradation of origin recognition complex containing Orc5p with a defective walker A motif and its suppression by over-production of Orc4p in yeast cells.	*Biochem. J.*	in press.		2007
Hoshino, T., Nakaya, T., Araki, W., Suzuki, K., Suzuki, T. and Mizushima, T.	Endoplasmic reticulum chaperones inhibit the production of amyloid-b peptides.	*Biochem. J.*	in press.		2007
Namba, T., Hoshino, T. Tanaka, K., Tsutsumi, S., Ishihara, T., Mima S., Suzuki, K., Ogawa, S. and Mizushima, T.	Up-regulation of 150-kDa oxygen-regulated protein (ORP150) by celecoxib in human gastric carcinoma cells.	*Mol. Pharmacol.*	in press.		2007
Tanaka, K., Tsutsumi, S., Arai, Y., Hoshino, T. Suzuki, K., Takai, E., Itoh, T., Takeuchi, K., Nakai, A. and Mizushima, T.	Genetic evidence for a protective role of heat shock factor 1 against irritant-induced gastric lesions.	*Mol. Pharmacol.*	in press.		2007

Mizushima, T.	Various stress proteins protect gastric mucosal cells against non-steroidal anti-inflammatory drugs.	*Inflammopharmacology*	in press.		2007
Namba, T., Ishihara, T., Tanaka, K., Hoshino, T. and Mizushima, T.	Transcriptional activation of ATF6 by endoplasmic reticulum stressors.	*Biochem. Biophys. Res. Commun.*	in press.		2007

Oncogene (2006) 25, 1018–1029

www.nature.com/onc

ORIGINAL ARTICLE

Celecoxib upregulates endoplasmic reticulum chaperones that inhibit celecoxib-induced apoptosis in human gastric cells

S Tsutsumi, T Namba, K-I Tanaka, Y Arai, T Ishihara, M Aburaya, S Mima, T Hoshino and T Mizushima

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan

Nonsteroidal anti-inflammatory drugs (NSAIDs) induce apoptosis in cancer cells and this effect is involved in their antitumor activity. We recently demonstrated that NSAIDs upregulate GRP78, an endoplasmic reticulum (ER) chaperone, in gastric mucosal cells in primary culture. In the present study, induction of ER chaperones by NSAIDs and the effect of those chaperones on NSAID-induced apoptosis were examined in human gastric carcinoma cells. Celecoxib, an NSAID, upregulated ER chaperones (GRP78 and its cochaperones ERdj3 and ERdj4) but also C/EBP homologous transcription factor (CHOP), a transcription factor involved in apoptosis. Celecoxib also upregulated GRP78 in xenograft tumors, accompanying with the suppression of tumor growth in nude mice. Celecoxib caused phosphorylation of eukaryotic translation initiation factor 2 kinase (PERK) and eukaryotic initiation factor-2α (eIF2α) and production of activating transcription factor (ATF)4 mRNA. Suppression of ATF4 expression by small interfering RNA (siRNA) partially inhibited the celecoxib-dependent upregulation of GRP78. Celecoxib increased the intracellular Ca^{2-} concentration, while 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′*N*′-tetraacetic acid, an intracellular Ca^{2+} chelator, inhibited the upregulation of GRP78 and ATF4. These results suggest that the Ca^{2+}-dependent activation of the PERK-eIF2α-ATF4 pathway is involved in the upregulation of ER chaperones by celecoxib. Overexpression of GRP78 partially suppressed the apoptosis and induction of CHOP in the presence of celecoxib and this suppression was stimulated by coexpression of either ERdj3 or ERdj4. On the other hand, suppression of GRP78 expression by siRNA drastically stimulated cellular apoptosis and production of CHOP in the presence of celecoxib. These results show that upregulation of ER chaperones by celecoxib protects cancer cells from celecoxib-induced apoptosis, thus may decrease the potential antitumor activity of celecoxib.
Oncogene (2006) **25**, 1018–1029. doi:10.1038/sj.onc.1209139; published online 3 October 2005

Correspondence: Professor T Mizushima, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University 5-1, Oe-honmachi, Kumamoto 862-0973, Japan.
E-mail: mizu@gpo.kumamoto-u.ac.jp
Received 7 April 2005; revised 23 August 2005; accepted 23 August 2005; published online 3 October 2005

Keywords: celecoxib; endoplasmic reticulum chaperones; apoptosis; calcium

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used therapeutic agents in the treatment of pain, inflammation and fever (Smalley *et al.*, 1995). In addition, recent epidemiological studies clearly show that prolonged NSAID use is associated with a reduced risk of cancer, while preclinical and clinical studies have shown that some NSAIDs are effective in the treatment and prevention of cancer. This effect is particularly well documented in relation to colon and rectal cancer, with recent studies showing that NSAID use reduces the risk of stomach cancer (Farrow *et al.*, 1998; Husain *et al.*, 2002; Sorensen *et al.*, 2003; Hu *et al.*, 2004). Of the various mechanisms proposed to explain the antitumor action of NSAIDs, such as cell growth suppression, inhibition of angiogenesis and inhibition of metastasis, NSAID-induced apoptosis in cancer cells is thought to play an important role (Gupta and Dubois, 2001; Kismet *et al.*, 2004).

The anti-inflammatory action of NSAIDs is mediated through the NSAID-induced inhibition of cyclooxygenase (COX). COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong propensity for inducing inflammation. PGs, such as PGE_2, inhibit cellular apoptosis and the overexpression of COX-2 (a subtype of COX) has been reported to play a role in the development of various types of tumors (Eberhart *et al.*, 1994; Piazza *et al.*, 1995; Ristimaki *et al.*, 1997; Hoshino *et al.*, 2003). Based on these reports, NSAID-induced apoptosis was believed to be based solely on its inhibitory effects on COX activity. However, several lines of evidence suggest that NSAID-induced apoptosis also involves COX-independent mechanisms. A derivative of the NSAID sulindac (sulindac sulfone), which has no COX-inhibitory activity, induced apoptosis in tumor cells and some NSAIDs have been shown to induce apoptosis in COX-null fibroblasts and in tumor cells in which COX expression was absent (Hanif *et al.*, 1996; Elder *et al.*, 1997; Zhang *et al.*, 1999). Therefore, it is important that the COX-independent mechanisms governing NSAID-induced apoptosis be identified.

