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萌芽的先端医療技術推進研究事業

医薬品等の毒性試験に用いるストレス遺伝子チップの開発

平成16年度　総括研究報告書

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医薬品等の毒性試験に用いるストレス遺伝子チップの開発

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研究要旨

昨年までの研究で開発したストレス遺伝子チップを用いて、臨床現場で問題になっている NSAIDs の副作用である、NSAIDs 潰瘍の発症機構の解明を行った。具体的には、NSAIDs 潰瘍の発症に、NSAIDs による細胞傷害作用（NSAIDs による CHOP 遺伝子の誘導による）が関与していること、及びそれが NSAIDs の膜傷害性に依存していることを明らかにした。

A. 研究目的

細胞は様々なストレスに対し、適切な遺伝子（ストレス遺伝子）を発現し自らの生存を保っている。従って、細胞がある物質（医薬品の候補化合物など）に対して誘導するストレス遺伝子を解析することにより、その物質がどのような種類のストレスとして細胞に作用しているか、即ちその物質の細胞毒性の分子機構を解明することができる。このような目的のためには、DNAチップ技術を活かして、ストレス遺伝子の網羅的な解析を可能にするストレス遺伝子チップ（全てのストレス遺伝子を乗せたDNAチップ）の開発が必要である。本研究提案は、様々なストレス遺伝子を様々な生物種において同定してきたという我々の実績の基に（ヒトに関しては既にストレス遺伝子チップの試作品を開発している）、様々な生物種において様々な方法でストレス遺伝子を同定し、ストレス遺伝子チップを各生物種において作成することを目標にしている。本研究の特徴は、ヒトだけでなく、様々な生物種でストレス遺伝子を同定することである。これは、ストレス遺伝子が種を越えてよく保存されていること、及び最近多くの生物種でゲノム情報が明らかになっていることを利用した独自の研究戦略である。即ち、ある生物種で新しいストレス遺伝子を同定した場合には、ゲノム情報を使って他の生物種でそのホモログを取るという研究戦略が本研究提案の特徴である。このような方法で得た全てのストレス遺伝子をチップ化し、それを指定研究（種々の毒性物質による遺伝子発現変化のデータベースを作成する）において使って頂くのが、本研究提案の最終目標である。また大腸菌、及び酵母などを使うことによって、単にストレスによって誘導される遺伝子だけでなく、細胞をストレス耐性化する遺伝子を遺伝学的手法を用いて網羅的に検索するのも本研究の特徴の一つである。それら遺伝子（及びそのホモログ）をストレス遺伝子チップに使用するだけでなく、このシステムを用いた全く新しい毒性試験の確立も本研究で目指したい。即ち、ある物質（医薬品の候補化合物など）に対して細胞を耐性化する遺伝子を同定することにより、その物質の細胞毒性の分子機構を解明するという方法を本研究において確立したいと考えている。

B. 研究方法

NSAIDs誘導性遺伝子の検索

昨年度までの本研究で開発したストレス遺伝子チップを使って、NSAIDs誘導性遺伝子の検索を行った。

NSAIDs潰瘍発症機構の解明

ラットを用いて、新しい実験動物モデルを確立し、動物実験を行った。

NSAIDsによるアポトーシス誘導機構

モルモット胃粘膜初代培養細胞を用いて、各種阻害剤の効果などを調べた。

C.研究結果

胃粘膜細胞をNSAIDsで処理し、誘導される遺伝子をストレス遺伝子チップを使って網羅的に解析した。その結果GRP78など、小胞体ストレス応答（小胞体が傷害を受け小胞体内に変性した蛋白質が蓄積すると誘導されるストレス応答）に関与する遺伝子を複数同定し、NSAIDsが小胞体ストレス応答を誘導することが初めて分かった。最近小胞体ストレス応答誘導により、アポトーシス誘導性を持つ転写因子CHOPが誘導されることが報告された。そこで我々はNSAIDsによるアポトーシス誘導がCHOPを介する可能性を考え実験を行った。まず我々は、mRNA、及び蛋白質レベルで、種々のNSAIDsがCHOPを誘導することを確認した。CHOPの誘導には、ATF6、ATF4、及びXBP-1という3種の転写因子が関与することが知られているが、我々は種々のNSAIDsによりこれら全ての転写因子が活性化されることを示した。さらにこのNSAIDsによるCHOP誘導がNSAIDsによるアポトーシス誘導に関与していること以下の二つの実験で示した。まずCHOPのドミナントネガティブ変異蛋白質を発現することにより、NSAIDsによるアポトーシス誘導が抑制されることを見出した。さらに、CHOPのノックアウトマウスから調製した細胞では、NSAIDsによるアポトーシス誘導が全く見られないことを示した。以上の結果は、NSAIDsは小胞体ストレス応答（CHOP）を誘導することにより、アポトーシスを起こすことを示唆している。

