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Low Direct Cytotoxicity of Nabumetone on Gastric Mucosal Cells

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Prodrugs of non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for clinical purposes because they are not harmful to the gastrointestinal mucosa. We recently showed that NSAIDs have direct cytotoxicity in NSAID-induced gastric lesions. We show here that under conditions where the NSAIDs indomethacin and celecoxib clearly induce cell death, an NSAID prodrug, nabumetone, and its active metabolite 6-methoxy-2-naphthylacetic acid (6MNA), did not have such effects. Moreover, nabumetone and 6MNA exhibited much lower membrane permeabilizing activities than did indomethacin and celecoxib. We recently reported that when an orally administered NSAID was used in combination with a low dose of intravenously administered indomethacin, the severity of gastric lesions produced in rats depended on the cytotoxicity of the orally administered NSAID. Using a similar protocol, we show here that gastric lesions were produced when the orally administered NSAID was celecoxib, but not when nabumetone was used. We thus propose that the low direct cytotoxicity of nabumetone observed in vitro is maintained in vivo, and that the use of nabumetone does not harm the gastric mucosa.

KEY WORDS: nabumetone; gastric mucosal cells; membrane permeabilization; gastric lesions.

Non-steroidal anti-inflammatory drugs (NSAIDs) are very popular and effective medicines used in the treatment of pain, inflammation and fever. The anti-inflammatory action of NSAIDs is mediated by their inhibition of cyclooxygenase (COX) activity. COX is an enzyme that is essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the downside, the use of NSAIDs is associated with gastrointestinal side-effects (1), with about 15–30% of chronic users of NSAIDs suffering from gastrointestinal ulcers and bleeding (2, 3). This negative aspect of NSAID use was previously thought to be due only to the inhibition of COX, because PGs have a strong protective effect on the gastrointestinal mucosa (4). In order to overcome the gastrointestinal side-effects of NSAID use, NSAIDs that inhibit COX activity in inflammatory tissues but not in the gastric mucosa are therefore required. Selective COX-2 inhibitors belong to such a category of NSAIDs. COX has two subtypes, COX-1 and COX-2, which are responsible for the majority of COX activity in the gastric mucosa and in inflamed tissues, respectively (5, 6). While a greatly reduced incidence of gastroduodenal lesions was reported for selective COX-2 inhibitors (such as rofecoxib and celecoxib) both in animal and clinical data (7, 8), their use however has been recently questioned because of their potential for causing cardiovascular thrombotic events owing to their specificity for COX-2 (9–12).

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NSAID prodrugs (such as loxoprofen sodium and nabumetone) are generally safe for use on the gastrointestinal mucosa and are widely used for clinical purposes, especially in Japan where highly specific COX-2 inhibitors (such as celecoxib) are not presently available in the market. Because most NSAID prodrugs do not possess any significant specificity for COX-2, these prodrugs may become very important as NSAIDs, considering the potential risk for cardiovascular thrombotic events of selective COX-2 inhibitors.

NSAIDs have a direct cytotoxic effect on gastrointestinal mucosal cells (13, 14) and we recently demonstrated that NSAIDs induce both necrosis and apoptosis in cultured gastric mucosal cells in a manner independent of COX inhibition (15–17). We also found that NSAIDs cause membrane permeabilization, which in turn is implicated in their direct cytotoxicity; that is, liposomal membranes are directly permeabilized by NSAIDs at concentrations closely related to those which result in cytotoxicity (18). Furthermore, we recently suggested that the combined effect of COX inhibition and the direct cytotoxic effect of NSAIDs (direct cell damage) on the gastric mucosa induces the production of gastric lesions (19). Therefore, the direct cytotoxicity of individual NSAIDs is a key factor to be determined in assessing their harmfulness on the gastric mucosa.

Since the direct cytotoxicity of NSAID prodrugs has not been studied at all, we examined here the direct cytotoxicity of nabumetone which, along with its active metabolite 6-methoxy-2-naphthylacetic acid (6MNA), was found to not harm the gastrointestinal mucosa in clinical studies on humans and in animal models (20, 21). Compared to indomethacin and celecoxib, both nabumetone and 6MNA showed very low activities for inducing necrosis, apoptosis and membrane permeabilization. Furthermore, in combination with the intravenous administration of a low dose of indomethacin (conditions under which gastric mucosal COX activity is completely inhibited), the oral administration of nabumetone did not result in the production of gastric lesions, which is in contrast to results obtained following the oral administration of celecoxib. Based on these observations, we consider that the low direct cytotoxicity of nabumetone will render its use safe on the gastrointestinal mucosa in vivo.

MATERIALS AND METHODS

Chemicals and Media. Fetal bovine serum (FBS) was from Gibco Co. 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma Co. Nabumetone and 6MNA were kindly gifted from Sanwa Kagaku Kenkyusho Co. Indomethacin was from Wako Co. Celecoxib was from LKT Laboratories Inc. Egg phosphatidylcholine (PC) was from Kanto Chemicals Co. The ELISA kit for PGE_2 quantitation was from Cayman Chemical Co. Male Wistar rats weighing 160–200 g and male guinea pigs weighing 200–300 g were purchased from Shimizu Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health and were approved by the Animal Care Committee of Kumamoto University.

In Vitro Assay of Cytotoxicity of NSAIDs

Gastric mucosal cells were isolated from guinea pig fundic glands as described previously (22, 23). Isolated gastric mucosal cells were cultured for 12 hr in RPMI 1640 containing 0.3% v/v FBS, 100 U/ml ampicillin 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, cells attached to the plate were used. Guinea pig gastric mucosal cells prepared under these conditions were previously characterized, with the majority (about 90%) of cells being identified as pit cells (22, 24).

NSAIDs were dissolved in DMSO. Cells were exposed to NSAIDs by changing the entire bathing medium.

We used MTT assay for monitoring cell viability. Cells were incubated for 2 hr with MTT solution at a final concentration of 0.5 mg/ml. Isopropanol and hydrochloric acid were added to the culture medium at final concentrations of 50% and 20 mM, respectively. The optical density of each sample at 570 nm was determined spectrophotometrically using a reference wavelength of 630 nm (25).

Gastric Damage Assay. Gastric damage assays were performed as described previously (19). Rats (24 hr fasted) were administered orally with NSAIDs in 1% methylcellulose in a volume of 5 ml/kg. In some experiments, indomethacin in PBS was administered intravenously 1 hr before the oral administration. Six hours after the oral administration, the rats were anesthetized and the stomach was removed and scored for hemorrhagic damage by an observer unaware of the treatment the rats had received. The score involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index. Determination of PGE_2 levels at the gastric mucosa was done by ELISA as previously described (26).

