厚生労働科学研究研究費補助金

長寿科学総合研究事業

蛋白質のフォールディング異常に着目した、分子シャペロンによる神経変性疾患の根本的治療法の開発

平成16年度　総括研究報告書

主任研究者　水島　徹

平成17（2005）年　3月

目次

厚生労働科学研究費補助金（長寿科学総合研究事業）
総括研究報告書

蛋白質のフォールディング異常に着目した、分子シャペロンによる神経変性疾患の根本的治療法の開発

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

漢方薬、及び天然物から、数多くの毒性のない分子シャペロン誘導剤を発見、単離した。またこれまでに整備した分子シャペロンに関する研究材料（ノックアウトマウスなど）を使って、分子シャペロンの神経変性疾患に対する効果を調べた。その結果、分子シャペロンの有効性を示す多くの結果を得た。従って、本研究で発見した種々の分子シャペロン誘導剤は、新しい神経変性弛緩の治療薬になることが期待される。

A. 研究目的

細胞内には蛋白質が正しいフォールディング（高次構造・折り畳み構造）をとるのを積極的に助けている一群の蛋白質（分子シャペロン）が存在する。分子シャペロンは、合成されたばかりの蛋白質が正しいフォールディングをとることを助けるだけでなく、ストレスによってそのフォールディングが異常になってしまった蛋白質を正常に戻すことも出来る。さらに分子シャペロンは、どうしてもそのフォールディングを正常に戻せない蛋白質に関しては、細胞に悪影響を及ぼすのを防ぐために、それを分解することもできる。このように分子シャペロンは細胞内で蛋白質の品質管理を行っている興味深い分子群である。

多くの疾患の根本的な原因は蛋白質の異常である。疾患の原因となる蛋白質の異常の関してこれまでは、発現異常や活性異常ばかりが注目されてきたが、最近の研究により、細胞内の蛋白質のフォールディング異常が原因になっている多くの疾患が明らかになり、蛋白質フォールディング異常病と呼ばれるようになっている。神経変性疾患は、代表的な蛋白質フォールディング異常病である。アルツハイマー病、ハンチントン舞踏症、パーキンソン病をはじめとする神経変性疾患の原因は、アルツハイマー病ではβアミロイド、ハンチントン舞踏症ではハンチンチン、パーキンソン病ではパーキンと一方本研究では、同じくストレス遺伝子を利用した全く新しい毒性試験の確立も目指す。細胞は毒物などのストレスに対し、適切な遺伝子（ストレス遺伝子）を発現することによって、自らの生存を保っている。そこで、ある物質に対しいう原因蛋白質のフォールディングが異常になり凝集し、その凝集体により神経細胞が死滅したり機能を失ったりすることによって発症する。この他にも、嚢胞性繊維症、肺気腫、白内障、家族性高コレステロール症、ある種の糖尿病など、多くの疾患においてその原因が蛋白質のフォールディング異常であることが分かっている。

蛋白質フォールディング異常病の共通点として、その根本的な治療法が確立されていないことがある。神経変性疾患などの蛋白質フォールディング異常病を根本的に治療するためには、異常なフォールディングの蛋白質を正常なフォールディングへ戻す、あるいは異常なフォールディングの蛋白質を速やかに分解することが必要であり、これらはいずれも分子シャペロンによって行うことができる。そこで我々は、分子シャペロンを利用して、蛋白質に正しいフォールディングをとらせる技術を開発し、神経変性疾患の根本的治療法を確立するための研究を提案する。

B. 研究方法

毒性のない分子シャペロン誘導剤のスクリーニング

漢方医薬研究振興財団から生薬成分、漢方薬などを供与して頂き、毒性のないシャペロン誘導剤のスクリーニングを行った。具体的には、HSP70, HSP90、GRP78と結合する物質を検索した。

分子シャペロンに関する研究材料の整備

種々の分子シャペロンに関して、抗体、過剰発現プラスミド、精製蛋白質などを整備した。

分子シャペロンの神経変性疾患への効果

HSF1ノックアウトマウスとAPP（βアミロイド前駆体蛋白質）の掛け合わせを行い、そのβアミロイドの状態を調べた。種々の分子シャペロン、及び昨年度発見した毒性のないHSP誘導剤のβアミロイド、及びポリグルタミンの凝集に対する効果を調べた。

C.研究結果

約400種の生薬成分、漢方薬などから、毒性のないシャペロン誘導剤のスクリーニングを行った。具体的には、HSP70, HSP90、GRP78と結合する物質を検索した。その結果、毒性のないHSP誘導剤４種（朝鮮あさがおなど由来）、毒性のないGRP誘導剤５種（オオバコなど由来）を発見した。

一方昨年度単離した毒性のないHSP誘導剤（ウワウルシ由来）を用いて、その神経変性疾患に対する効果を細胞、及び動物レベルで検討した。まず、APP（アルツハイマー病の原因蛋白質、βアミロイドの前駆体）を発現している細胞に作用させたところ、培地中へのβアミロイドの放出が顕著に抑制された。またAPPノックインマウスにこの誘導剤を投与したところ、脳でHSPを誘導するとともに、脳内のβアミロイドの量の低下が見られた。さらに細胞内で再現したポリグルタミンの凝集に対しても、抑制効果を示した。

分子シャペロンに関する研究材料の整備に関しては、この２年間の研究でHSF1 のノックアウトマウスの作成に成功し、またHSP105、HSP90、HSP70、HSP60、HSP47、HSP28、GRP94、GRP78、ORP150、カルレティキュリン、カルネキシンに対して、精製蛋白質、特異的な抗体、細胞内で発現するためのプラスミド、siRNA の整備を完了した。さらに現在、これらの遺伝子のノックアウトマウスの作成を行っている。

これらの材料を用いて、分子シャペロンの神経変性疾患に対する効果を検討した。その結果

βアミロイドの産生を抑制するもの

HSP105、HSP70、GRP94、GRP78、ORP150、カルレティキュリン、カルネキシン

ポリグルタミンの凝集を抑制するもの

HSP70、HSP28、GRP94、GRP78、ORP150、

カルレティキュリン、カルネキシン。

という結果を得た。

またHSF1ノックアウトマウスとAPP（βアミロイド前駆体蛋白質）の掛け合わせを行い、そのβアミロイドの状態を調べたところ、野生型に比べ、HSF1ノックアウトマウスではよりβアミロイドの蓄積、老人班の形成、行動異常が早く見られることが分かった。

D.考察

以上の研究から、分子シャペロンが神経変性疾患に有効であることが示唆された。

E.結論

本研究で発見した種々の分子シャペロン誘導剤は、新しい神経変性弛緩の治療薬になることが期待される。

F.健康危険情報

該当なし

G.研究発表

1. 論文発表

1. 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.
2. Tanaka, K., Nishimoto, K., Tomisato, W., Tsutsumi, S., Hoshino, T., Tsuchiya, T. and Mizushima, T. (2004) Adaptive cytoprotection induced by pre-treatment with ethanol protects against gastric cell damage by NSAIDs. ***Dig. Dis. Sci.*** 49, 210-217.
3. Tsutsumi, S., Gotoh, T., Mima, S., Tomisato, W., Hoshino, T., Hwang, H-J., Mori, M., Tsuchiya, T. and Mizushima, T. (2004) NSAIDs induce apoptosis through endoplasmic reticulum stress response in gastric mucosal cells. ***Cell Death Differ.*** 11, 1009-1016.
4. Tanaka, K., Tomisato, W., Tsutsumi, S., Hoshino, T., Tsuchiya, T. and Mizushima, T. (2004) Cytotoxic synergy between indomethacin and hydrochloric acid in gastric mucosal cells. ***Biol. Pharm. Bull.***27, 1188-1192.
5. Tomisato, W., Tanaka, K., Katsu, T., Kakuta, H., Sasaki, K., Tsutsumi, S., Hoshino, T., Aburaya, M., Li, D., Tsuchiya, T., Suzuki, K., Yokomizo, K., and Mizushima, T. (2004) Membrane permeabilization by non-steroidal anti-inflammatory drugs. ***Biochem. Biophys. Res. Commun.*** 323, 1032-1039.
6. Mima, S., Tsutsumi, S., Ushijima, H., Takeda, M., Fukuda, I., Yokomizo, K., Suzuki, K., Sano, T., Nakanishi, T., Tomisato, W., Tsuchiya, T. and

Mizushima, T. (2004) Induction of claudin-4 by non-steroidal anti-inflammatory drugs and its contribution to their chemopreventive effect. ***Cancer Res.*** in press.

7. Arai, Y., Tanaka, K., Ushijima, H., Tomisato, W., Tsutsumi, S., Aburaya, M., Hoshino, T., Yokomizo, K., Suzuki, K., Katsu, T., Tsuchiya, T. and Mizushima, T. (2004) Low direct cytotoxicity of nabumetone on gastric mucosal cells. ***Dig. Dis. Sci.*** in press.
8. Tomisato, W., Tanaka, K., Tsutsumi, S., Hoshino, T., Yokomizo, K., Suzuki, K., Katsu, T., and Mizushima, T. (2005) Low direct cytotoxicity and cytoprotective effects of nitric oxide-releasing indomethacin. ***Dig. Dis. Sci.*** in press.
9. Sone, M., Hayashi, H., Yamamoto, H., Hoshino, T., Mizushima, T., and Nakashima, T. (2005) Upregulation of HSP by geranylgeranylacetone protects the cochlear lateral wall from endotoxin-induced inflammation. ***Hearing Research*** in press.