NSAID-induced apoptosis in normal gastric mucosal cells seems to be involved in the production of gastric lesions by NSAIDs. We recently suggested that, in addition to COX inhibition by NSAIDs, the direct cytotoxicity of NSAIDs (induction of necrosis and apoptosis) contributes to the production of NSAID-induced gastric lesions (Tomisato *et al*., 2001, 2004b). We examined the mechanism of NSAID-induced apoptosis in guinea pig gastric mucosal cells in primary culture and found that NSAIDs induce apoptosis by acting as endoplasmic reticulum (ER) stressors. Various NSAIDs induce C/EBP homologous transcription factor (CHOP), which is known to be important for the induction of apoptosis by ER stressors. Further to this, we showed, using CHOP-deficient mice or a dominant-negative form of CHOP, that this CHOP induction is essential for NSAID-induced apoptosis (Tsutsumi *et al*., 2004).

In addition to inducing apoptosis, ER stressors cause upregulation of ER chaperones, which protect the ER against ER stressor activity by refolding unfolded proteins in the ER (Lee, 2001). In fact, we reported that various NSAIDs induced the expression of glucose-regulated protein (GRP)-78, a representative ER chaperone, in gastric mucosal cells in primary culture (Tsutsumi *et al*., 2004). However, it is not known if NSAIDs upregulate other ER chaperones such as ERdj3 and Erdj4, which act as cochaperones for GRP78 and activate the ATPase and refolding activity of GRP78 (Yu *et al*., 2000; Shen *et al*., 2002b). Furthermore, it is also not known if NSAIDs induce ER chaperones in other types of cells, such as tumor cells. It was reported that overexpression of GRP78 makes cells resistant to apoptosis induced by anticancer drugs (topoisomerase inhibitors) and ER stressors (tunicamycin and Ca^{2+} ionophores) (Morris *et al*., 1997; Reddy *et al*., 2003). Therefore, it is possible that the induction of GRP78 by NSAIDs contributes to the protection of cells from NSAID-induced apoptosis. In the present study, we have examined perturbations to ER chaperones by NSAIDs and the effect of such chaperones on NSAID-induced apoptosis in human gastric carcinoma cells. Several NSAIDs upregulated not only GRP78 but also ERdj3 and ERdj4. We suggest that this upregulation is mediated by an increase in intracellular Ca^{2+} concentration. Furthermore, the contribution of ER chaperones to the protection of cells from celecoxib (a NSAID)-induced apoptosis was supported by experiments using overexpression plasmid and small interfering RNA (siRNA) for GRP78.

Results

NSAIDs upregulate ER chaperones

In a previous report, we showed that NSAIDs (such as celecoxib, indomethacin and diclofenac) upregulated GRP78 expression in guinea pig gastric mucosal cells in primary culture (Tsutsumi *et al*., 2004). Here, we used immunoblotting techniques to examine the increase in GRP78 production in AGS cells caused by a number of different NSAIDs. As shown in Figure 1a, all NSAIDs tested clearly increased cellular levels of GRP78. The concentrations of celecoxib, indomethacin and diclofenac required for these increases in AGS cells were similar to those previously reported to have caused similar effects in guinea pig gastric mucosal cells (Tsutsumi *et al*., 2004). COX exists as two subtypes, COX-1 and COX-2, for which celecoxib and nimesulide are COX-2 selective in their action. The results shown in Figure 1a suggest that NSAIDs increased cellular GRP78, irrespective of their COX-2 specificity. Furthermore, although celecoxib and nimesulide have similar IC_{50} values for COX-inhibition (Riendeau *et al*., 1997; Ben-Chetrit *et al*., 2005), higher concentrations of nimesulide than celecoxib were required for similar increases in GRP78 production, suggesting that NSAIDs increase GRP78 independently of COX inhibition. Of the NSAIDs tested (see results in Figure 1a), we selected celecoxib for use in most of subsequent experiments because it increased GRP78 at the lowest concentration