Assay for Erythrocyte Hemolysis. Hemolysis in erythrocytes were monitored as described (18). 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). After incubation with NSAIDs for 10 min at 30 C, hemolysis was estimated by measuring the absorbance at 540 nm.

Membrane Permeability Assay. Membrane permeability assays were performed as described previously (18). Liposomes were prepared using the reversed-phase evaporation method. Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2, v/v), dried, and dissolved in 1.5 ml of 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 homogenous 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 (10 mM phosphate buffer, containing 150 mM NaCl) 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 of buffer A, following which 400 l of this suspension was incubated at 30 C for 10 min in the presence of the NSAID under investigation. The release of calcein from liposomes (the amount of calcein outside the liposomes) was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm), because the calcein fluoresces very weakly when at high concentrations (when calcein is trapped in liposomes) due to self-quenching.

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

RESULTS AND DISCUSSION

Necrosis- and Apoptosis-Inducing Activities of Nabumetone and 6MNA. We previously reported that NSAIDs induced either necrosis or apoptosis depending on treatment conditions; short-term (1 hr) treatment of primary cultures of guinea pig gastric mucosal cells with relatively high concentrations of NSAIDs (2.5 mM for indomethacin and 0.2 mM for celecoxib) and long-term (16 hr) treatment of these cells with relatively low concentrations of NSAIDs (1 mM for indomethacin and 0.05 mM for celecoxib) induces necrosis and apoptosis, respectively (15, 18, 19). Nabumetone and 6MNA were tested here for their ability to induce necrosis and apoptosis. Consistent with previous reports (15, 18, 19), cell viability decreased in a dose-dependent manner when guinea pig gastric mucosal cells in primary culture were treated with indomethacin or celecoxib for 1 hr. In contrast, nabumetone and 6MNA decreased cell viability to a much lesser extent under the same experimental conditions (Figure 1A), with the necrosis- and apoptosis-inducing effects of nabumetone being slightly but significantly lower than those of 6MNA (Figure 1B). We confirmed that cell death highlighted in Figure 1 was mediated by necrosis given that no accompanying apoptotic DNA fragmentation or chromatin condensation were evident (data not shown).

Similar results to the above were obtained when apoptosis was induced. Treatment of cells for 16 hr with indomethacin or celecoxib decreased cell viability in a dose-dependent manner (Figure 2A), which is also consistent with previous reports (15, 18, 19). Nabumetone and 6MNA showed very low activities for decreasing cell viability under these conditions (Figure 2A). and nabumetone was again slightly but significantly less damaging than 6MNA (Figure 2B). Because cell death as highlighted in Figure 2 was accompanied by apoptotic DNA fragmentation and chromatin condensation (data not shown), it is

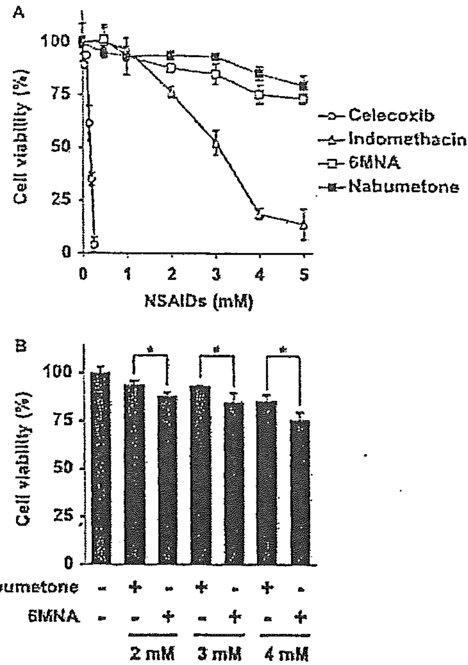

Fig 1. Necrosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NSAIDs for 1 hr. Cell viability was determined by the MTT method. Values are mean ± SD ($n = 3$). $*P < 0.05$.

most likely to have been mediated by apoptosis. Overall, the results in Figures 1 and 2 show that nabumetone and 6MNA induce necrosis and apoptosis to a lesser extent than do indomethacin and celecoxib. Furthermore, although the metabolic conversion of nabumetone to 6MNA drastically increases the inhibition of COX activity, this conversion does not seem to be associated with a similar increase in direct cytotoxicity.

Membrane Permeabilization Activities of Nabumetone and 6MNA. The ability of nabumetone and 6MNA to permeabilize the membranes of calcein-loaded liposomes was examined. Calcein fluoresces very weakly when at high concentrations due to self-quenching. Thus, the addition of membrane permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by releasing calcein trapped within the liposomes and thus lower the calcein concentration (18). As shown in Figure 3, indomethacin and celecoxib increased the calcein fluorescence in a dose-dependent manner, which is consistent with previous findings (18). Nabumetone and 6MNA also increased the calcein fluorescence, suggesting that they

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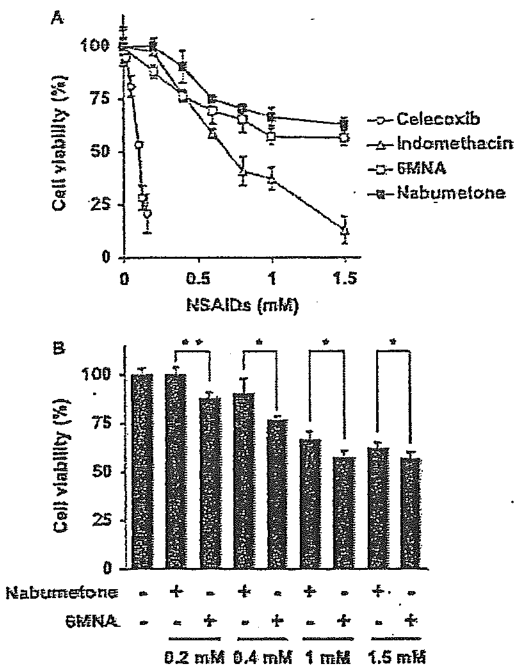

Fig 2. Apoptosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NSAIDs for 16 hr. Cell viability was determined by the MTT method. Values are mean ± SD ($n = 3$). ** $P < 0.01$; * $P < 0.05$.

caused membrane permeabilization; however, as the concentrations of nabumetone and 6MNA required for membrane permeabilization were much higher than those of indomethacin and celecoxib, their abilities to permeabilize membranes were thus very weak. The results shown in Figure 3 suggest that the low direct cytotoxicity of

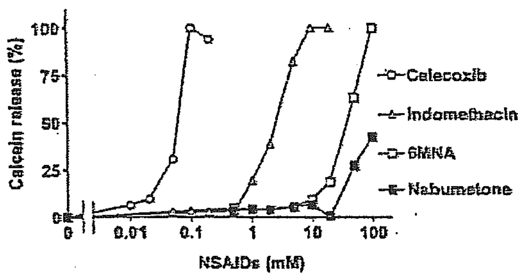

Fig 3. Membrane permeabilization by NSAIDs. Calcein-loaded liposomes were incubated for 10 min at 30 C with indicated concentrations of each NSAID. The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin (10 μM) was used to determine the 100% level of membrane permeabilization.