学会発表（招待講演のみ）

1. 水島徹　NSAIDs 研究の新展開　岡山大学医学部での招待講演　(2004) (岡山)
2. 水島徹　NSAIDs 潰瘍発症の分子機構と HSP 誘導薬による保護　Heart Protection Forum 特別講演　(2004) (佐賀)
3. 水島徹　NSAIDs 潰瘍発症の分子機構と HSP 誘導薬による保護　実験潰瘍学会イブニングセミナー　(2004) (大津)
4. Tohru Mizushima Mechanism of NSAID-induced gastric lesions and its protection by HSP inducers. International Symposium in Biohealth Products Research Center (2004) (Pusan)
5. 水島徹　NSAIDs 潰瘍発症の分子機構と HSP 誘導薬による保護　熊本大学医学部麻酔科研究会特別講演 (2004) (熊本)
6. 水島徹　NSAIDs 研究の新展開　愛媛県病院薬剤師会学術講演会 (2005) (愛媛)
7. 水島徹　NSAIDs 研究の新展開　北海道大学での特別講演　(2005) (札幌)
8. 水島徹　NSAIDs 研究の新展開　大正富山製薬（株）研究所での特別講演　(2005) (さいたま)

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
Tomisato, W., Tsutsumi, S., Hoshino, T., Hwang, H-J., Mio, M., Tsuchiya, T. Mizushima, T.	Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions.	***Biochem. Pharmacol.***	67	575-585	2004
Tanaka, K., Nishimoto, K., Tomisato, W., Tsutsumi, S., Hoshino, T., Tsuchiya, T. Mizushima, T.	Adaptive cytoprotection induced by pre-treatment with ethanol protects against gastric cell damage by NSAIDs.	***Dig. Dis. Sci.***	49	210-217	2004
Tsutsumi, S., Gotoh, T., Mima, S., Tomisato, W., Hoshino, T., Hwang, H-J., Mori, M., Tsuchiya, T. Mizushima, T.	NSAIDs induce apoptosis through endoplasmic reticulum stress response in gastric mucosal cells.	***Cell Death Differ.***	11	1009-1016	2004
Tanaka, K., Tomisato, W., Tsutsumi, S., Hoshino, T., Tsuchiya, T. Mizushima, T.	Cytotoxic synergy between indomethacin and hydrochloric acid in gastric mucosal cells.	***Biol. Pharm. Bull.***	27	1188-1192	2004
Tomisato, W., Tanaka, K., Katsu, T., Kakuta, H., Sasaki, K., Tsutsumi, S., Hoshino, T., Aburaya, M., Li, D., Tsuchiya, T., Suzuki, K., Yokomizo, K., Mizushima, T.	Membrane permeabilization by non-steroidal anti-inflammatory drugs.	***Biochem. Biophys. Res. Commun.***	323	, 1032-1039	2004
Mima, S., Tsutsumi, S., Ushijima, H., Takeda, M., Fukuda, I., Yokomizo, K., Suzuki, K., Sano, T., Nakanishi, T., Tomisato, W., Tsuchiya, T. Mizushima, T.	Induction of claudin-4 by non-steroidal anti-inflammatory drugs and its contribution to their chemopreventive effect.	***Cancer Res.***	in press.		2005
Arai, Y., Tanaka, K., Ushijima, H., Tomisato, W.,	Low direct cytotoxicity of nabumetone on gastric	***Dig. Dis. Sci.***	in press.		2005

Tsutsumi, S., Aburaya, M., Hoshino, T., Yokomizo, K., Suzuki, K., Katsu, T., Tsuchiya, T. Mizushima, T.	mucosal cells.				
Tomisato, W., Tanaka, K., Tsutsumi, S., Hoshino, T., Yokomizo, K., Suzuki, K., Katsu, T., Mizushima, T.	Low direct cytotoxicity and cytoprotective effects of nitric oxide-releasing indomethacin.	***Dig. Dis. Sci.***	in press.		2005

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 323 (2004) 1032–1039
BBRC
www.elsevier.com/locate/ybbrc

Membrane permeabilization by non-steroidal anti-inflammatory drugs

Wataru Tomisato[a,b], Ken-ichiro Tanaka[a], Takashi Katsu[b], Hiroki Kakuta[b], Kenji Sasaki[b], Shinji Tsutsumi[a], Tatsuya Hoshino[a], Mayuko Aburaya[a], Daiwei Li[b], Tomofusa Tsuchiya[b], Keitarou Suzuki[a], Kazumi Yokomizo[a], 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*

Received 26 August 2004
Available online 11 September 2004

Abstract

The cytotoxicity of non-steroidal anti-inflammatory drugs (NSAIDs) is involved in the formation of NSAID-induced gastric lesions. The mechanism(s) behind these cytotoxic effects, however, is not well understood. We found here that several NSAIDs tested caused hemolysis when employed at concentrations similar to those that result in cytotoxicity. Moreover, these same NSAIDs were found to directly permeabilize the membranes of calcein-loaded liposomes. Given the similarity in NSAID concentrations for cytotoxic and membrane permeabilization effects, the cytotoxic action of these NSAIDs may be mediated through the permeabilization of biological membranes. Increase in the intracellular Ca^{2+} level can lead to cell death. We here found that all of NSAIDs tested increased the intracellular Ca^{2+} level at concentrations similar to those that result in cytotoxicity. Based on these results, we consider a possibility that membrane permeabilization by NSAIDs induces cell death through increase in the intracellular Ca^{2+} level.

Keywords: Membrane permeabilization; Cytotoxicity; Membrane fluidity; Gastric mucosal cells; NSAIDs; Intracellular Ca^{2+} level

Because of their efficacy in the treatment of pain, inflammation, and fever, non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently used classes of medicines in the world and account for nearly 5% of all prescribed medications [1]. The action of NSAIDs is mediated via their capacity to inhibit cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong propensity for inducing inflammation. On the reverse side, NSAID use is associated with gastrointestinal complications, such as gastric lesions [2]. About 15–30% of chronic users of NSAIDs have gastrointestinal ulcers and bleeding [3–6]. In the United States, about 16,500 people die per year as a result of NSAID-associated gastrointestinal complications [7].

The inhibition of COX activity by NSAIDs was previously thought to be fully responsible for their gastrointestinal side effects [8]. This is because PGs have a strong cytoprotective effect on the gastrointestinal mucosa [9]. However, the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs are not always linked with each other [10,11], this would suggest that additional mechanisms are involved in the induction of gastrointestinal lesions by NSAIDs [12]. It is well known that NSAIDs have a direct cytotoxicity (topical irritant property) on gastric mucosal cells [12–14]. We recently demonstrated, using primary cultures of guinea pig gastric mucosal cells, that NSAIDs were able to induce both necrosis and apoptosis of cells [15]. Further to this, we also found that this direct cytotoxicity of NSAIDs is independent of the inhibition of COX activity and suggested that both the inhibition of COX activity and the direct cytotoxicity of NSAIDs are required for the induction

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

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.08.205

of gastric lesions in vivo [16]. Therefore, the mechanism(s) governing the direct cytotoxicity of NSAIDs needs to be elucidated in order for the overall molecular mechanism of NSAID-induced gastric lesions to be understood and for safer NSAIDs to be developed. Previous reports suggested that various factors, such as bcl-2 family proteins and mitogen-activated protein kinases (MAPKs), are involved in NSAID-induced apoptosis [17,18]. However, the primary target of NSAIDs in relation to their direct cytotoxicity remains unknown. One such target candidate is the cell membrane. It has been reported that NSAIDs interact with phospholipids and that phospholipid liposomes reduce the direct cytotoxicity of NSAIDs in vivo [13,19–21]. In the experiments described here, we found that a range of NSAIDs have membrane permeabilization activity. Since the concentrations of NSAIDs required to induce apoptosis and necrosis in gastric mucosal cells were closely related with those required to increase membrane permeability, we propose that the primary target of these NSAIDs in relation to their direct cytotoxicity is the cell membrane.

Materials and methods

Chemicals, media, and animals. Fetal bovine serum (FBS) and trypsin were purchased from Gibco (Grand Island, New York). RPMI 1640 was obtained from Nissui Pharmaceutical (Tokyo, Japan). Pronase E and type I collagenase were purchased from Kaken Pharmaceutical (Kyoto, Japan) and Nitta Gelatin (Osaka, Japan), respectively. Nimesulide and flurbiprofen were from Cayman Chemical (Ann Arrbor, Michigan). Cholesterol, dicetyl phosphate (DCP), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), mefenamic acid, and flufenamic acid were from Sigma (Tokyo, Japan). Egg phosphatidylcholine (PC) was from Kanto Chemicals (Tokyo, Japan). Celecoxib was from LKT Laboratories (St. Paul, Minnesota). Fluo-3/acetoxymethyl ester (AM) and Pluronic F127 were from Dojindo Lab (Tokyo, Japan). Male guinea pigs (4 weeks of age) were purchased from Shimizu (Kyoto, Japan). All experiments and procedures described here were approved by the Animal Care Committee of Kumamoto University.