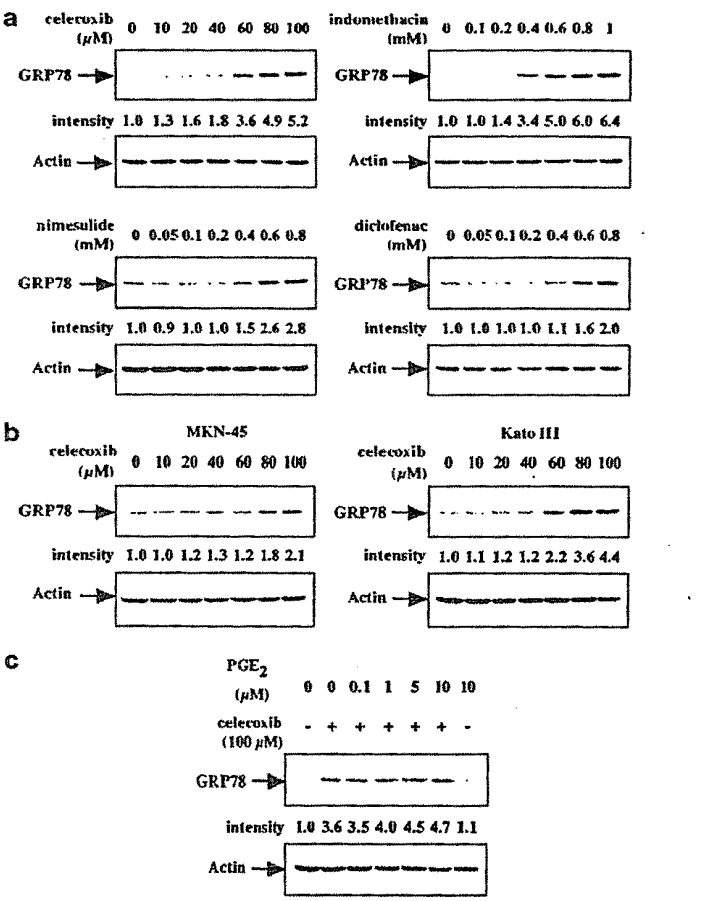

Figure 1 Upregulation of GRP78 by NSAIDs. AGS (**a** and **c**) or MKN-45 and Kato III (**b**) cells were incubated with indicated concentrations of stated NSAIDs for 12 h (celecoxib) or 24 h (NSAIDs other than celecoxib). Cells were pretreated with indicated concentrations of PGE_2 for 2 h before the celecoxib treatment (**c**). Whole cell extracts (5 μg protein) were analysed by immunoblotting with an antibody against GRP78 or actin. Band intensity of GRP78 was determined by densitometric scanning, gel-loading levels compensated against the band intensity of actin, and expressed relative to the control sample (i.e. without NSAIDs).

and its effectiveness in cancer therapy has been well established (Koki and Masferrer, 2002).

We also examined the upregulation of GRP78 by celecoxib in other cell types. The MKN-45 and Kato III cell lines are derived from gastric cancer cells (Okada *et al*., 2000). As shown in Figure 1b, celecoxib increased GRP78 mRNA in both of these cell lines at concentrations similar to those used for the AGS cells. It has been reported that both COX-1 and COX-2 mRNA are expressed in AGS and MKN-45 cells, whereas COX-1 but not COX-2 mRNA expression is detectable in KATO-III cells (Kawai *et al*., 1998; Fan *et al*., 2001; Lim *et al*., 2001). We confirmed these phenotypes by RT–PCR, that is, COX-1 mRNA expression was confirmed in each of the cell lines tested, whereas COX-2 mRNA was detected only in AGS and MKN-45 cells (data not shown). Thus, the results in Figure 1 show that the COX-2-selective NSAID, celecoxib, upregulated GRP78 mRNA not only in COX-2-expressing cells but also in cells lacking COX-2 expression, again suggesting that GRP78 upregulation can be induced by NSAIDs independently of COX inhibition. For further confirmation of this independence, we examined the effect of PGE_2 on the GRP78 upregulation by celecoxib. As shown in Figure 1c, PGE_2 did not affect the expression of GRP78 in both presence and absence of celecoxib, suggesting that the GRP78 upregulation by celecoxib cannot be explained by decrease in PGE_2 by COX inhibition.

GRP78 belongs to the HSP70 family of proteins for which cochaperones are also known (Lee, 2001). For example, HSP40 binds to HSP70 and stimulates its ATPase and refolding activities (Landry, 2003). Various cochaperones have been suggested for GRP78, among which ERdj3 and ERdj4 have been shown to bind to GRP78 and activate its ATPase activity (Yu *et al*., 2000; Shen *et al*., 2002b). We found, using real-time RT–PCR analysis, that not only *GRP78* but also *ERdj3* and *ERdj4* mRNAs were upregulated by celecoxib (see Figure 2). The concentrations of celecoxib required for the increase of both *ERdj3* and *ERdj4* mRNAs were similar to that required for the increase of *GRP78* mRNA (Figure 2a). Moreover, the curve describing the time course for the upregulation by celecoxib of *GRP78* mRNA was indistinguishable from those for *ERdj3* and *ERdj4* mRNAs (Figure 2b), showing that celecoxib simultaneously upregulates *GRP78*, *ERdj3* and *ERdj4*.

We also examined the effect of treatment with celecoxib on GRP78 expression in xenograft tumors in nude mice. Tumors were developed in nude mice by inoculation (s.c.) of MKN-45 cells and were treated with celecoxib by its oral administration. Xenograft tumor growth was clearly inhibited by the oral administration of celecoxib (Figure 3a), being consistent with results in a previous report (Williams *et al*., 2000; Leahy *et al*., 2002; Zweifel *et al*., 2002; Kulp *et al*., 2004). As shown in Figure 3b and c, the amount of GRP78 in tumors was increased by this celecoxib treatment. Results showed that celecoxib upregulates GRP78 also in tumors *in vivo*, accompanying with the suppression of tumor growth by this drug.