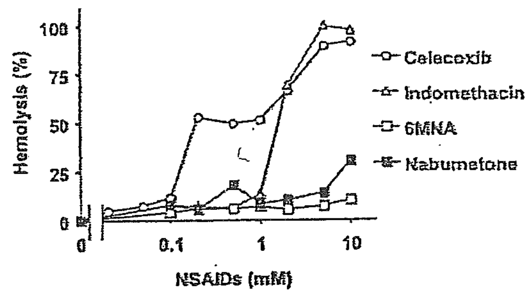

Fig 4. NSAID-induced hemolysis from erythrocytes. Human erythrocytes were incubated in the presence of each of NSAIDs for 10 min at 30 C. Hemolysis was estimated by measuring the absorbance at 540 nm. Melittin (10 μM), a membrane permeabilizing reagent, was used to determine the 100% level of hemolysis (33).

nabumetone and 6MNA is due to their low membrane permeabilizing effects. When nabumetone and 6MNA were compared, 6MNA had a higher membrane permeabilizing effect than nabumetone (Figure 3), which is consistent with the results describing their direct cytotoxicity (Figures 1 and 2).

We also examined the membrane permeabilization activities of NSAIDs by measuring hemolysis. As shown in Figure 4, results similar to calcein release (Figure 3) were obtained, suggesting that NSAIDs cause membrane permeabilization not only in liposomes but also in cells. Membrane permeabilization (Figures 3 and 4) was observed at relatively higher concentrations of NSAIDs than those required for decrease in cell viability (Figures 1 and 2), being consistent with our previous report (18). This may be due to the difference in assay conditions.

Activities of Nabumetone and 6MNA for Production of Gastric Lesions. As described in the Introduction, we recently found that gastric lesions develop in a manner that depends both on intravenously administered low doses of indomethacin and on orally administered cytotoxic NSAIDs, such as celecoxib (19). Using this model, the ability of nabumetone to produce gastric lesions was compared to that of celecoxib. As shown in Figure 5, in the absence of prior intravenous administration of indomethacin, the oral administration of either nabumetone or celecoxib did not clearly produce gastric lesions, which is consistent with previous results (27, 28). Oral administration of either nabumetone or celecoxib did not significantly reduce the level of PGE_2 (Table 1). The intravenous administration of a low dose (5 mg/kg) of indomethacin alone also did not produce gastric lesions (Figure 5), but did bring about a reduction of more than 90% in the level of PGE_2 (Table 1). A combination of the oral administration of celecoxib and the intravenous administration of

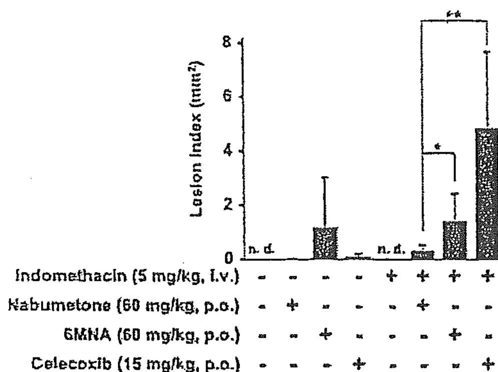

Fig 5. Production of gastric lesions by NSAIDs. Rats were intravenously administered with indomethacin or vehicle. After 1 hr, animals were administered orally with nabumetone, celecoxib, 6MNA or vehicle. After 6 hr, the stomach was removed and scored for hemorrhagic damage as described in Materials and Methods section. Values are mean ± SD (n = 5–6). ** $P < 0.01$; * $P < 0.05$. n.d.; not detected.

indomethacin clearly gave rise to the production of gastric lesions (Figure 5) as previously reported (19). In contrast, gastric lesions were not so evident when the oral administration of nabumetone and the intravenous administration of indomethacin were used in combination (Figure 5). Oral administration of 6MNA produced a little but significant gastric lesions both in the presence or absence of intravenous administration of indomethacin (Figure 5). This may be due to that oral administration of 6MNA, itself, significantly reduced the level of PGE_2 (Table 1). We consider from the results presented in Figure 5 that nabumetone also has a low level of direct cytotoxicity in vivo.

In summary, we show here that nabumetone has a very low level of direct cytotoxicity on gastric mucosal cells in vitro and suggest that this is also the case in vivo. As described earlier, it is well known that nabumetone is experimentally and clinically safe and that its use is not as harmful to the gastric mucosa compared to other NSAIDs such as indomethacin and aspirin (20, 29). In addition to its inability to inhibit gastric mucosal COX activity soon after oral administration, its inhibitory effect on neutrophil functions was also recently suggested (30). We propose here that in addition to these mechanisms, the low direct cytotoxicity of nabumetone make it far less harmful on the gastric mucosa and therefore much safer for clinical use.

It is known that non-selective NSAIDs modulate the gastric acid secretion and inhibit bicarbonate secretion (31, 32). Although we did not examine the effect of nabumetone on these processes, it is possible that these process also involve the safety of this drug on gastric mucosa in vivo.

TABLE 1. INHIBITION OF GASTRIC PGE_2 SYNTHESIS BY NSAIDs

NSAIDs	*Gastric PGE_2 (ng/g tissue)*
Control	29.3 ± 2.6
5 mg/kg indomethacin i.v.	2.4 ± 0.4***
15 mg/kg celecoxib p.o.	32.4 ± 11.8
60 mg/kg nabumetone p.o.	19.1 ± 9.6
60 mg/kg 6MNA p.o.	2.8 ± 0.6***

Note. Rats were intravenously (i.v.) or orally (p.o.) administered with indicted doses of NSAIDs. After 6 hr (p.o.) or 7 hr (i.v.), the level of PGE_2 in gastric mucosa was determined by ELISA. Values are mean ± SEM (n = 3).
*** $P < 0.001$.