Preparation and culture of gastric mucosal cells. Gastric mucosal cells were isolated from guinea pig fundic glands as described previously [22–24]. Isolated gastric mucosal cells (3×10^6 cells/dish) were cultured for 12 h in RPMI 1640 medium containing 0.3% FBS, 100 U/ml penicillin, 2% BSA, and 100 μg/ml streptomycin in type-I collagen-coated plastic culture plates under the conditions of 5% CO_2/95% air and 37 °C. After removing non-adherent cells by washing with RPMI 1640, cells that were attached to plates at about 50% confluence were used. Guinea pig gastric mucosal cell preparations cultured under these conditions have been previously characterized, with the majority (about 90%) of cells being identified as pit cells [22,25].

Treatment of cells with NSAIDs. Cells were exposed to NSAIDs by replacement of the entire bathing medium with fresh medium containing the NSAID under investigation. NSAIDs were dissolved in DMSO and control experiments (without NSAIDs) were performed in the presence of same concentrations of DMSO. It was known that NSAIDs have high affinity for proteins, however, BSA and FBS in medium did not affect the cell death by NSAIDs in our system (data not shown).

For monitoring cell viability, cells were incubated for 2 h with MTT solution at a final concentration of 1 mg/ml. Isopropanol and hydrochloric acid were added to the culture medium at the final concentrations of 50% and 20 mM, respectively. The optical density of each sample at 570 nm was determined by spectrophotometer using a reference wavelength of 630 nm [26].

Assay for erythrocyte hemolysis and K^+ efflux. Hemolysis and K^+ efflux in erythrocytes were monitored as described [27,28] with some modifications. Human erythrocytes were washed twice with buffer A (5 mM Hepes/NaOH (pH 7.4) and 150 mM NaCl) and then suspended in fresh buffer A at a final concentration of 0.5% hematocrit (5×10^7 cells/ml). The concentration of phospholipids in this suspension was 30 μM in egg PC equivalent. After incubation with NSAIDs for 10 min at 30 °C, hemolysis was estimated by measuring the absorbance at 540 nm. K^+ efflux was measured with a K^+ ion-selective electrode.

Membrane permeability assay. Liposomes were prepared using reversed-phase evaporation method [29,30]. Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2, v/v) in the presence or absence of cholesterol (7.5 μmol, 2.9 mg) or DCP (1 μmol, 0.547 mg), dried, and dissolved in 1.5 ml diethyl ether. This was followed by the addition of 1 ml of 100 mM calcein–NaOH (pH 7.4). The mixture was sonicated to obtain a homogeneous emulsion. The diethyl ether solvent was removed using a conventional rotary evaporator under reduced pressure at 25 °C. The resulting suspension of liposome was centrifuged and washed twice with fresh buffer A to remove untrapped calcein. The final liposome precipitate was re-suspended in 5 ml buffer A. A 0.3 ml aliquot of this suspension was diluted with 19.7 ml buffer A, following which 500 μl of this suspension was incubated at 30 °C for 10 min in the presence of the NSAID under investigation. The final PC concentration was 30 μM. The release of calcein from liposomes was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm).

Fluorescence polarization. Membrane fluidity was measured by the fluorescence polarization technique [31]. Diphenylhexatriene (1 mol% of egg PC) was used as a fluorescence probe. Liposomes were prepared using reversed-phase evaporation method similar to membrane permeability experiments, except for the addition of 1 ml buffer A instead of 100 mM calcein–NaOH. The final PC concentration was 30 μM. Measurements were carried out using a Hitachi F-4500 fluorospectrophotometer. The degree of polarization (P) was calculated according to the following equation:

$$P = (I_{VV} - C_f I_{VH})/(I_{VV} + C_f I_{VH}),$$

where I is the fluorescence intensity, and subscripts V and H refer, respectively, to the vertical and horizontal orientations of the excitation (first) and emission (second) polarizers. C_f ($=I_{HV}/I_{HH}$) is a correction factor.

Measurement of the intracellular Ca^{2+} level. The intracellular Ca^{2+} level was monitored according to manufacturer's protocols (Dojindo Lab) [32]. Cells were detached by trypsin and washed with the assay buffer containing 115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM Hepes, and 13.8 mM glucose. Then cells were incubated with 4 μM fluo-3/AM in the assay buffer containing 0.1% BSA, 0.04% Pluronic F127, and 2 mM probenecid for 40 min at 37 °C. After washing twice with the assay buffer, cells were suspended with the assay buffer containing 2 mM probenecid. Fluo-3 fluorescence was measured in a water-jacketed cuvette (1.6×10^6 cells/cuvette) with a HITACHI F-2000 spectrofluorophotometer by recording excitation signals at 490 nm and emission signal at 530 nm at 1-s intervals. Maximum and minimum fluorescence values (F_{max} and F_{min}) were obtained by adding 10 μM ionomycin and 10 μM ionomycin plus 5 mM EGTA (in Ca^{2+} free medium), respectively. The intracellular Ca^{2+} level was calculated according to the equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the apparent dissociation constant (400 nM) of the fluorescence dye–Ca^{2+} complex [32].

Results and discussion

Induction of necrosis and apoptosis by NSAIDs in gastric mucosal cells

Fig. 1 shows structures of the five different NSAIDs that were used in this study. We previously reported that short-term (1 h) treatment of primary cultures of guinea pig gastric mucosal cells with relatively high concentrations of NSAIDs and long-term (16 h) treatment of these cells with relatively low concentrations of NSAIDs induced necrosis and apoptosis, respectively [15]. We first tested the ability of the different NSAIDs (Fig. 1) to induce necrosis and apoptosis, and found that cell viability was decreased following short-term (1 h) treatment with each NSAID tested (Fig. 2A). Since cell death in this manner was not associated with apoptotic DNA fragmentation and chromatin condensation (data not shown), it is likely to have been mediated by necrosis. In contrast, the decrease in cell viability with long-term (16 h) NSAID treatment (Fig. 2B) was associated with apoptotic DNA fragmentation and chromatin condensation (data not shown), suggesting that it is mediated by apoptosis. Higher concentrations of NSAIDs were required to induce necrosis compared to those required for apoptosis (Fig. 2), which is consistent with previous reports [15]. Among all of the NSAIDs tested, celecoxib showed the most potent necrosis- and apoptosis-inducing activity, followed by flufenamic acid (Fig. 2). The cytotoxicity of NSAIDs is not directly related to their potency to produce gastric lesions in vivo. This is because both the inhibition of COX activity and the direct cytotoxicity of NSAIDs are involved in the induction of gastric lesions in vivo [16]. Furthermore, various other factors, such as mucosal blood flow and gastric motility, are also involved in the induction of gastric lesions in vivo.

Two subtypes of COX, COX-1 and COX-2, are responsible for the majority of COX activity in gastric mucosal and inflammatory tissues, respectively, and recently a number of COX-2-selective NSAIDs were developed [33]. Among the NSAIDs whose data are graphed in Fig. 2, nimesulide and celecoxib have selectivity for COX-2. No relationship was evident between NSAID cytotoxicity and selectivity for COX-2, supporting the idea that the direct cytotoxicity of NSAIDs is independent of COX inhibition. We also confirmed that exogenously added PGE_2 (either native PGE_2 or 16,16-dimethyl-PGE_2) did not affect the extent of cell death by short-term and long-term treatment with NSAIDs even at a higher concentration of PGE_2 in the culture medium than is present endogenously (10^{-9} M) (data not shown).

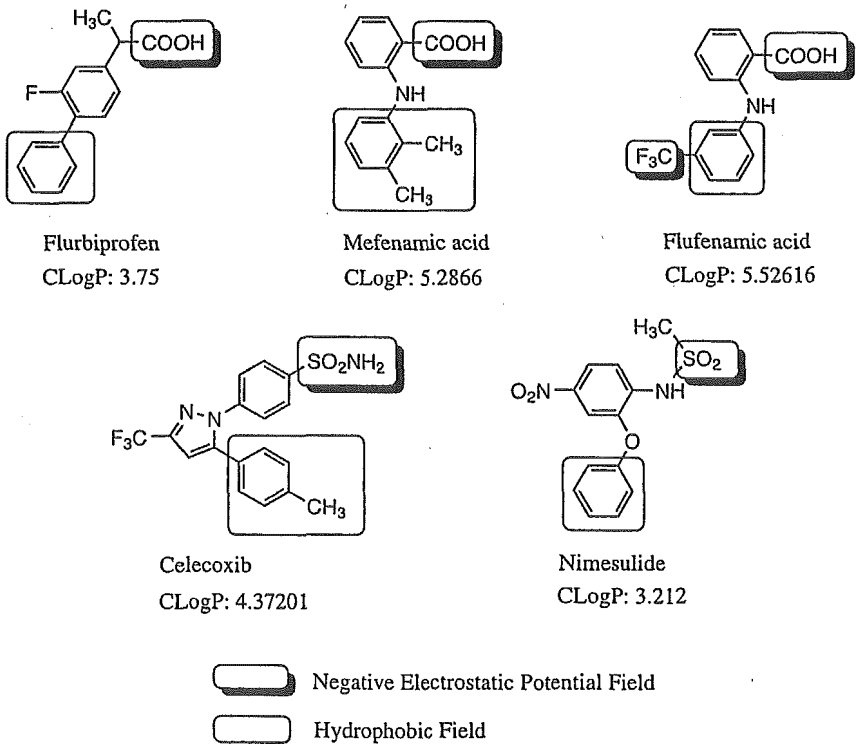

Fig. 1. Molecular structures and CLogP values of NSAIDs. Discriminative negative electrostatic potential fields are shown with bold-lined boxes, and discriminative hydrophobic fields (phenyl groups) are shown with normal-lined boxes. CLogP values were calculated with CLOGP3 program (Pomona MedChem Software 3.6).