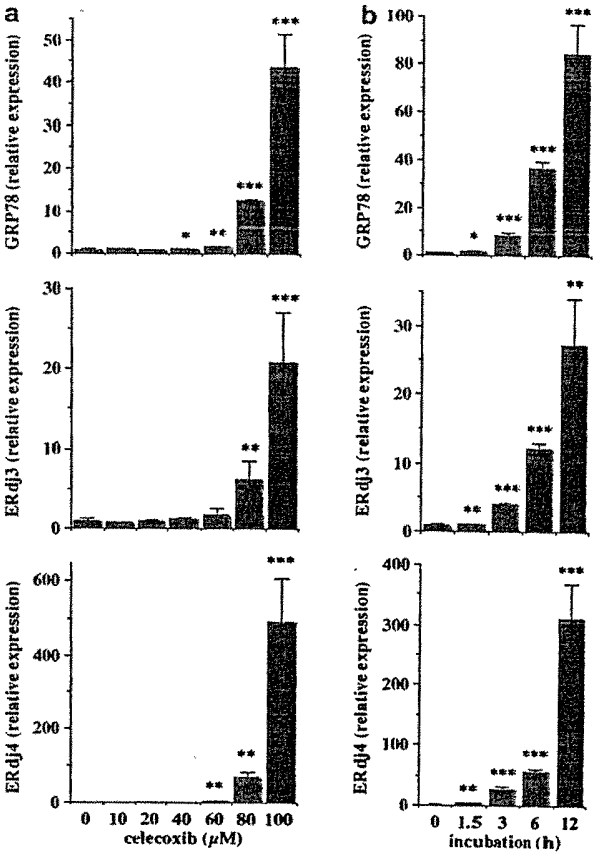

Figure 2 Upregulation of ER chaperone genes by celecoxib. AGS cells were incubated with indicated concentrations (**a**) or 100 μM (**b**) of celecoxib for 12 h (**a**) or the time periods indicated (**b**) and total RNA extracted. Samples were subjected to real-time RT–PCR by use of a specific primer for each gene. Values were normalized to actin gene expression and expressed relative to the control sample (i.e. without celecoxib). Values given are mean±s.d. ($n=3$). ***$P<0.001$; **$P<0.01$; *$P<0.05$.

Mechanism for upregulation of ER chaperones by celecoxib

Eukaryotic translation initiation factor 2 kinase (PERK) is an ER transmembrane protein that plays an important role in ER chaperone induction by ER stressors. Previous studies revealed that ER stressors activate PERK by its phosphorylation, the PERK then activates eukaryotic initiation factor-2α (eIF2α) by its phosphorylation, the eIF2α induces activating transcription factor (ATF)4 expression, and finally, ATF4 binds to the promoter of the GRP78 gene, resulting in the increased production of GRP78 (Harding *et al*., 2000; Luo *et al*., 2003). We used DNA microarray techniques to search for genes whose expression is stimulated by NSAIDs in AGS cells (Mima *et al*., 2005). *ATF4* was identified as one such gene, suggesting that its upregulation is involved in the induction of ER chaperones by NSAIDs. As shown in Figure 4a, both PERK and eIF2α were phosphorylated in the presence of celecoxib. The PERK phosphorylation was transient; it decreased after 3 h and we have no

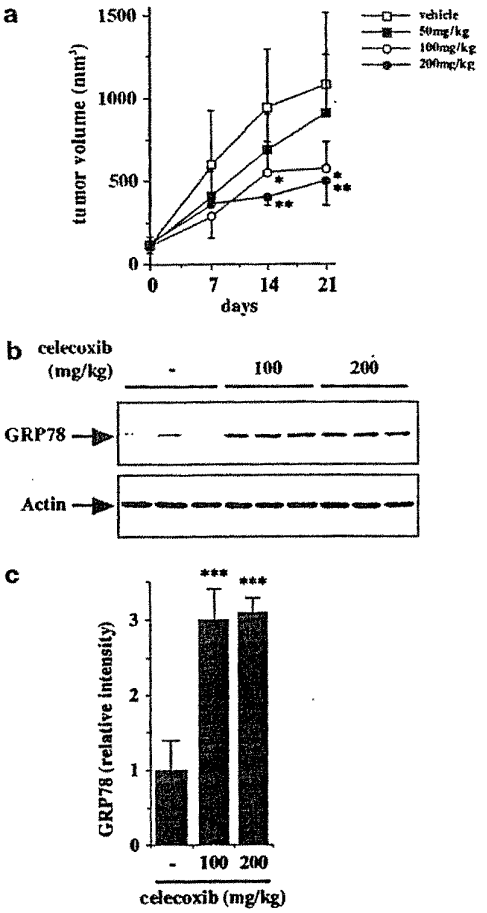

Figure 3 Effect of celecoxib on growth of xenograft tumor and expression of GRP78 in nude mice. Each nude mouse was inoculated s.c. with MKN-45 cells and tumors were developed until size of tumors reached a mean volume of $116 \pm 34\,mm^3$. Then indicated dose of celecoxib was administered single daily orally for the duration of the study. Tumors were measured weekly and their volumes calculated (**a**). After 4 days from the start of celecoxib administration, cell lyzates prepared from tumors were analysed by immunoblotting with an antibody against GRP78 or actin (**b**). Band intensity of GRP78 was determined by densitometric scanning, compensated against the band intensity of actin, and expressed relative to the control sample (i.e. without celecoxib) (**c**). Values given are mean ± s.d. ($n=6$ for (**a**) and $n=3$ for (**b** and **c**)). $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