REFERENCES

1. Hawkey CJ: Nonsteroidal anti-inflammatory drug gastropathy. Gastroenterology 119:521–535, 2000
2. Gabriel SE, Jaakkimainen L, Bombardier C: Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs. A meta-analysis. Ann Int Med 115:787–796, 1991
3. Kurata JH, Abbey DE: The effect of chronic aspirin use on duodenal and gastric ulcer hospitalizations. J Clin Gastroenterol 12:260–266, 1990
4. Miller TA: Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. Am J Physiol 245:G601–G623, 1983
5. Vane J: Towards a better aspirin. Nature 367:215–216, 1994
6. Smith CJ, Zhang Y, Koboldt CM, Muhammad J, Zweifel BS, Shaffer A, Talley JJ, Masferrer JL, Seibert K, Isakson PC: Pharmacological analysis of cyclooxygenase-1 in inflammation. Proc Natl Acad Sci USA 95:13313–13318, 1998
7. Chan CC, Boyce S, Brideau C, Charleson S, Cromlish W, Ethier D, Evans J, Ford HA, Forrest MJ, Gauthier JY, Gordon R, Gresser M, Guay J, Kargman S, Kennedy B, Leblanc Y, Leger S, Mancini J, O'Neill GP, Ouellet M, Patrick D, Percival MD, Perrier H, Prasit P, Rodger I, *et al.*: Rofecoxib [Vioxx, MK-0966; 4-(4'-methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone]: a potent and orally active cyclooxygenase-2 inhibitor. Pharmacological and biochemical profiles. J Pharmacol Exp Ther 290:551–560, 1999
8. FitzGerald GA, Patrono C: The coxibs, selective inhibitors of cyclooxygenase-2. N Engl J Med 345:433–442, 2001
9. Mukherjee D, Nissen SE, Topol EJ: Risk of cardiovascular events associated with selective COX-2 inhibitors. JAMA 286:954–959, 2001
10. Mukherjee D: Selective cyclooxygenase-2 (COX-2) inhibitors and potential risk of cardiovascular events. Biochem Pharmacol 63:817–821, 2002
11. McAdam BF, Catella LF, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA: Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci USA 96:272–277, 1999
12. Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald DJ: Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. Circulation 102:840–845, 2000

13. Lichtenberger LM: Where is the evidence that cyclooxygenase inhibition is the primary cause of nonsteroidal anti-inflammatory drug (NSAID)-induced gastrointestinal injury? Topical injury revisited. Biochem Pharmacol 61:631–637, 2001
14. Somasundaram S, Rafi S, Hayllar J, Sigthorsson G, Jacob M, Price AB, Macpherson A, Mahmod T, Scott D, Wrigglesworth JM, Bjarnason I: Mitochondrial damage: a possible mechanism of the "topical" phase of NSAID induced injury to the rat intestine. Gut 41:344–353, 1997
15. Tomisato W, Tsutsumi S, Rokutan K, Tsuchiya T, Mizushima T: NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. Am J Physiol Gastrointest Liver Physiol 281:G1092–G1100, 2001
16. Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, 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
17. Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ, Takenaka H, Tsuchiya T, Mori M, Mizushima T: Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. Cell Death Differ 11:1009–1016, 2004
18. 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
19. Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, 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
20. Melarange R, Gentry C, O'Connell C, Blower PR, Neil C, Kelvin AS, Toseland CD: Anti-inflammatory and gastrointestinal effects of nabumetone or its active metabolite, 6MNA (6-methoxy-2-naphthylacetic acid): comparison with indomethacin. Agents Actions Spec No: C82–C83, 1992
21. Bernhard GC: Worldwide safety experience with nabumetone. J Rheumatol Suppl 36:48–57, 1992
22. Hirakawa T, Rokutan K, Nikawa T, Kishi K: Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. Gastroenterology 111:345–357, 1996
23. Tomisato W, Takahashi N, Komoto C, Rokutan K, Tsuchiya T, Mizushima T: Geranylgeranylacetone protects cultured guinea pig gastric mucosal cells from indomethacin. Dig Dis Sci 45:1674–1679, 2000
24. Tomisato W, Hoshino T, Tsutsumi S, Tsuchiya T, Mizushima T: Maturation-associated increase in sensitivity of cultured guinea pig gastric pit cells to hydrogen peroxide. Dig Dis Sci 47:2125–2133, 2002
25. Tsutsumi S, Tomisato W, Takano T, Rokutan K, Tsuchiya T, Mizushima T: Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. Biochim Biophys Acta 1589:168–180, 2002
26. Futaki N, Arai I, Hamasaka Y, Takahashi S, Higuchi S, Otomo S: Selective inhibition of NS-398 on prostanoid production in inflamed tissue in rat carrageenan-air-pouch inflammation. J Pharm Pharmacol 45:753–755, 1993
27. Spangler RS: Gastrointestinal damage demonstrated with nabumetone or etodolac in preclinical studies. Am J Med 95:35S–39S, 1993
28. Laudanno OM, Cesolari JA, Esnarriaga J, Rista L, Piombo G, Maglione C, Aramberry L, Sambrano J, Godoy A, Rocaspana A: Gastrointestinal damage induced by celecoxib and rofecoxib in rats. Dig Dis Sci 46:779–784, 2001
29. Huang JQ, Sridhar S, Chen Y, Hunt RH: Meta-analysis of the relationship between Helicobacter pylori seropositivity and gastric cancer. Gastroenterology 114:1169–1179, 1998
30. Ishiwata Y, Okamoto M, Yokochi S, Hashimoto H, Nakamura T, Miyachi A, Naito Y, Yoshikawa T: Non-steroidal anti-inflammatory drug, nabumetone, prevents indometacin-induced gastric damage via inhibition of neutrophil functions. J Pharm Pharmacol 55:229–237, 2003
31. Mertz-Nielsen A, Hillingso J, Bukhave K, Rask-Madsen J: Indomethacin decreases gastroduodenal mucosal bicarbonate secretion in humans. Scand J Gastroenterol 30:1160–1165, 1995
32. Borrelli F, Tavares IA: Effect of nimesulide on gastric acid secretion in the mouse stomach in vitro. Life Sci 72:885–896, 2003
33. Katsu T, Kobayashi H, Hirota T, Fujita Y, Sato K, Nagai U: Structure-activity relationship of gramicidin S analogues on membrane permeability. Biochim Biophys Acta 899:159–170, 1987