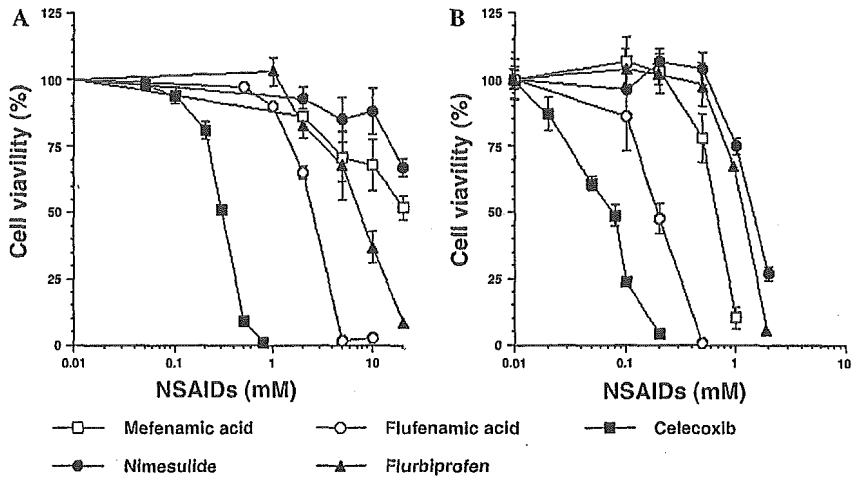

Fig. 2. Necrosis and apoptosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with NSAID under investigation for 1 h (A) or 16 h (B). Cell viability was determined by the MTT method. Values are means ± SEM ($n = 3$).

NSAID-induced hemolysis and K^+ efflux

Measurement of hemolysis is a standard method for testing the membrane permeabilization activities of drugs. As shown in Fig. 3A, all of the tested NSAIDs caused hemolysis of erythrocytes; a finding which strongly suggests that NSAIDs increase the permeability of cell membranes. The relative potency of each NSAID for hemolysis was approximately similar to that for cytotoxicity. For example, celecoxib showed the most potent activity for hemolysis, followed by flufenamic acid (Fig. 3A), which is in relative accordance with the cytotoxic potency of each NSAID (Fig. 2). Therefore, it would appear that NSAID-induced cell death (necrosis and apoptosis) is mediated by membrane permeabilization.

We also measured K^+ efflux from erythrocytes in the presence of each NSAID and found that most of them stimulated K^+ efflux (Fig. 3B) at similar concentrations to those required for inducing hemolysis (Fig. 3A). However, the increase in K^+ efflux induced by celecoxib was observed at a lower concentration than that at which hemolysis was observed (Fig. 3). This result suggests that, in a manner different from that of other NSAIDs, celecoxib causes initially the formation of small pores in the cytoplasmic membrane that are able to mediate the efflux of K^+ but not of hemoglobin.

Membrane permeabilization by NSAIDs

We next examined the ability of each NSAID to permeabilize calcein-loaded liposomes prepared from PC. Calcein fluoresces very weakly at high concentrations due to self-quenching. Thus, the addition of membrane permeabilizing drugs to a medium containing calcein-

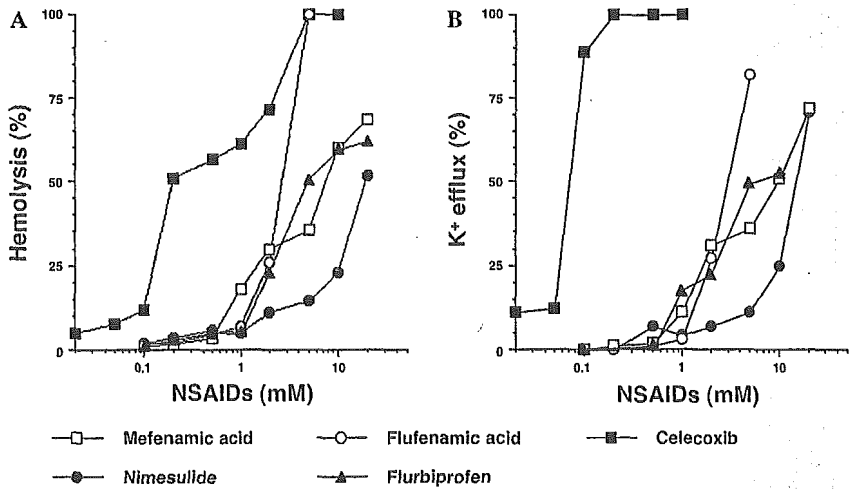

Fig. 3. NSAID-induced hemolysis and K^+ efflux from erythrocytes. Human erythrocytes were incubated in the presence of each NSAID for 10 min at 30 °C. Hemolysis was estimated by measuring the absorbance at 540 nm (A). The level of K^+ efflux was measured with a K^+ ion-selective electrode (B). Melittin (10 μM), a membrane permeabilizing reagent, was used to determine the 100% level of hemolysis and K^+ efflux [28].

loaded liposomes should cause an increase in fluorescence by releasing calcein trapped inside the liposomes [29,30]. Calcein fluorescence increased in the presence of each of all the NSAIDs tested, thereby showing that NSAIDs have membrane permeabilization effects on PC liposomes (Fig. 4). The target of NSAIDs in terms of their membrane permeabilization effects thus appears to be phospholipids. The relative potency of each NSAID for calcein release (Fig. 4) was approximately similar to that for cytotoxicity (Fig. 2). For example, celecoxib showed the most potent activity for calcein release, followed by flufenamic acid (Fig. 4). It would appear that the cytotoxic action of NSAIDs is mediated through their ability to permeabilize membranes.

As for the mechanism of membrane permeabilization-dependent cell death (necrosis and apoptosis), we considered the contribution of intracellular Ca^{2+} level, based on previous results; permeabilization of cytoplasmic membrane causes increase in intracellular Ca^{2+} level, increase in intracellular Ca^{2+} level can cause cell death through induction of both necrosis and apoptosis [34], and some NSAIDs increased the intracellular Ca^{2+} level [35–41]. Therefore, we examined the effect of each NSAID on the intracellular Ca^{2+} level by use of fluo-3/AM assay system. As shown in Fig. 5, each NSAID tested significantly increased the intracellular Ca^{2+} level at concentrations of ED_{50} value for apoptosis (concentrations required for inducing apoptosis in 50% cells), in other words, accompanying induction of apoptosis. This increase in the intracellular Ca^{2+} level by NSAIDs may contribute to their activity for inducing cell death.

The endoplasmic reticulum (ER) stress response is a cellular mechanism that aids in protecting the ER against ER stressors and is involved in ER stressor-induced apoptosis. We recently reported that exposure of cells to NSAID induced GRP78 that protects cells from ER stressor as well as CHOP, a transcription factor involved in apoptosis. Since NSAID-induced apoptosis was suppressed in cultured guinea pig gastric mucosal cells by expression of the dominant negative form of CHOP, or in peritoneal macrophages from CHOP-deficient mice, we proposed that ER stress response-related proteins, particularly CHOP, are involved in NSAID-induced apoptosis [42]. However, the upstream pathway for NSAID-induced ER stress response (induction of CHOP) remained unknown. In this study, we found that NSAID-induced apoptosis is related to their activity for membrane permeabilization and increase in the intracellular Ca^{2+} level. It is known that increase in intracellular Ca^{2+} level induces ER stress