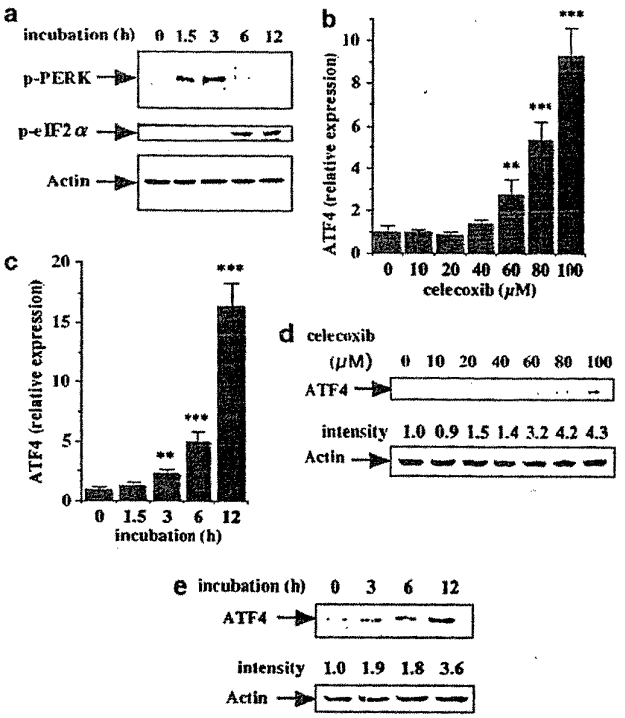

Figure 4 Activation of PERK, eIF2α and ATF4 by celecoxib. AGS cells were incubated with 100 μM (**a**, **c** and **e**) or indicated concentrations (**b** and **d**) of celecoxib for the time periods indicated (**a**, **c** and **e**) or 12 h (**b** and **d**). For (**a**, **d** and **e**), whole-cell extracts (5 μg protein for actin, 10 μg protein for ATF4 and 20 μg protein for PERK and eIF2α) were analysed by immunoblotting with an antibody against phosphorylated PERK (p-PERK), phosphorylated eIF2α (p-eIF2α), ATF4 or actin. For (**b** and **c**), total RNA was extracted and subjected to real-time RT–PCR by use of a specific primer for ATF4. Values were analysed and expressed as previously described in the legend of Figure 2. Values shown are mean ± s.d. ($n=3$). $^{***}P<0.001$; $^{**}P<0.01$.

clear explanation for this phenomenon. Furthermore, upregulation of *ATF4* mRNA and ATF4 protein by celecoxib was confirmed by real-time RT–PCR analysis and immunoblotting analysis, respectively (Figure 4b–e). Both time–course and dose–response curves for upregulation of *ATF4* mRNA were similar to those observed for the increase of *GRP78* mRNA by celecoxib (Figures 2 and 4). Interestingly, phosphorylation of PERK was detected within 1.5 h of the addition of celecoxib, maximal eIF2α was reached 6 h after addition and peak *ATF4* mRNA and protein was observed 12 h after addition (Figure 4), suggesting that the sequential activation of PERK, eIF2α and ATF4 is involved in the upregulation of GRP78. To test this possibility, we examined the effect of siRNA for ATF4 on the celecoxib-dependent upregulation of GRP78. Transfection of siRNA for ATF4 clearly inhibited the expression of *ATF4* mRNA, both in the presence and absence of celecoxib (Figure 5a). As shown in Figure 5b, transfection of siRNA for ATF4 partially suppressed the increase of *GRP78* mRNA production caused by celecoxib, suggesting that ATF4 is involved in this celecoxib-dependent GRP78 upregulation. In order to estimate the specificity of this siRNA, we examined its effect on the expression of mRNA of other CREB protein family member (ATF2 and ATF3). As shown in Figure 5c, transfection of siRNA for ATF4 did not affect the celecoxib-dependent induction of *ATF2* mRNA so clearly as that of *ATF4*, suggesting that this siRNA specifically inhibited the expression of *ATF4* mRNA. On the other hand, transfection of this siRNA inhibited the induction of *ATF3* mRNA by celecoxib (Figure 5d). This may be due to the dependence of ATF3 expression on ATF4; the upregulation of ATF3 by thapsigargin was significantly suppressed in ATF4 knockout cells (Jiang *et al*., 2004).

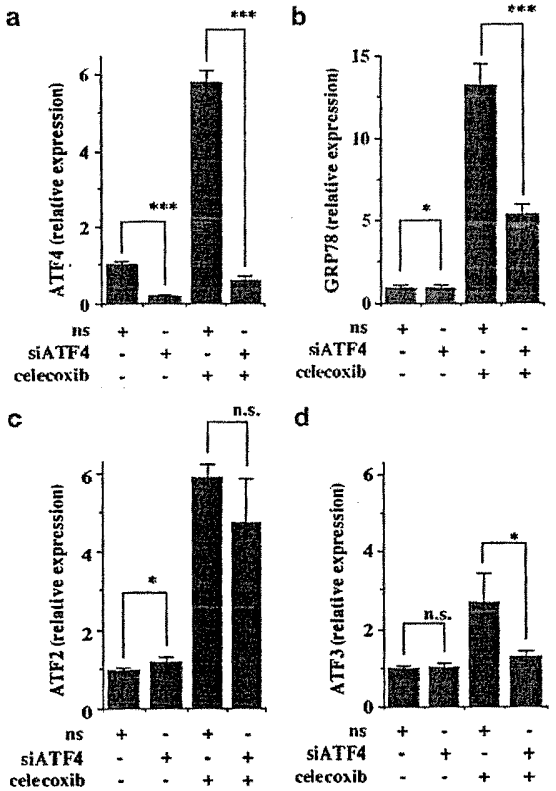

Figure 5 Effect of siRNA for ATF4 on the celecoxib-dependent upregulation of GRP78. AGS cells transfected with siRNA for ATF4 (siATF4) or nonsilencing (ns) siRNA were incubated with or without 100 μM celecoxib for 12 h. Total RNA was extracted and subjected to real-time RT–PCR by use of a specific primer for ATF4 (**a**), GRP78 (**b**), ATF2 (**c**) and ATF3 (**d**). Values were analysed and expressed as previously described in the legend of Figure 2. Values shown are mean$\pm$s.d. ($n=3$). ***$P<0.001$; *$P<0.05$. n.s., not significant.