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Low Direct Cytotoxicity and Cytoprotective Effects of Nitric Oxide Releasing Indomethacin

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Nitric oxide (NO) releasing non-steroidal anti-inflammatory drugs (NSAIDs) have shown a marked reduction of gastrointestinal side effects and we here examined the cytotoxicity of NCX 530 (NO-indomethacin). Under conditions where indomethacin clearly induced both necrosis and apoptosis, NCX 530 induced neither. NCX 530 protected cells from celecoxib-induced necrosis and apoptosis. NCX 530 partially suppressed celecoxib-dependent membrane permeabilization and an inhibitor for guanylate cyclase suppressed the cytoprotective effect of NCX 530 against celecoxib. In vivo, NCX 530 alone produced fewer gastric lesions in rats than did indomethacin. A combination of the oral administration of celecoxib together with the intraperitoneal administration of indomethacin, but not of NCX 530, clearly resulted in the production of gastric lesions. The low direct cytotoxicity and the cytoprotective effect of NCX 530 observed in vitro may also act in vivo, thus ensuring that NCX 530 is safe for use on the gastric mucosa.

KEY WORDS: direct cytotoxicity; cyclooxygenase; NO-NSAID; indomethacin; gastric lesion; selective cyclooxygenase-2 inhibitor.

Non-steroidal anti-inflammatory drugs (NSAIDs) are a very popular class of medicines that account for nearly 5% of all prescribed medications (1). The anti-inflammatory action of NSAIDs is mediated through their inhibition of cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the reverse side, NSAID use is associated with gastrointestinal complications (2), with about 15–30% of chronic users of NSAIDs suffering from 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 by NSAIDs was previously thought to be the sole explanation for their associated gastrointestinal side effects (8), given that PGs have a strong protective effect on the gastrointestinal mucosa (9). However, since the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs do not always occur in parallel (10, 11), it was proposed that the induction of gastrointestinal lesions by NSAIDs involved additional mechanisms (12). It is well known that NSAIDs have a direct cytotoxic effect on gastrointestinal mucosal cells (12–14). We recently demonstrated that NSAIDs induce both necrosis and apoptosis in cultured gastric mucosal cells in a manner independent of COX inhibition (15, 16). Furthermore, we recently proposed that both COX inhibition and the direct cytotoxic effect of NSAIDs (direct cell damage) on the gastric mucosa are involved in the production of gastric lesions in vivo; gastric lesions develop in a manner that depends on both an intravenously administered low dose of indomethacin (inhibition of COX activity without direct gastric mucosal cell damage) and an orally administered cytotoxic

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COX-2-selective inhibitor, such as celecoxib (direct cell damage without inhibition of COX at the gastric mucosa) (16). Therefore, the direct cytotoxicity of individual NSAIDs is one of key factors to determine the safety of their use on the gastric mucosa.

Nitric oxide (NO) releasing NSAIDs (NO-NSAIDs) are a newly developed group of NSAIDs, consisting of a traditional NSAID to which a group donating NO has been covalently attached via spacers. NO-NSAIDs show a marked reduction of gastrointestinal side effects but maintain, and in some cases extend, anti-inflammatory properties both in clinical studies on humans and in animal models (17–21). Further to this, given that the anti-thrombotic effect of NO-aspirin was reported to be superior to that of aspirin (22), much attention is now being paid to NO-NSAIDs as alternatives in treatment protocols.

Various mechanisms have been proposed for the gastrointestinal safety of NO-NSAIDs, such as inhibition of neutrophil adherence, promotion of mucosal blood flow and stimulation of mucin and bicarbonate secretion by NO (21, 23). In addition to these mechanisms, a lower cytotoxicity on gastric mucosal cells was reported for one NO-NSAID, NO-flurbiprofen, whose use resulted in reduced apoptosis compared to that seen with standard flurbiprofen (24). Furthermore, NO-flurbiprofen suppressed the extent of TNFα- or ceramide-induced apoptosis by inhibiting caspases in vitro (24, 25). However, necrosis induced by NO-NSAIDs and apoptosis induced by NO-NSAIDs other than NO-flurbiprofen are yet to have been studied. Furthermore, although more than two species of NSAIDs are usually used simultaneously in a clinical setting, the effect of NO-NSAIDs on cell death induced by other NSAIDs is also yet to be examined.

In this study, we used primary cultures of guinea pig gastric mucosal cells to examine the direct cytotoxicity of NCX 530 (NO-indomethacin), which according to one animal study (17) is safe from the perspective of the gastric mucosa. We also examined the effect of NCX 530 on cell death induced by other gastric irritants, including NSAIDs. NCX 530 not only showed a lower propensity than indomethacin for inducing both necrosis and apoptosis, but also protected cells from necrosis and apoptosis induced by celecoxib or ethanol. In vivo, NCX 530 alone or in combination with orally administered celecoxib or ethanol resulted in the production of fewer gastric lesions than did indomethacin. Furthermore, NCX 530 suppressed the production of gastric lesions caused by other gastric irritants. These results concerning the safety of NCX 530 use *in vivo* are probably related to the low direct cytotoxicity and cytoprotective effects of NCX 530 observed in vitro.

MATERIALS AND METHODS

Chemicals and Media. Fetal bovine serum (FBS) was from Gibco Co. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 1H-[1,2,4-]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and cycloheximide were from Sigma Co. Celecoxib was from LKT Laboratories Inc. Indomethacin and NCX 530 were kindly provided by NicOx S. A. Egg phosphatidylcholine (PC) was from Kanto Chemicals Co. Male Wistar rats weighing 160–200 g and male guinea pigs weighing 200–300 g were purchased from Shimizu Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health and were approved by the Animal Care Committee of Kumamoto University.

In vitro Assay of Cytotoxicity and DNA Fragmentation. Gastric mucosal cells were isolated from guinea pig fundic glands as described previously (26, 27). Isolated gastric mucosal cells were cultured for 12 hr in RPMI 1640 containing 0.3% v/v FBS, 100 U/mL penicillin, 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, cells attached to the plate were used. Guinea pig gastric mucosal cells prepared under these conditions were previously characterized, with the majority (about 90%) of cells being identified as pit cells (26, 28). NSAIDs were dissolved in DMSO and control experiments (without NSAIDs) were performed in the presence of same concentrations of DMSO. Cells were exposed to NSAIDs by changing the entire bathing medium. Cell viability was determined by the MTT method.