次にNSAIDsによるCHOP誘導メカニズムについて検討した。我々は細胞内カルシウム濃度上昇の関与を考えた。それはカルシウムイオノフォアなどにより細胞内カルシウム濃度を上昇させた時、小胞体ストレス応答が誘導されることを根拠としている。実際に我々は調べた限り全てのNSAIDsにより細胞内カルシウム濃度が上昇すること、及び細胞内でカルシウムをキレートしその効果を消失させるBAPTA-AMにより、NSAIDsによるアポトーシス誘導が抑制されることを見出した。以上の結果は、NSAIDsによるアポトーシス誘導に、細胞内カルシウム濃度の上昇が関与していることを示している。

次に我々はNSAIDsによる細胞内カルシウム濃度の上昇メカニズムを検討した。我々は、我々はNSAIDsが膜リン脂質と相互作用するという論文に注目し、NSAIDsが膜傷害性を持ち、これが細胞内カルシウム濃度の上昇の原因ではないかと考えた。そこで10種類以上のNSAIDsを用いて、その膜傷害性とアポトーシス誘導性（細胞内カルシウム濃度

の上昇）の相関性を調べた。その結果、調べた限り全てのNSAIDsはアポトーシスを誘導するだけでなく、赤血球からのヘモグロビンの漏出を促進し膜傷害性を持つことが分かった。さらに蛍光物質であるカルセインを封入したリポソーム（リン脂質のみから成る）を用いて、カルセインの漏出を指標とした膜傷害試験を行った。その結果、全てのNSAIDsがカルセインを漏出させ、NSAIDsはリン脂質（細胞膜上の蛋白質ではなく）をターゲットとして、膜傷害を起こすことが分かった。さらに、これらNSAIDsによる膜傷害性と細胞内カルシウム濃度の上昇は大変よく相関しており、NSAIDsはその膜傷害性を介して、細胞内カルシウム濃度の上昇させることが強く示唆された。

それでは、このNSAIDsによる直接細胞傷害（胃粘膜細胞死）は、胃潰瘍発症に本当に関与しているのだろうか？我々は、NSAIDsが胃潰瘍を導くためには、胃粘膜でCOXを阻害しPGを低下させることに加え、NSAIDsによる胃粘膜細胞死が必要であるという新しい仮説を考えた。この仮説を証明するために我々は、新しいNSAIDs潰瘍に関する動物モデルを考案した。それは低用量インドメタシンの静脈注射と、細胞傷害性のあるCOX-2選択的NSAIDsの経口投与を組み合わせるモデルである。前述のように細胞傷害に必要な濃度に比べ、インドメタシンのCOXを阻害するために必要な濃度は低い。そのため低用量インドメタシンの静脈注射により、胃粘膜でCOXを阻害しPGを低下させながら、細胞傷害は起こらないようにすることが出来る。逆に細胞傷害性のあるCOX-2選択的NSAIDsの経口投与では、経口投与なので胃内でのNSAIDsの濃度はかなり高くなり細胞傷害は起こしながら、胃粘膜のPGを低下させないようにすることが出来る（胃粘膜で主に発現しているCOX-1は阻害しないため）。そこで仮に我々の仮説が正しく、即ちNSAIDsが胃潰瘍を導くためには、胃粘膜でCOXを阻害しPGを低下させることに加え、NSAIDsによる胃粘膜細胞死が必要であるならば、低用量インドメタシンの静脈注射、及び細胞傷害性のあるCOX-2選択的NSAIDsの経口投与、それぞれ単独では潰瘍は起こさないが、両者を同時に投与すると胃潰瘍が発症することが予想される。実際、低濃度インドメタシンの静脈注射、及びセレコキシブ（最も細胞傷害性の強いCOX-2選択的NSAIDs）の経口投与を同時に行ったときのみ、胃潰瘍の発症が見られた。またセレコキシブの代わりにロフェコキシブ（ほとんど細胞傷害性を持たない）を用いた場合には、ほとんど胃潰瘍が発症しなかった。以上の結果は我々の仮説の妥当性、即ちNSAIDsが胃潰瘍を導くためには、胃粘膜でCOXを阻害しPGを低下させることに加え、