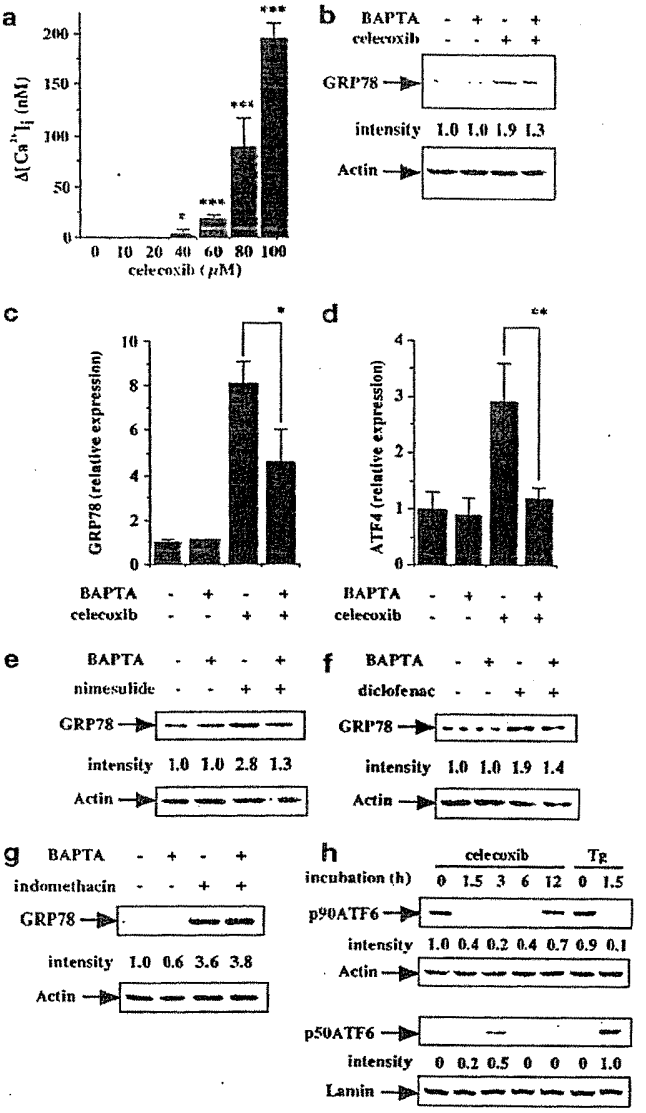

Figure 6 Changes in intracellular Ca^{2+} concentration in the NSAID-dependent upregulation of GRP78. The intracellular Ca^{2+} concentration was monitored by a fluo-3/AM assay system. Indicated concentrations of celecoxib were added to fluo-3/AM-loaded cells and the time–course of fluo-3 fluorescence change monitored. The maximum value for the increase in the intracellular Ca^{2+} level ($\Delta[Ca^{2+}]_i$) is shown (**a**). AGS cells were preincubated with or without 2 μM BAPTA-AM for 1 h and further incubated with or without 80 μM celecoxib (**b–d**). 800 μM nimesulide (**e**), 800 μM diclofenac (**f**) or 400 μM indomethacin (**g**) in the presence or absence of 2 μM BAPTA-AM for 6 h (celecoxib) or 12 h (other NSAIDs). The levels of GRP78 protein (**b**, **e–g**), GRP78mRNA (**c**) and ATF4 mRNA (**d**) were estimated by immunoblotting or real-time RT–PCR experiments as described in the legends of Figures 1 and 2. AGS cells were incubated with 100 μM celecoxib or 2 μM thapsigargin for indicated periods (**h**). Whole cell extracts (25 μg protein for ATF6 and 10 μg protein for actin) (upper panel in (**h**)) or nuclear extracts (20 μg protein for p50 ATF6 and 5 μg protein for lamin B) (lower panel in (**h**)) were analysed by immunoblotting with an antibody against ATF6, actin or lamin B as described in the legends of Figure 1. As for p50 ATF6 band, intensity of each band was expressed relative to the positive control sample (i.e. cells treated with thapsigargin for 1.5 h). Values shown are mean$\pm$s.d. ($n=3$). ***$P<0.001$; **$P<0.01$; *$P<0.05$.

Some NSAIDs have been reported to increase intracellular Ca^{2+} concentrations (Johnson *et al.*, 2002; Tomisato *et al.*, 2004a). We recently found that all of the NSAIDs tested can cause membrane permeabilization, resulting in an increase in intracellular Ca^{2+} levels. This activity correlates well with the NSAID-induced apoptosis (Tomisato *et al.*, 2004a). On this basis, we have tested whether the increase in intracellular Ca^{2+} by celecoxib is responsible for the induction of ER chaperones. First, we confirmed the presence of an increase in intracellular Ca^{2+} concentration in the presence of celecoxib under the same conditions as those used for the upregulation of GRP78 in AGS cells. As shown in Figure 6a, celecoxib increased intracellular Ca^{2+} concentration in a dose-dependent manner, similar to that observed for the increase in *GRP78* mRNA (Figure 2a). Furthermore, 1,2-bis(2-aminophenoxy)-ethane-*N,N,N′N′*-tetraacetic acid (BAPTA-AM), an intracellular Ca^{2+} chelator, inhibited the celecoxib-dependent upregulation of GRP78, *GRP78* mRNA and *ATF4* mRNA (Figure 6b–d), but had no effect when celecoxib was not present. At the concentrations used, BAPTA-AM did not affect the cell viability (data

not shown). These results strongly suggest that upregulation of GRP78 and ATF4 by celecoxib is mediated, at least in part, through an increase in intracellular Ca^{2+} concentration.

We also examined the effect of BAPTA-AM on the upregulation of GRP78 induced by other NSAIDs. As is the case of celecoxib, BAPTA-AM inhibited the upregulation of GRP78 by nimesulide or diclofenac (Figure 6e and f). On the other hand, BAPTA-AM did not affect the upregulation of GRP78 by indomethacin (Figure 6g).

ATF6 is another type of ER transmembrane protein that also plays an important role in ER chaperone induction by ER stressors. We previously suggested that ATF6 is activated in the presence of NSAIDs in gastric mucosal cells in primary culture (Tsutsumi *et al*., 2004), and it was recently reported that ATF6 is activated by nitric oxide through an increase in the intracellular Ca^{2+} level (Xu *et al*., 2004). Therefore, we examined the effect of celecoxib on the activation of ATF6. In the presence of ER stressors, such as thapsigargin, p90 ATF6 (the inactive form of ATF6 for ER stress response) is cleaved into p50 ATF6, which translocates to the nucleus where it specifically activates transcription of genes related to ER stress response (Yoshida *et al*., 2000). As shown in Figure 6h, as well as thapsigargin, treatment of cells with celecoxib caused appearance of p50 ATF6 and disappearance of p90 ATF6, suggesting that celecoxib activated ATF6. This activation was transient; both appearance of p50 ATF6 and disappearance of p90 ATF6 was apparent 3 h but not observed 12 h after the addition of celecoxib.