Apoptotic DNA fragmentation was monitored as previously described (29). Cells were collected using a rubber policeman and suspended in 50 μL of lysis buffer, consisting of 50 mM Tris–HCl (pH 7.8), 10 mM EDTA, and 0.5% sodium-*N*-lauroylsarcosinate. Proteinase K was added to a final concentration of 1 mg/mL, and the lysate incubated at 50°C for 2 hr. RNaseA was then added to a final concentration of 0.5 mg/mL and incubated at 50°C for 30 min. These samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 μg/mL ethidium bromide.

Gastric Damage Assay. Gastric damage assays were performed as described previously (16). Rats (24 hr fasted) were administered orally with ethanol or NSAIDs in 1% methylcellulose in a volume of 5 mL/kg. In some experiments, indomethacin or NCX 530 in 1% methylcellulose was administered intraperitoneally 1 hr before the oral administration. Six hours after the oral administration, the rats were anesthetized and the stomach was removed and scored for hemorrhagic damage by an observer unaware of the treatment the rats had received. The score involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index.

Membrane Permeability Assay. Liposomes were prepared using the reversed-phase evaporation method (30, 31). Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2, v/v), dried, and dissolved in 1.5 mL of 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 homogenous 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 (10 mM potassium buffer, containing 150 mM

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NaCl) 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 of buffer A, following which 400 L 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).

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

RESULTS

Comparison of Necrosis- and Apoptosis-Inducing Activities of NCX 530 and Indomethacin. We previously reported that short-term (1 hr) treatment of primary cultures of guinea pig gastric mucosal cells with relatively high concentrations of NSAIDs and long-term (16 hr) treatment of these cells with relatively low concentrations of NSAIDs induces necrosis and apoptosis, respectively (15). The results of experiments in which guinea pig gastric mucosal cells in primary culture were treated with NCX 530 or indomethacin for 1 hr are shown in Figure 1A. Indomethacin decreased cell viability in a dose-dependent manner, while NCX 530 did not affect cell viability at concentrations lower than 5 mM. We confirmed that cell death induced by indomethacin (Figure 1A) was mediated by necrosis as no accompanying apoptotic DNA fragmentation or chromatin condensation was evident (data not shown). The findings of experiments in which cells were treated with NCX 530 or indomethacin for 16 hr are summarized in Figure 1B. Again, indomethacin but not NCX 530 induced cell death. Because cell death under these conditions was accompanied by apoptotic DNA fragmentation (Figure 1C), it is most likely to have been mediated by apoptosis. Overall, the results in Figure 1 show that NCX 530 had a lower capacity than indomethacin for inducing necrosis and apoptosis.

Effect of NCX 530 on Cell Death Induced by Other Gastric Irritants. We next examined the cytoprotective effect of NCX 530 by pre-treating gastric mucosal cells with NCX 530 for 1 hr (pre-incubation step) and then with one of several of gastric irritants (celecoxib, ethanol, indomethacin or hydrogen peroxide) for 16 hr (apoptotic conditions) (Figure 2) or 1 hr (Figure 3) (necrotic conditions) (incubation step). As shown in Figure 2A, treatment of cells with 0.1 mM celecoxib for 16 hr induced cell death which could be partially suppressed by the pre-treatment of cells with NCX 530. A similar cytoprotective effect of NCX 530 was observed for cell death induced by exposure of cells to 3% ethanol for 16 hr (Figure 2B). Under these conditions (Figure 2), the cell death was mediated by apoptosis given that apoptotic DNA fragmentation and chromatin condensation were also present (data not shown).

As shown in Figure 3A and B, NCX 530 partially suppressed the cell death induced by treatment of cells with 0.18 mM celecoxib or 8% ethanol for 1 hr. Cell death under these conditions was mediated by necrosis as apoptotic DNA fragmentation and chromatin condensation were not in evidence (data not shown). NCX 530 was included in both the pre-incubation and incubation steps of experiments whose results are detailed in Figures 2 and 3. When NCX 530 was omitted in the pre-incubation step, its cytoprotective effect was similar to that when it was used in both the pre-incubation and incubation steps (data not shown). On the other hand, when NCX 530 was omitted from the incubation step (but included in the pre-incubation step), its cytoprotective effect was not as clear-cut. Thus, it seems that NCX 530 must be present simultaneously with celecoxib or ethanol in order to exert fully its cytoprotective effect.

The cytoprotective effect of NCX 530 was not observed for all gastric irritants tested. For example, NCX 530 affected neither apoptosis (Figure 2C and D) nor necrosis (Figure 3C and D) induced by indomethacin or hydrogen peroxide. Similar results were obtained when NCX 530 was omitted in the pre-incubation or incubation steps (data not shown). Based on these findings, it appears that NCX 530 protects gastric mucosal cells from necrosis and apoptosis induced by celecoxib or ethanol but not by indomethacin or hydrogen peroxide.

Mechanism for the Low Cytotoxicity and Cytoprotective Effect of NCX 530. We recently found that NSAIDs cause membrane permeabilization, which, in turn, is implicated in their cytotoxicity (induction of necrosis and apoptosis); that is, NSAIDs directly permeabilize the membranes of liposomes, with concentrations of NSAIDs required for this effect being closely related to those which result in cytotoxicity (32). In further experiments, the ability of NCX 530 and indomethacin to permeabilize the membranes of calcein-loaded liposomes was compared. Calcein fluoresces very weakly at high concentrations due to self-quenching. Thus, the addition of membrane permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by releasing calcein trapped within the liposomes (30, 31). As shown in Figure 4A, NCX 530 and indomethacin showed similar dose–response curves for the increase in calcein fluorescence, suggesting that their ability to cause membrane permeabilization is virtually indistinguishable. Therefore, it seems that the low cytotoxicity