NSAIDs による胃粘膜細胞死が必要であることを示唆している。

D.考察

以上の我々の研究から、胃潰瘍を起こさないNSAIDs を開発するためには、膜傷害性（細胞傷害性）のないNSAIDs、あるいは胃粘膜で PG を低下させないNSAIDs を開発すればいいことが分かる。後者、即ち胃粘膜で PG を低下させないNSAIDs が、COX-2 選択的NSAIDs である。しかし前述のように最近、COX-2 選択的NSAIDs は心筋梗塞を誘発するという衝撃的な論文が相次いで発表された。血液凝固調節系において、COX-1 によって合成されるトロンボキサン A2 が血液凝固を促進するのに対し、COX-2 によって合成されるプロスタサイクリンは血液凝固を阻害する。そこで、COX-2 だけを阻害する COX-2 選択的 NSAIDs は、血液凝固を阻害するプロスタサイクリンだけを減少させるために、血栓を出来やすくするのである。実際、COX-2 選択性のない従来の NSAIDs を使用している患者に比べ、COX-2 選択的 NSAIDs を使用している患者の心筋梗塞を起こす危険性は 5 倍以上であるという臨床試験の結果が公表されている。また最近北米では、「心筋梗塞を起こす可能性のある患者には、COX-2 選択的 NSAIDs を使用しないように」という注意文書が配布された。さらにごく最近、代表的な COX-2 選択的 NSAIDs であるロフェコキシブがその心筋梗塞誘発副作用のために販売停止となった。そこで COX-2 選択性を高める以外の方法で、NSAIDs 潰瘍を起こさないNSAIDs を開発する必要がある。そこで注目されるのが膜傷害作用のないNSAIDs である。即ち、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。我々はこのようなNSAIDs の発見を目指して現在そのスクリーニングを行っている。

E.結論

本研究により、医薬品により誘導される遺伝子の解析が医薬品の副作用機構の解明、及び副作用のない新しい医薬品開発に役立つことが示された。

F.健康危険情報

該当なし

G.研究発表

1. 論文発表

1. 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

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Membrane permeabilization by non-steroidal anti-inflammatory drugs

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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

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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 Arbor, 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).

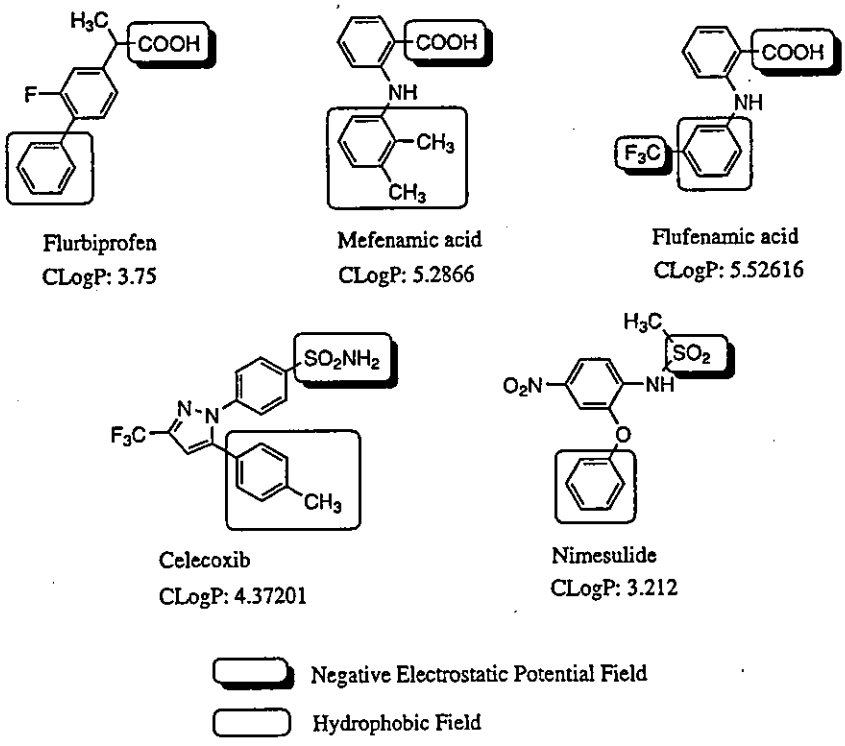

Fig. 1. Molecular structures and CLogP values of NSAIDs. Discriminative negative electrostatic potential fields are shown with bold-lined boxes, and discriminative hydrophobic fields (phenyl groups) are shown with normal-lined boxes. CLogP values were calculated with CLOGP3 program (Pomona MedChem Software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