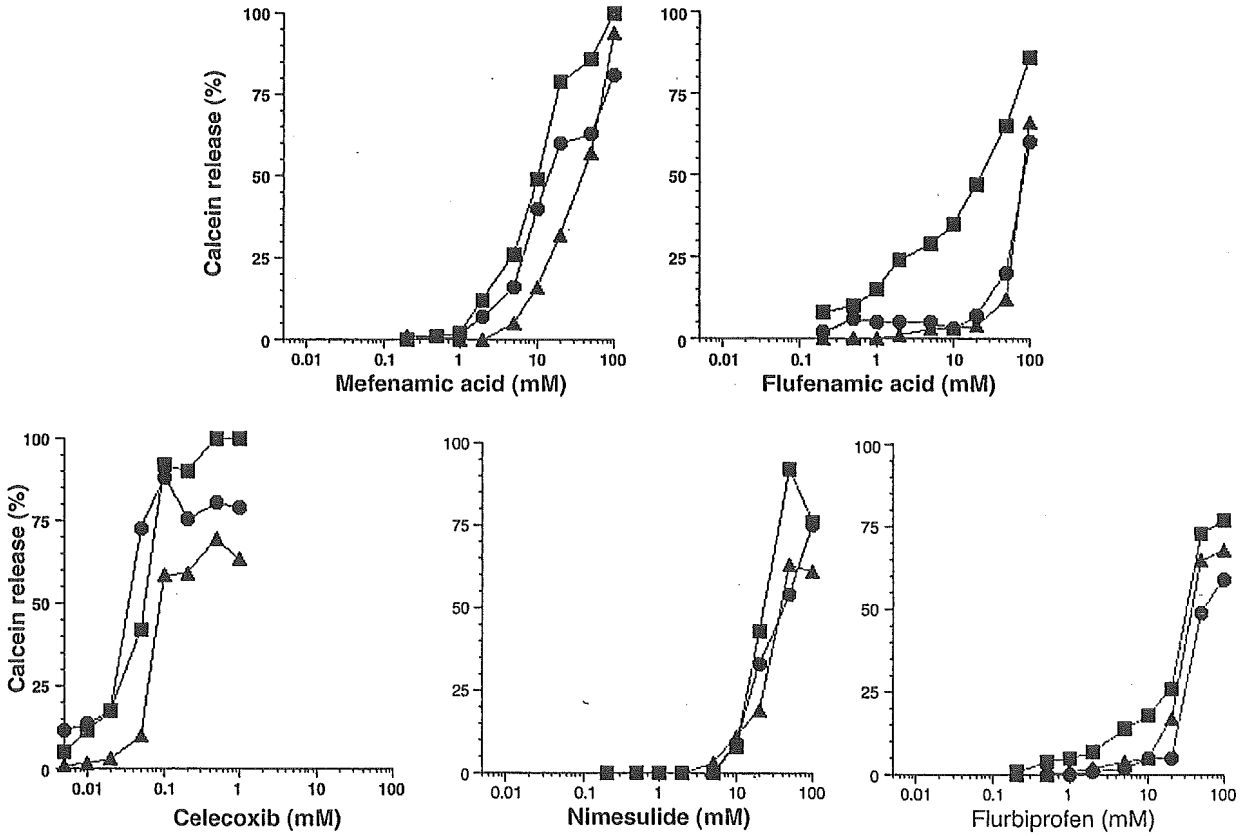

Fig. 4. Membrane permeabilization induced by NSAIDs. Calcein-loaded liposomes prepared from PC (squares), PC/cholesterol (circles), or PC/DCP (triangles) were incubated with each NSAID for 10 min at 30 °C. The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin (10 μM) was used to determine the 100% level of membrane permeabilization [28].

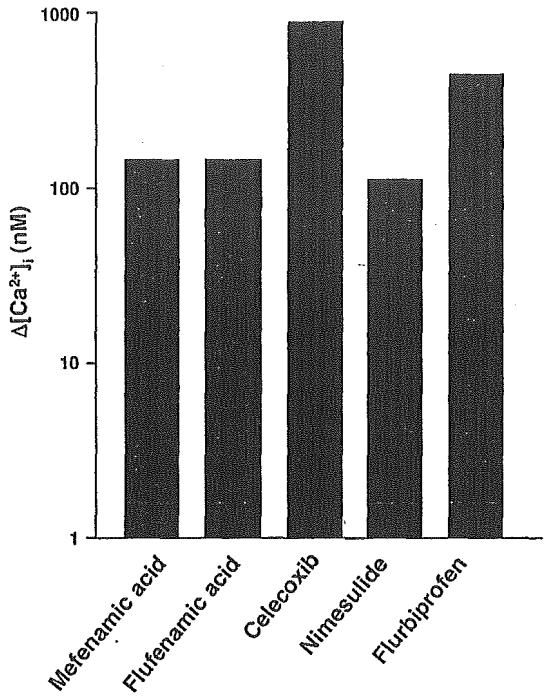

Fig. 5. Increase in the intracellular Ca^{2+} level by NSAIDs. The intracellular Ca^{2+} level was monitored by fluo-3/AM assay system as described in Materials and methods. NSAIDs were added to fluo-3/AM-loaded cells at concentrations of ED_{50} values for apoptosis (mefenamic acid, 0.7 mM; flufenamic acid, 0.2 mM; celecoxib, 0.08 mM; nimesulide, 1.6 mM; and flurbiprofen, 1.2 mM) and time course of fluo-3 fluorescence change was monitored. The maximum value for increase in the intracellular Ca^{2+} level ($\Delta[Ca^{2+}]_i$) of each NSAID was shown.

response; Ca^{2+} ionophore induces ER stress response [43,44]. Therefore, we assume that the increase in intracellular Ca^{2+} level is located in the upstream pathway of NSAID-induced ER stress response. In other words, permeabilization of cytoplasmic membrane by NSAIDs increases intracellular Ca^{2+} level, which in turn induced ER stress response (induction of CHOP), resulting in induction of apoptosis. Since celecoxib (but not other NSAIDs) was reported to inhibit sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [37], apoptosis by celecoxib may also involve this SERCA inhibition.

Although the chemical structures of the NSAIDs used in the experiments reported here are quite different, we attempted to identify a structure–activity relationship by focusing on common structural features between the various compounds. As shown in Fig. 1, all of the tested NSAIDs have hydrophobic field (phenyl groups) and negative electrostatic potential field (sulfonamide or carboxyl group). The partition coefficient is the equilibrium concentration of solute in a non-polar solvent divided by the concentration of the same species in a polar solvent. In this and most other applications, the polar solvent is water. The logarithm of the partition coefficient, log *P*, has been successfully used as a hydrophobic parameter in 'extrathermo-dynamic' Hammett methodology. 1-Octanol has much to recommend it as the choice for the non-polar phase and log *P* has been used successfully in quantitative structure–activity relationships (QSAR). By now many efficient methods of measurement of octanol/water partition coefficients have been developed, and the first attempt to reduce log *P* calculation to computer algorithm was done by Chou and Jurs [45]. It was called CLOGP. In this paper we calculated the CLogP (calculated log *P* by CLOGP program) values of the compounds, which indicate their hydrophobicity (the larger the CLogP value the higher the hydrophobicity) using CLOGP3 program (Pomona MedChem Software 3.6) (http://clogp.pomona.edu/medchem/chem/papers/14-clogp.html). This program is one of the efficient methods to calculate log *P* from structure by an additive-constitutive procedure. As can also be deduced from Fig. 1, there is a slight tendency for compounds with larger CLogP values to have higher cytotoxic (or membrane permeabilization) activity. For example, compounds with a CLogP value higher than 4.0 coincide with those with an ED_{50} value (for apoptosis induction) lower than 1.0 mM (Fig. 1). We consider that the common structural features described above and high CLogP values may be important if NSAIDs are to have potent cytotoxic (or membrane permeabilization) activity.

Mechanism of membrane permeabilization by NSAIDs

The effect of cholesterol on membrane permeabilization (calcein release) by NSAIDs was also examined in this study. As shown in Fig. 4, cholesterol made PC liposomes resistant to a NSAID (flufenamic acid) but not so evidently to others. We also examined the effect of anionic lipids (DCP) on membrane permeabilization (calcein release) by NSAIDs. DCP also made liposomes resistant to some NSAIDs (mefenamic acid, flufenamic acid, and celecoxib), but again not so clearly to others. These data suggest that the mechanism of membrane permeabilization by NSAIDs is different depending on the NSAID under investigation.

Some NSAIDs (for example, indomethacin and naproxen) are known to affect membrane fluidity [19]. We therefore examined the effect of each NSAID on membrane fluidity using the fluorescence polarization technique. In such experiments, the higher the calculated *P* value, the lower the membrane fluidity. As shown in Table 1, most of the NSAIDs tested (mefenamic acid, flufenamic acid, celecoxib, and nimesulide) decreased membrane fluidity. However, this effect of NSAIDs was not closely related with their cytotoxicity and membrane permeabilization activity. Flurbiprofen did not significantly affect membrane fluidity (Table 1). We also examined the effect of the different NSAIDs on the membrane fluidity of liposomes containing cholesterol. As shown in Table 1, cholesterol inhibited the decrease

Table 1
Effect of various NSAIDs on membrane fluidity

NSAID (mM)	Degree of polarization (*P*)	
	PC	PC + cholesterol
Control	0.095	0.306
Mefenamic acid		
0.1	0.170	0.298
Flufenamic acid		
0.1	0.134	0.302
1	0.259	0.335
10	0.304	0.341
Celecoxib		
0.01	0.106	0.273
0.1	0.117	0.236
1	0.391	0.288
Nimesulide		
0.1	0.209	0.316
1	0.196	
Flurbiprofen		
0.1	0.101	0.308
1	0.105	0.290
10	0.107	0.262

The degree of polarization (*P*) of PC or PC/cholesterol liposomes in the presence of various NSAIDs was measured as described under Materials and methods.

in membrane fluidity caused by NSAIDs as described above. Cholesterol, itself, decreased membrane fluidity in the absence of NSAIDs. All these data suggest that permeabilization activity of NSAIDs cannot be simply explained by a decrease in membrane fluidity.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan, as well as by the Suzuken Memorial Foundation, the Japan Research Foundation for Clinical Pharmacology, and a Sasakawa Scientific Research Grant from the Japan Science Society.