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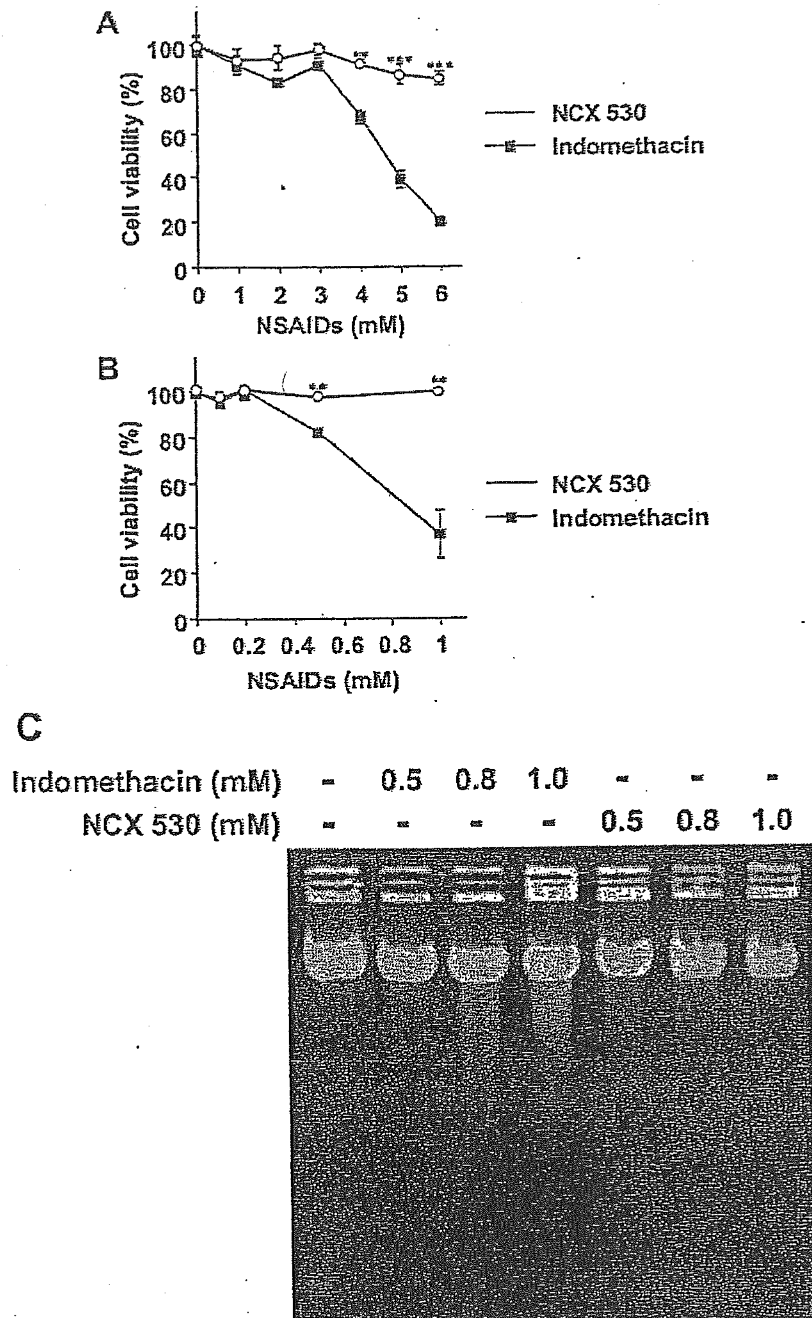

Fig 1. Necrosis and apoptosis induced by NCX 530 or indomethacin. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NCX 530 or indomethacin for 1 hr (A) (necrotic conditions) or 16 hr (B, C) (apoptotic conditions). Cell viability was determined by the MTT method (A, B). Values are mean ± S.E.M. ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (C).

of NCX 530 cannot be explained on the basis of its membrane permeabilization activity. Celecoxib caused membrane permeabilization at very low concentrations, as found previously (32), which could be partially suppressed by NCX 530 (Figure 4B) but not by indomethacin (data not shown). On the other hand, NCX 530 did not affect the indomethacin-dependent membrane permeabilization (data not shown). Therefore, the cytoprotective effect of

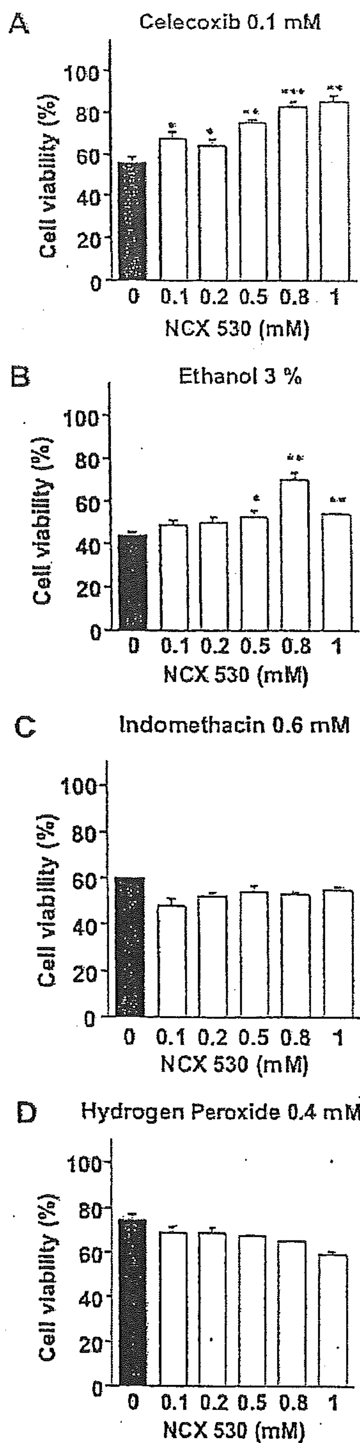

Fig 2. Effect of NCX 530 on apoptosis induced by various gastric irritants. Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of NCX 530 for 1 hr and further incubated with 0.1 mM celecoxib (A), 3% ethanol (B), 0.6 mM indomethacin (C) or 0.4 mM hydrogen peroxide (D) in the presence of indicated concentrations of NCX 530 for 16 hr (apoptotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. ($n = 3$). $***P < 0.001$; $**P < 0.01$; $*P < 0.05$.