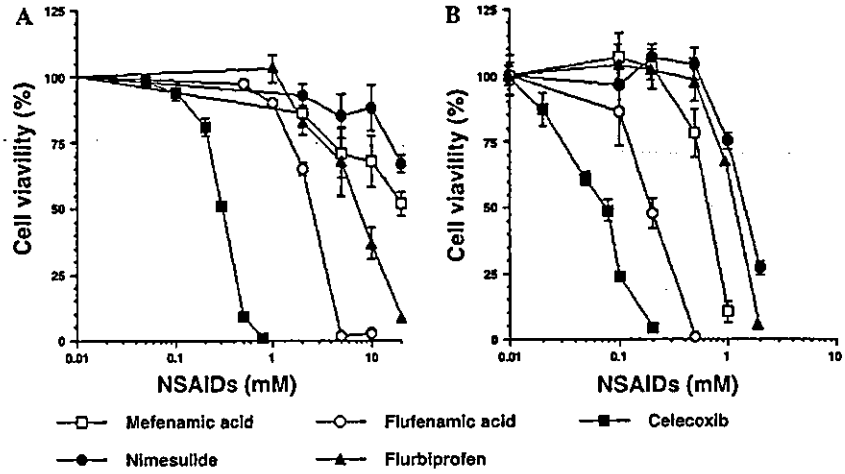

Fig. 2. Necrosis and apoptosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with NSAID under investigation for 1 h (A) or 16 h (B). Cell viability was determined by the MTT method. Values are means ± SEM ($n = 3$).

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-

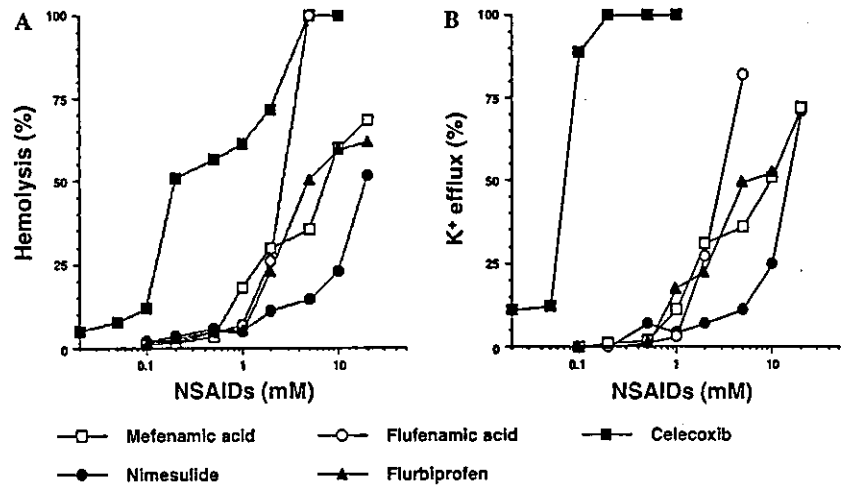

Fig. 3. NSAID-induced hemolysis and K^+ efflux from erythrocytes. Human erythrocytes were incubated in the presence of each NSAID for 10 min at 30 °C. Hemolysis was estimated by measuring the absorbance at 540 nm (A). The level of K^+ efflux was measured with a K^+ ion-selective electrode (B). Melittin (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