References

[1] W.E. Smalley, W.A. Ray, J.R. Daugherty, M.R. Griffin, Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons, Am. J. Epidemiol. 141 (1995) 539–545.
[2] C.J. Hawkey, Nonsteroidal anti-inflammatory drug gastropathy, Gastroenterology 119 (2000) 521–535.
[3] C.H. Barrier, B.I. Hirschowitz, Controversies in the detection and management of nonsteroidal antiinflammatory drug-induced side effects of the upper gastrointestinal tract, Arthritis Rheum. 32 (1989) 926–932.
[4] S.E. Gabriel, L. Jaakkimainen, C. Bombardier, Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs. A meta-analysis, Ann. Intern. Med. 115 (1991) 787–796.
[5] J.F. Fries, S.R. Miller, P.W. Spitz, C.A. Williams, H.B. Hubert, D.A. Bloch, Toward an epidemiology of gastropathy associated with nonsteroidal antiinflammatory drug use, Gastroenterology 96 (1989) 647–655.
[6] J.H. Kurata, D.E. Abbey, The effect of chronic aspirin use on duodenal and gastric ulcer hospitalizations, J. Clin. Gastroenterol. 12 (1990) 260–266.
[7] G. Singh, Recent considerations in nonsteroidal anti-inflammatory drug gastropathy, Am. J. Med. 105 (1998) 31S–38S.
[8] J.R. Vane, R.M. Botting, Mechanism of action of anti-inflammatory drugs, Scand. J. Rheumatol. Suppl. 102 (1996) 9–21.
[9] T.A. Miller, Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms, Am. J. Physiol. 245 (1983) G601–G623.
[10] M. Ligumsky, E.M. Golanska, D.G. Hansen, G.J. Kauffman, Aspirin can inhibit gastric mucosal cyclo-oxygenase without causing lesions in rat, Gastroenterology 84 (1983) 756–761.
[11] M. Ligumsky, M. Sestieri, F. Karmeli, J. Zimmerman, E. Okon, D. Rachmilewitz, Rectal administration of nonsteroidal antiinflammatory drugs. Effect on rat gastric ulcerogenicity and prostaglandin E2 synthesis, Gastroenterology (1990) 1245–1249.
[12] L.M. Lichtenberger, 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 (2001) 631–637.
[13] L.M. Lichtenberger, Z.M. Wang, J.J. Romero, C. Ulloa, J.C. Perez, M.N. Giraud, J.C. Barreto, Non-steroidal anti-inflammatory drugs (NSAIDs) associate with zwitterionic phospholipids: insight into the mechanism and reversal of NSAID-induced gastrointestinal injury, Nat. Med. 1 (1995) 154–158.
[14] S. Somasundaram, S. Rafi, J. Hayllar, G. Sigthorsson, M. Jacob, A.B. Price, A. Macpherson, T. Mahmod, D. Scott, J.M. Wrigglesworth, I. Bjarnason, Mitochondrial damage: a possible mechanism of the "topical phase of NSAID induced injury to the rat intestine, Gut 41 (1997) 344–353.
[15] W. Tomisato, S. Tsutsumi, K. Rokutan, T. Tsuchiya, T. Mizushima, NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture, Am. J. Physiol. Gastrointest. Liver Physiol. 281 (2001) G1092–1100.
[16] W. Tomisato, S. Tsutsumi, T. Hoshino, H.J. Hwang, M. Mio, T. Tsuchiya, T. Mizushima, Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions, Biochem. Pharmacol. 67 (2004) 575–585.
[17] X.H. Liu, S. Yao, A. Kirschenbaum, A.C. Levine, NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells, Cancer Res. 58 (1998) 4245–4249.
[18] P. Schwenger, P. Bellosta, I. Vietor, C. Basilico, E.Y. Skolnik, J. Vilcek, Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-Jun N-terminal kinase/stress-activated protein kinase activation, Proc. Natl. Acad. Sci. USA 94 (1997) 2869–2873.
[19] M.N. Giraud, C. Motta, J.J. Romero, G. Bommelaer, L.M. Lichtenberger, Interaction of indomethacin and naproxen with gastric surface-active phospholipids: a possible mechanism for the gastric toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs), Biochem. Pharmacol. 57 (1999) 247–254.
[20] B.S. Anand, J.J. Romero, S.K. Sanduja, L.M. Lichtenberger, Phospholipid association reduces the gastric mucosal toxicity of aspirin in human subjects, Am. J. Gastroenterol. 94 (1999) 1818–1822.
[21] H. Aki, M. Yamamoto, Biothermodynamic characterization of erythrocyte hemolysis induced by phenothiazine derivatives and anti-inflammatory drugs, Biochem. Pharmacol. 39 (1990) 396–398.

[22] K. Rokutan, M. Yamada, J. Torigoe, T. Saito, Transforming growth factor-beta inhibits proliferation and maturation of cultured guinea pig gastric pit cells, Am. J. Physiol. 275 (1998) G526–533.
[23] S. Tsutsumi, W. Tomisato, T. Takano, K. Rokutan, T. Tsuchiya, T. Mizushima, Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture, Biochim. Biophys. Acta 1589 (2002) 168–180.
[24] T. Hoshino, S. Tsutsumi, W. Tomisato, H.J. Hwang, T. Tsuchiya, T. Mizushima, Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation, J. Biol. Chem. 278 (2003) 12752–12758.
[25] W. Tomisato, T. Hoshino, S. Tsutsumi, T. Tsuchiya, T. Mizushima, Maturation-associated increase in sensitivity of cultured guinea pig gastric pit cells to hydrogen peroxide, Dig. Dis. Sci. 47 (2002) 2125–2133.
[26] B.B. Wolf, D.R. Green, Suicidal tendencies: apoptotic cell death by caspase family proteinases, J. Biol. Chem. 274 (1999) 20049–20052.
[27] T. Katsu, M. Kuroko, T. Morikawa, K. Sanchika, H. Yamanaka, S. Shinoda, Y. Fujita, Interaction of wasp venom mastoparan with biomembranes, Biochim. Biophys. Acta 1027 (1990) 185–190.
[28] T. Katsu, H. Kobayashi, T. Hirota, Y. Fujita, K. Sato, U. Nagai, Structure–activity relationship of gramicidin S analogues on membrane permeability, Biochim. Biophys. Acta 899 (1987) 159–170.
[29] T. Katsu, Application of calcein-loaded liposomes for the determination of membrane channel size, Biol. Pharm. Bull. 22 (1999) 978–980.
[30] R.R.C. New, Liposomes: A Practical Approach, IRL Press, Oxford, 1990.
[31] M. Makise, S. Mima, T. Katsu, T. Tsuchiya, T. Mizushima, Acidic phospholipids inhibit the DNA-binding activity of DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*, Mol. Microbiol. 46 (2002) 245–256.
[32] J.P. Kao, A.T. Harootunian, R.Y. Tsien, Photochemically generated cytosolic calcium pulses and their detection by fluo-3, J. Biol. Chem. 264 (1989) 8179–8184.
[33] J. Vane, Towards a better aspirin, Nature 367 (1994) 215–216.
[34] S. Orrenius, B. Zhivotovsky, P. Nicotera, Regulation of cell death: the calcium-apoptosis link, Nat. Rev. Mol. Cell Biol. 4 (2003) 552–565.
[35] W.J. Murdoch, Differential effects of indomethacin on the sheep ovary: prostaglandin biosynthesis, intracellular calcium, apoptosis, and ovulation, Prostaglandins 52 (1996) 497–506.
[36] B.L. Tepperman, B.D. Soper, Effect of extracellular Ca^{2+} on indomethacin-induced injury to rabbit dispersed gastric mucosal cells, Can. J. Physiol. Pharmacol. 72 (1994) 63–69.
[37] A.J. Johnson, A.L. Hsu, H.P. Lin, X. Song, C.S. Chen, The cyclo-oxygenase-2 inhibitor celecoxib perturbs intracellular calcium by inhibiting endoplasmic reticulum Ca^{2+}-ATPases: a plausible link with its anti-tumour effect and cardiovascular risks, Biochem. J. 366 (2002) 831–837.
[38] J.L. Wang, K.L. Lin, J.S. Chen, Y.C. Lu, B.P. Jiann, H.T. Chang, S.S. Hsu, W.C. Chen, J.K. Huang, C.M. Ho, C.R. Jan, Effect of celecoxib on Ca^{2+} movement and cell proliferation in human osteoblasts, Biochem. Pharmacol. 67 (2004) 1123–1130.
[39] S. Fiorucci, E. Antonelli, G. Migliorati, L. Santucci, O. Morelli, B. Federici, A. Morelli, TNFalpha processing enzyme inhibitors prevent aspirin-induced TNFalpha release and protect against gastric mucosal injury in rats, Aliment. Pharmacol. Ther. 12 (1998) 1139–1153.
[40] R.A. Levine, J. Nandi, R.L. King, Aspirin potentiates prestimulated acid secretion and mobilizes intracellular calcium in rabbit parietal cells, J. Clin. Invest. 86 (1990) 400–408.
[41] U.K. Murthy, R.A. Levine, Aspirin induces morphological transformation to the secretory state in isolated rabbit parietal cells, Gastroenterology 101 (1991) 404–409.
[42] S. Tsutsumi, T. Gotoh, W. Tomisato, S. Mima, T. Hoshino, H.J. Hwang, H. Takenaka, T. Tsuchiya, M. Mori, T. Mizushima, Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis, Cell Death Differ. 11 (2004) 1009–1016.
[43] S. Nozaki, G.W. Sledge Jr., H. Nakshatri, Repression of GADD153/CHOP by NF-kappaB: a possible cellular defense against endoplasmic reticulum stress-induced cell death, Oncogene 20 (2001) 2178–2185.
[44] H. Zinszner, M. Kuroda, X. Wang, N. Batchvarova, R.T. Lightfoot, H. Remotti, J.L. Stevens, D. Ron, CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum, Genes Dev. 12 (1998) 982–995.
[45] J.T. Chou, P.C. Jurs, Computer-assisted computation of partition coefficients from molecular structures using fragment constants, J. Chem. Inf. Comput. Sci. 19 (1979) 172–178.