Effect of ER chaperones on celecoxib-induced apoptosis

It is well known that celecoxib induces apoptosis in various types of tumor cells (Koki and Masferrer, 2002). As shown in Figure 7a and b, celecoxib induced apoptosis in AGS cells in both a dose- and time-dependent manner. Real-time RT–PCR analysis showed that celecoxib induced *CHOP* mRNA production, with the dose–response and time–course curves for this response (Figure 7c and d) being similar to those seen for the induction of apoptosis (Figure 7a and b). This finding suggests that the induction of CHOP expression is responsible for the celecoxib-induced apoptosis, as reported previously (Tsutsumi *et al*., 2004).

The dose–response and time–course curves for the upregulation of GRP78 by celecoxib (Figure 2) were also similar to those for the induction of apoptosis (Figure 7a and b), showing that GRP78 upregulation and apoptosis occur simultaneously. Previous reports showed that overexpression of GRP78 in cells suppresses apoptosis induced by topoisomerase inhibitors and ER stressors (Morris *et al*., 1997; Reddy *et al*., 2003). Therefore, it is possible that celecoxib-induced GRP78 protects cells from celecoxib-induced apoptosis. In order to test this possibility, we examined the effect of overexpression of GRP78 on celecoxib-induced apoptosis. Transfection of pcDNA3.1 containing the *GRP78* gene caused both an increase in the level of GRP78 in

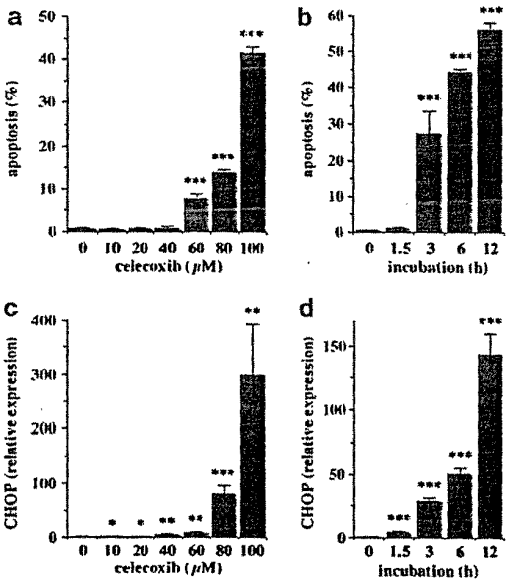

Figure 7 Induction of apoptosis by celecoxib. AGS cells were incubated with the indicated concentrations (**a** and **c**) or 100 μM (**b** and **d**) of celecoxib for 12 h (**a** and **c**) or indicated periods (**b** and **d**). For (**a** and **b**), apoptotic cell numbers were determined by FACS (**a** and **b**). For (**c** and **d**) total RNA was extracted and subjected to real-time RT–PCR by use of a specific primer for CHOP. Values were analysed and expressed as previously described in the legend of (Figure 2c and d). Values shown are mean ± s.d. ($n=3$). $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

cells and partial suppression of celecoxib-induced apoptosis in a manner that depended on the dose of transfected DNA (Figure 8a and b). Real-time RT–PCR analysis revealed that the transfection increased *GRP78* mRNA both in the presence and absence of celecoxib (Figure 8c). Furthermore, the transfection partially suppressed the celecoxib-dependent induction of *CHOP* mRNA (Figure 8d). We confirmed that overexpression of GRP78 did not affect the spontaneous apoptosis (apoptosis in the absence of celecoxib) (Figure 9d). These results suggest that the celecoxib-induced increase in GRP78 expression protects cells from celecoxib-induced apoptosis by repressing the expression of *CHOP* mRNA. Overexpression of GRP78 did not diminish the celecoxib-dependent *GRP78* upregulation (Figure 8c), which is inconsistent with previous results showing that overexpression of GRP78 diminished tunicamycin-dependent GRP78 production (Morris *et al*., 1997). This discrepancy may be explained by differences in stressors or in the extent of overexpression (the extent of overexpression of GRP78 in the paper by Morris *et al*. was much higher than that found here).

It was recently reported that overexpression of ERdj4 in cells inhibits apoptosis induced by tunicamycin (Kurisu *et al*., 2003). We here examined the effect of overexpression of ERdj4, ERdj3, or their coexpression with GRP78 on celecoxib-induced apoptosis. As shown in Figure 9a, transfection of plasmid resulting in overexpression of *ERdj3* partially suppressed the cele-