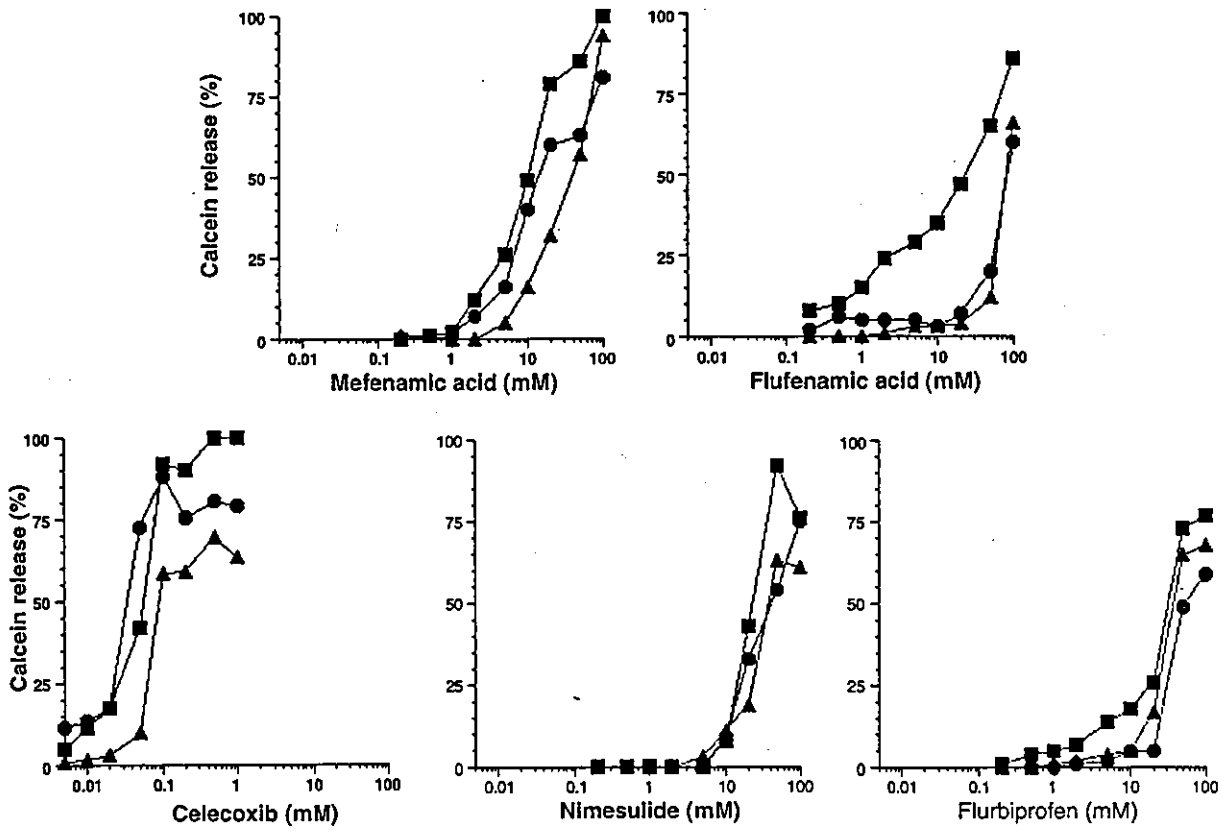

Fig. 4. Membrane permeabilization induced by NSAIDs. Calcein-loaded liposomes prepared from PC (squares), PC/cholesterol (circles), or PC/DCP (triangles) were incubated with each NSAID for 10 min at 30 °C. The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin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

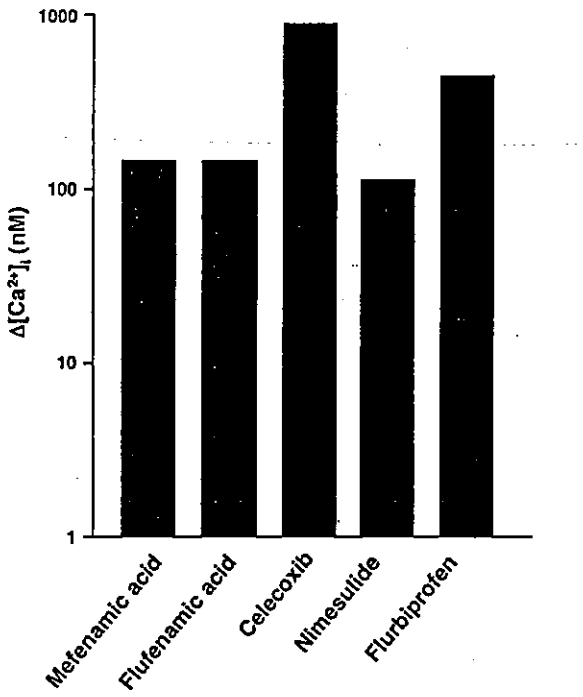

Fig. 5. Increase in the intracellular Ca^{2+} level by NSAIDs. The intracellular Ca^{2+} level was monitored by fluo-3/AM assay system as described in Materials and methods. NSAIDs were added to fluo-3/AM-loaded cells at concentrations of ED_{50} values for apoptosis (mefenamic acid, 0.7 mM; flufenamic 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

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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.