Cell Death and Differentiation (2004) 11, 1009–1016

www.nature.com/cdd

Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis

S Tsutsumi[1], T Gotoh[2], W Tomisato[1], S Mima[1], T Hoshino[1], H-J Hwang[1], H Takenaka[1], T Tsuchiya[1], M Mori[2] and T Mizushima*[,1]

[1] Faculty of Pharmaceutical Sciences, Okayama University, Okayama, Japan
[2] Department of Molecular Genetics, Kumamoto University School of Medicine, Kumamoto, Japan
* Corresponding author: T Mizushima, Faculty of Pharmaceutical Sciences, Okayama University, 1-1-1, Tsushima-naka, Okayama 700-8530, Japan
E-mail: mizushima@pharm.okayama-u.ac.jp

Received 19.9.03; revised 05.2.04; accepted 06.2.04; published online 07.5.04
Edited by H Ichijo

Abstract

Apoptosis induced by nonsteroidal anti-inflammatory drugs (NSAIDs) is involved not only in the production of NSAID-induced gastric lesions but also in the antitumor activity of these drugs. The endoplasmic reticulum (ER) stress response is a cellular mechanism that aids in protecting the ER against ER stressors and is involved in ER stressor-induced apoptosis. Here, we examine the relationship between this response and NSAID-induced apoptosis in cultured guinea-pig gastric mucosal cells. Exposure of cells to indomethacin, a commonly used NSAID, induced GRP78 as well as CHOP, a transcription factor involved in apoptosis. Three factors that positively regulate CHOP expression (ATF6, ATF4 and XBP-1) were activated and/or induced by indomethacin. NSAIDs other than indomethacin (diclofenac, ibuprofen and celecoxib) also induced CHOP. Monitoring of the transcriptional activities of ATF6 and CHOP by luciferase assay revealed that both were stimulated in the presence of indomethacin. Furthermore, indomethacin-induced apoptosis was suppressed in cultured guinea-pig gastric mucosal cells by expression of the dominant-negative form of CHOP, or in peritoneal macrophages from CHOP-deficient mice. These results suggest that ER stress response-related proteins, particularly CHOP, are involved in NSAID-induced apoptosis.
Cell Death and Differentiation (2004) **11**, 1009–1016.
doi:10.1038/sj.cdd.4401436
Published online 7 May 2004

Keywords: NSAIDs; apoptosis; endoplasmic reticulum; CHOP; gastric mucosal cells

Abbreviations: ASK1, apoptosis signal-regulating kinase 1; ATF, activating transcription factor; CHOP, C/EBP homologous transcription factor; COX, cyclooxygenase; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; ERSE, ER stress response element; FBS, fetal bovine serum; GRP, glucose-regulated protein; IRE1, protein-kinase and site-specific endoribonuclease; JNK, c-jun NH_2-terminal kinase; MTT, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NSAIDs, nonsteroidal anti-inflammatory drugs; PEK, pancreatic eIF2 kinase; PERK, protein kinase R-like ER kinase; PG, prostaglandin; TRAF2, tumor necrosis factor receptor-associated factor; UPR, unfolded protein response; XBP-1, X box binding protein

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently used classes of medicines in the world and account for nearly 5% of all prescribed medications.[1] NSAIDs have great efficacy in the treatment of pain, inflammation and fever on account of their inhibition of cyclooxygenase (COX) activity. COX is essential for the synthesis of prostaglandins (PGs), which have a strong involvement in the induction of inflammation. Recently, clinically beneficial properties of NSAIDs other than anti-inflammation have been revealed. Epidemiological studies revealed that prolonged use of aspirin or other NSAIDs reduces the risk of cancer and Alzheimer's disease.[2–4] These activities of NSAIDs were confirmed by a number of *in vivo* and *in vitro* experiments. Since these activities of NSAIDs cannot be fully explained by the COX-inhibition,[5,6] COX-independent actions of NSAIDs are necessary to be identified.

NSAID administration is associated with gastrointestinal complications, such as gastric ulcers.[7] In the United States, about 16 500 people die per year as a result of NSAID-associated gastrointestinal complications.[8] We recently suggested that direct cytotoxic effects of NSAIDs (such as induction of apoptosis), that is, COX-independent are involved in NSAID-induced gastric lesions *in vivo*.[9] Furthermore, in addition to inhibition of angiogenesis and cell growth by NSAIDs, induction of apoptosis by NSAIDs is also involved in their antitumor activities.[6] Therefore, elucidation of the mechanism of NSAID-induced apoptosis is important to understand the mechanism of both NSAID-induced gastric lesions and their antitumor activities. We previously reported that activation of caspases (caspase-3, -8 and -9) is accompanied with induction of apoptosis by NSAIDs.[10] However, the upstream pathways of apoptosis induced by NSAIDs remain unknown.

Accumulation of unfolded protein in the endoplasmic reticulum (ER) induces the ER stress response, otherwise known as the unfolded protein response (UPR). In the mammalian ER stress response, three types of ER transmembrane proteins are important: protein-kinase and site-specific endoribonuclease (IRE1), protein kinase R-like ER kinase/pancreatic eIF2 kinase (PERK/PEK) and activating transcription factor 6 (ATF6).[11–13] The mammalian ER stress response can be separated into two phases, adaptation and

apoptosis. Cells initially adapt to the accumulation of unfolded proteins by inducing ER-resident stress proteins (molecular chaperons) such as glucose-regulated protein (GRP) 78 and GRP94.[14–17] These proteins refold the unfolded proteins in an attempt to maintain homeostasis in the ER. However, if this adaptation does not prove sufficient, the apoptotic response is initiated, by both ATF6- and ATF4-dependent activation of C/EBP homologous transcription factor (CHOP).[18] In this study, we revealed that NSAIDs induce ER stress response. We found that indomethacin induces both GRP78 and CHOP. It also causes activation of ATF6, ATF4 and X box binding protein (XBP-1). Experiment using the dominant-negative form of CHOP and cells from CHOP-deficient mice implicated that the activation of CHOP is involved in NSAID-induced apoptosis.

Results

Induction of GRP78 and CHOP by indomethacin associated with apoptosis

We have previously reported that long-term (16 h) treatment of primary cultures of guinea-pig gastric mucosal cells with NSAIDs (1 mM for indomethacin) induces apoptosis.[10] In order to reveal the pathway underlying this apoptosis, we used DNA microarray techniques to search for genes whose expression is stimulated by indomethacin under these apoptotic conditions, successfully identifying *GRP78* as one such gene (S Mima *et al.*, unpublished results). In the present study, we first confirmed the indomethacin-dependent induction of GRP78 in gastric mucosal cells. Figure 1 shows the time-course and dose–response of apoptosis induced by indomethacin. A decrease in cell viability and apoptotic DNA fragmentation were observed when cells were treated with 0.5–1 mM indomethacin for 4–16 h (Figure 1), this being consistent with our previously reported results.[10] We also confirmed the induction of apoptosis by showing the chromatin condensation and caspase-3 activation under the conditions (data not shown). Furthermore, we showed that almost all cells were not stained with propidium iodide,[10] showing that necrosis was not induced under the conditions (less than 1 mM) (data not shown). In subsequent experiments, we treated cells with 1 mM indomethacin for 16 h in order to examine the mechanism underlying this apoptosis.

The effect of indomethacin on the expression of GRP78 was examined by immunoblotting analysis (Figure 2). GRP78 was present in nontreated control cells and increased in response to treatment with thapsigargin (an inhibitor of sarco ER Ca^{2+} ATPase and a representative inducer of the ER stress response), as previously described.[19] Indomethacin slightly but significantly increased the amount of GRP78 (Figure 2). We also examined the effect of indomethacin on the expression of another ER stress response-related protein, CHOP, a transcription factor, which induces apoptosis. CHOP was not present in nontreated cells, but was induced by thapsigargin, this being consistent with previously reported results.[19] Indomethacin clearly induced CHOP, and to a greater extent than thapsigargin (Figure 2).