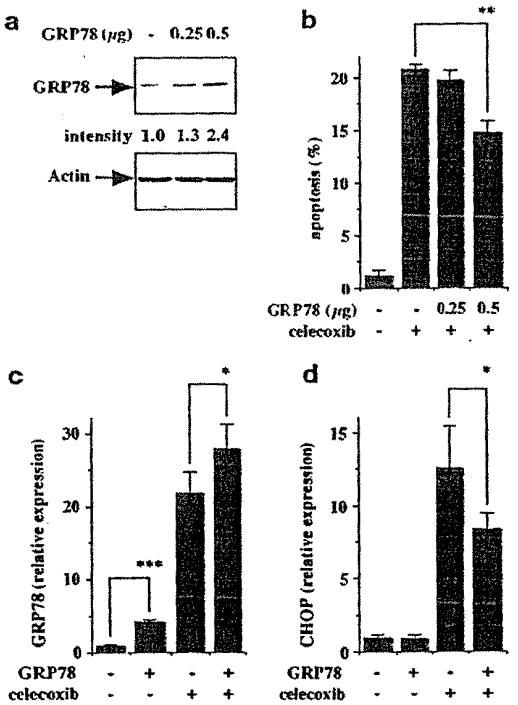

Figure 8 Effect of overexpression of GRP78 on celecoxib-induced apoptosis. AGS cells were transfected with the indicated amount (**a** and **b**) or 0.5 μg (**c** and **d**) of plasmid for the overexpression of GRP78 and pcDNA3.1 vector (total DNA amounts were fixed at 4 μg). After 48 h, cells were incubated with or without 100 μM celecoxib for 6 h (**b–d**). The levels of GRP78 protein (**a**), GRP78mRNA (**c**) and CHOP mRNA (**d**) were estimated by immunoblotting or real-time RT-PCR experiments as previously described in the legends of Figures 1 and 2. Apoptotic cell numbers were determined by FACS as described in the legend of Figure 7(b). Values shown are mean±s.d. ($n=3$). ***$P<0.001$; **$P<0.01$; *$P<0.05$.

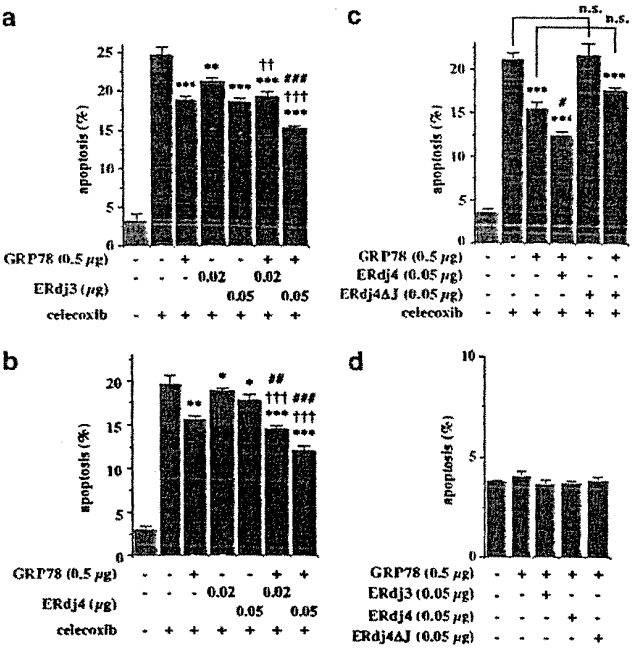

Figure 9 Stimulation of the antiapoptotic effect of GRP78 against celecoxib by coexpression of ERdj3 or ERdj4. AGS cells were transfected with the indicated amounts of each expression plasmid and pcDNA3.1 vector (total DNA amounts were fixed at 4 μg). After 48 h, AGS cells were incubated with or without 100 μM celecoxib for 6 h. Apoptotic cell numbers were determined by FACS as described in the legend of Figure 7. Values shown are mean±s.d. ($n=3$). ***, ††† and ###$P<0.001$; **, †† and ##$P<0.01$; * and #<0.05. * (versus celecoxib-treated cells only), # (versus celecoxib-treated and GRP78 overexpressing cells), † (versus celecoxib-treated and ERdj3 (or ERdj4) overexpressing cells). n.s., not significant (**a–d**).

coxib-induced apoptosis in a manner that was dependent on the amount of transfected DNA. Furthermore, the cotransfection of plasmids for the overexpression of both GRP78 and ERdj3 caused a more clear-cut suppression of celecoxib-induced apoptosis than did transfection of each plasmid alone (Figure 9a). Similar results were obtained for ERdj4 (Figure 9b). We confirmed that overexpression of both GRP78 and ERdj3 or ERdj4 did not affect the spontaneous apoptosis (apoptosis in the absence of celecoxib) (Figure 9d). These results suggest that the ERdj4 and ERdj3 cochaperones stimulate the antiapoptotic effect of GRP78 against the actions of celecoxib.

The J domain of HSP40 family proteins is responsible for their interaction with HSP70 family proteins (Landry, 2003). It was shown that J domain-deleted ERdj4 (ERdj4ΔJ) could not interact with GRP78 and activate the ATPase activity of GRP78 (Shen *et al.*, 2002b). As shown in Figure 9c, in contrast to the results obtained with wild-type ERdj4, transfection of plasmid for the overexpression of ERdj4ΔJ caused neither the suppression of celecoxib-induced apoptosis nor stimulation of an antiapoptotic effect of GRP78 against celecoxib. These findings suggest that the antiapoptotic effects of ERdj4 are achieved via its interaction with GRP78.

The siRNA technique was used to further confirm that celecoxib-induced GRP78 protects cells from celecoxib-induced apoptosis. Transfection of siRNA for GRP78 decreased the expression of GRP78 protein (Figure 10a) and *GRP78* mRNA (Figure 10b), both in the presence and absence of celecoxib, and also stimulated celecoxib-induced apoptosis and *CHOP* mRNA expression (Figure 10e and f). In order to estimate the specificity of siRNA for GRP78, we examined its effect on the expression of mRNA of other ER chaperons (*ERdj3* and *ERdj4*). As shown in Figure 10c and d, transfection of siRNA for GRP78 significantly increased the *ERdj3* or *ERdj4* mRNA in both presence and absence of celecoxib, suggesting that this siRNA specifically inhibit the expression of GRP78. The stimulation of the *ERdj3* or *ERdj4* mRNA expression by this siRNA may be due to that GRP78 negatively regulated the ER stress response; GRP78 binds to PERK and protein-kinase and site-specific endoribonuclease (IRE1) and inhibits their activity for inducing ER stress response (Bertolotti *et al.*, 2000). These results strongly suggest that celecoxib-induced GRP78 protects