We also examined the effect of indomethacin on the expression of *GRP78* and *CHOP* mRNA by Northern blotting analysis (Figure 3). Similar to thapsigargin, indomethacin increased the amount of *GRP78* and *CHOP* mRNA, suggesting that these ER stress response-related proteins are induced at the level of transcription. Given that CHOP is a

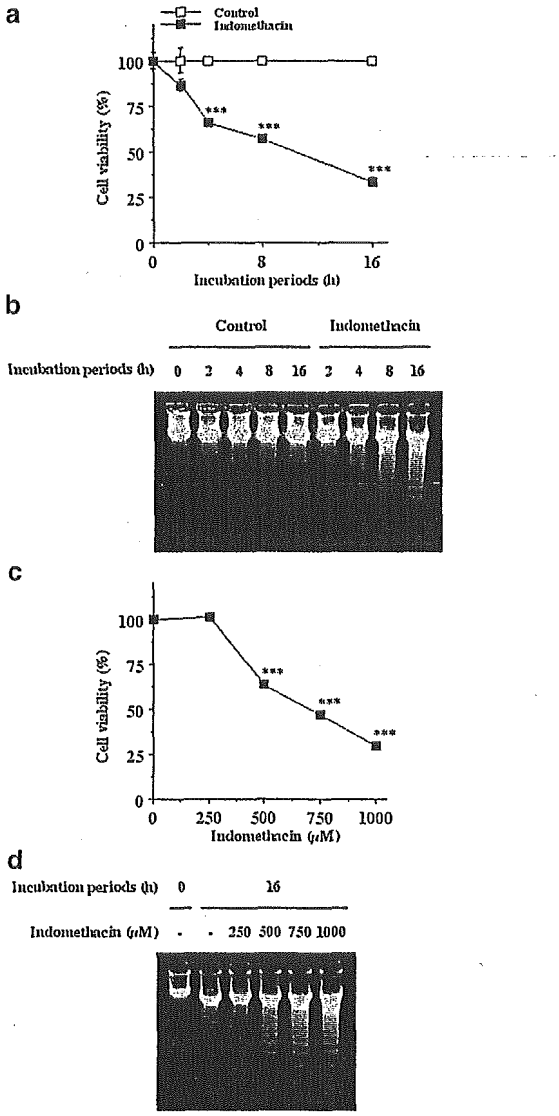

Figure 1 Apoptosis induced by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM (**a, b**) or the indicated concentrations (**c, d**) of indomethacin for the indicated periods (**a, b**) or 16 h (**c, d**). Cell viability was determined by the MTT method. Values are expressed as mean ± S.E.M. ($n=4$). ***$P<0.001$ (**a, c**). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (**b, d**)

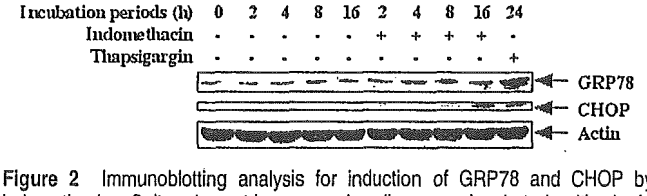

Figure 2 Immunoblotting analysis for induction of GRP78 and CHOP by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin or 2 μM thapsigargin (positive control) for the indicated periods. Whole cell extracts (20 μg protein for GRP78, 5 μg protein for actin and 30 μg protein for CHOP) were analyzed by immunoblotting with an antibody against GRP78, actin or CHOP

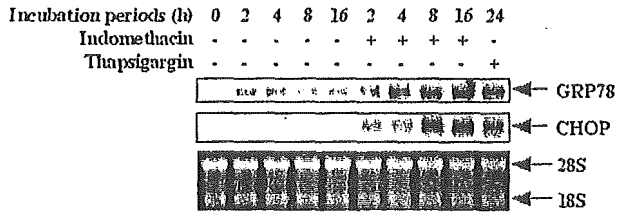

Figure 3 Northern blotting analysis for induction of *GRP78* and *CHOP* mRNA by indomethacin. Cultured gastric mucosal cells were incubated with 1 mM indomethacin or 2 μM thapsigargin (positive control) for the indicated periods. The level of the *GRP78* and *CHOP* mRNA was monitored by Northern blotting analysis. Bands of ribosomal RNA (28S and 18S) stained with ethidium bromide are shown

transcription factor involved in the induction of apoptosis, we then focused on CHOP induction by indomethacin in order to understand the mechanism of NSAID-induced apoptosis.

Induction of apoptosis is not specific for indomethacin, but is observed in response to NSAIDs in general. Given that we have previously found that aspirin, diclofenac, ibuprofen and celecoxib also induce apoptosis,[9] we examined the effect of some of these agents on *CHOP* mRNA induction. As well as indomethacin (1 mM), treatment of cells with diclofenac (1 mM), ibuprofen (2 mM) or celecoxib (80 μM) caused a decrease in cell viability and apoptotic DNA fragmentation, confirming that apoptosis is induced under these conditions (Figure 4a and b). These NSAIDs increased the amount of *CHOP* mRNA (Figure 4c). There are two subtypes of COX, these being COX-1 and COX-2, but COX activity in gastric mucosal cells is mainly derived from COX-1. With the exception of celecoxib, which is COX-2 specific, NSAIDs used in Figure 4 can inhibit both subtypes.[20] Therefore, if the induction of *CHOP* mRNA by indomethacin is caused by COX inhibition in gastric mucosal cells (in other words, inhibition of COX-1), higher concentrations of celecoxib would be required to reveal an effect. Given that the reverse was the case, it appears that CHOP induction by NSAIDs is not related to inhibition of COX and PG synthesis in gastric mucosal cells. Furthermore, we found that the addition of PGE_2 to the culture medium did not attenuate apoptosis and CHOP induction by indomethacin (data not shown).

We have previously established conditions under which other gastric stressors (ethanol, hydrogen peroxide and hydrochloric acid) induce apoptosis in primary cultures of guinea-pig gastric mucosal cells.[21] We therefore examined whether these gastric stressors induce CHOP under apoptotic conditions. Treatment of cells with 4% ethanol for 6 h, 300 μM hydrogen peroxide for 6 h or 20 mM hydrochloric acid for 8 h caused a decrease in cell viability and apoptotic DNA fragmentation (Figure 5a and b). However, none of these gastric stressors induced *CHOP* mRNA (Figure 5c). Therefore, induction of CHOP (and possibly the ER stress response) is not generally observed for gastric stressor-induced apoptosis, but is specific for NSAID-induced apoptosis.

Activation of ATF6 by indomethacin

ER stress response element (ERSE) has been identified in the promoter of ER chaperons and *CHOP* genes.[22] ER trans-

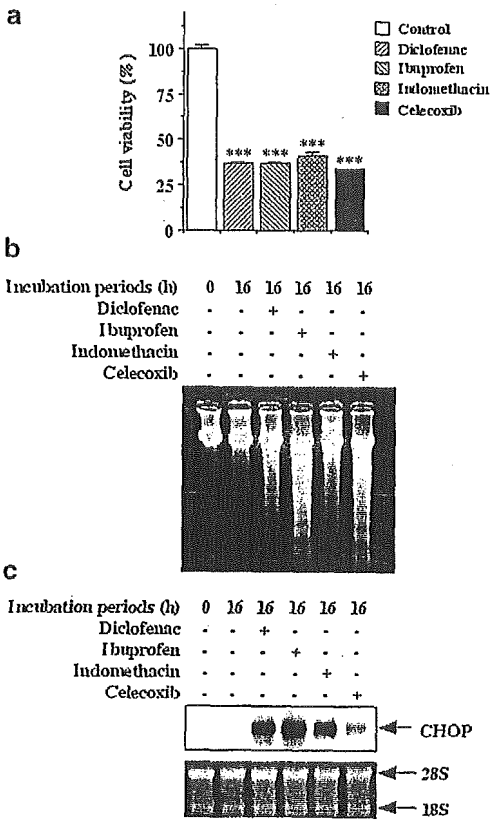

Figure 4 Induction of *CHOP* mRNA by NSAIDs other than indomethacin. Cultured gastric mucosal cells were incubated with 1 mM diclofenac, 2 mM ibuprofen, 1 mM indomethacin or 80 μM celecoxib for 16 h. Cell viability was determined by the MTT method. Values are expressed as mean $\pm$ S.E.M. ($n=4$). ***$P<0.001$ (a). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (b). The level of *CHOP* mRNA was monitored by Northern blotting analysis. Bands of ribosomal RNA (28S and 18S) stained with ethidium bromide are shown (c)

membrane-localized p90-ATF6 (the inactive form of ATF6 for ERSE-dependent transcription) is cleaved into p50-ATF6, which translocates to the nucleus where it specifically binds to ERSE to activate the transcription from ERSE.[13] The induction of *GRP78* and *CHOP* mRNA by indomethacin (Figure 3) suggests that p90-ATF6 is activated (cleaved) into p50-ATF6. Immunoblotting analysis was used to confirm this point. Immunoblotting of whole cell extracts revealed that the p90-ATF6 band disappeared following treatment of cells with indomethacin, as well as with thapsigargin (Figure 6a). Unfortunately, due to the presence of cross-reaction bands around p50-ATF6, we could not detect p50-ATF6 in whole cell extracts (data not shown). We therefore prepared nuclear extracts in which p50-ATF6 should be enriched. As shown in Figure 6b, the p50-ATF6 band was indeed detected in extracts prepared from indomethacin- or thapsigargin-treated cells, but not from control cells. As expected, the p90-ATF6 band was not detected in any nuclear extracts (data not shown). These results suggest that ATF6 is activated in the presence of indomethacin.

For further confirmation of this point, the transcriptional activity of ATF6 was measured using a reporter plasmid where the ERSE of the *CHOP* gene was inserted.[23]