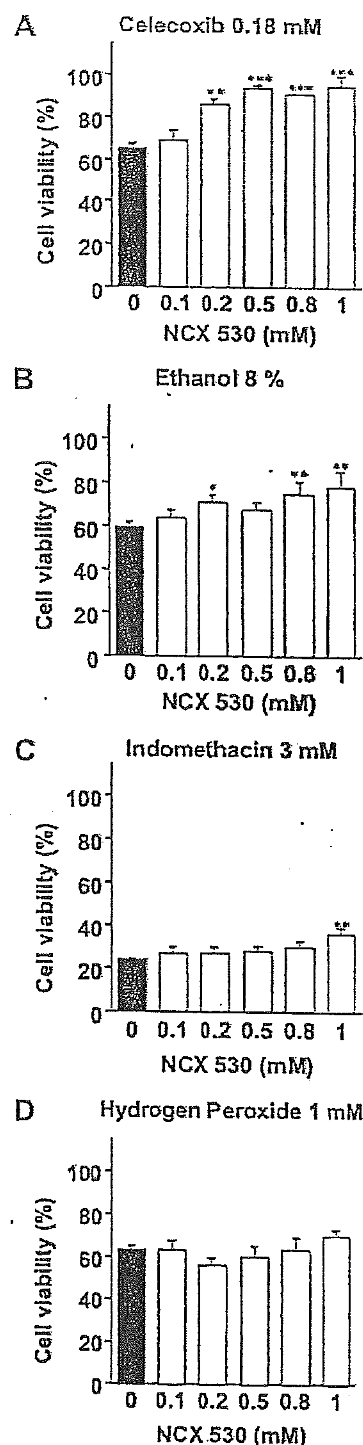

Fig 3. Effect of NCX 530 on necrosis induced by various gastric irritants. Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of NCX 530 for 1 hr and further incubated with 0.18 mM celecoxib (A), 8% ethanol (B), 3 mM indomethacin (C) or 1 mM hydrogen peroxide (D) in the presence of indicated concentrations of NCX 530 for 1 hr (necrotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. ($n = 3$). $***P < 0.001$; $**P < 0.01$; $*P < 0.05$.

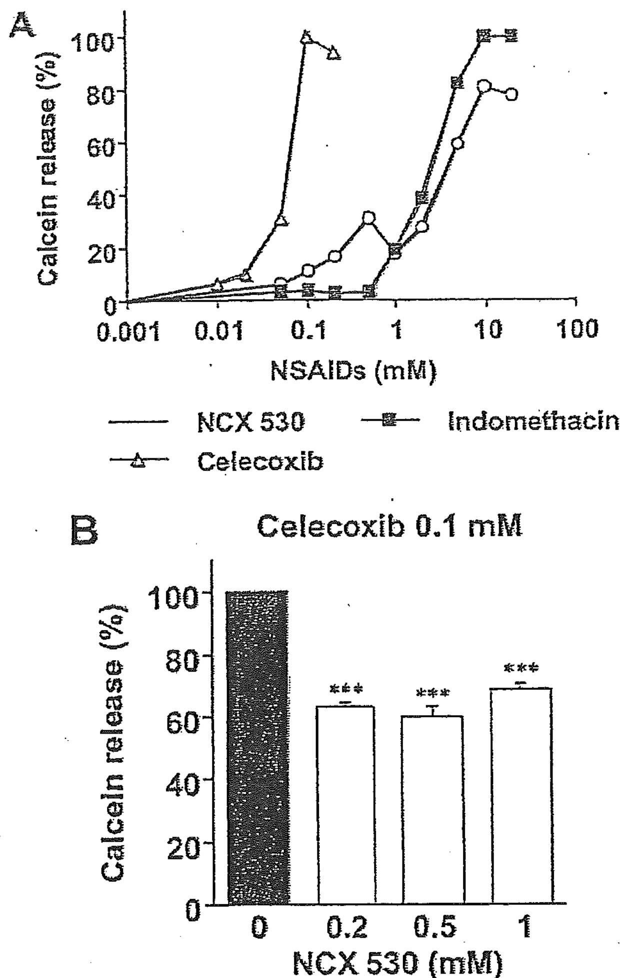

Fig 4. Membrane permeabilization by NSAIDs. Calcein-loaded liposomes were incubated for 10 min at 30°C with indicated concentrations of each NSAID (A) or 0.1 mM celecoxib plus indicated concentrations of NCX 530 (B). The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin (10 μM) was used to determine the 100% level of membrane permeabilization (47).

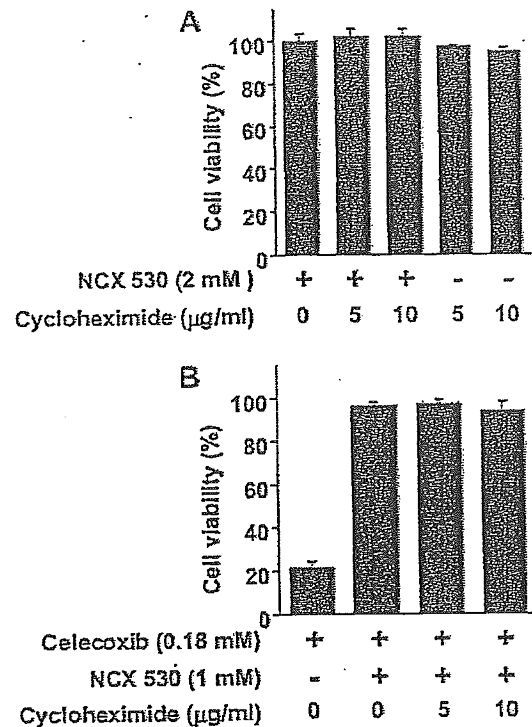

Fig 5. Effect of cycloheximide on cell viability in the presence of NCX 530. Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of cycloheximide for 1 hr. Cells were further incubated with 2 mM NCX 530 and indicated concentrations of cycloheximide for 1 hr (A) (necrotic conditions). Cells were pre-incubated with indicated concentrations of cycloheximide and 1 mM NCX 530 for 1 hr. Cells were further incubated with 1 mM NCX 530, 0.18 mM celecoxib and indicated concentrations of cycloheximide for 1 hr (B) (necrotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. ($n = 3$).

NCX 530 on celecoxib-induced cell death probably involves changes to the membrane permeabilizing capacity of celecoxib. However, at present, it is unclear why NCX 530 with membrane permeabilizing activity itself, protects membrane from celecoxib but not from indomethacin.

NSAIDs have been shown to stimulate the induction of some protective proteins such as heat shock proteins (33) and endoplasmic reticulum chaperons (34). It is possible that induction of protective proteins mediates the low cytotoxicity and the cytoprotective effect of NCX 530. In order to test this possibility, we examined the effect of an inhibitor of protein synthesis (cycloheximide) on the low cytotoxicity and cytoprotective effects of NCX 530. Pre-treatment of cells with cycloheximide did not affect cell viability after incubation with NCX 530 for 1 hr (necrotic conditions) (Figure 5A). Furthermore, pretreatment of cells with cycloheximide did not affect the protective action of NCX 530 against celecoxib-induced cell death (necrotic conditions) (Figure 5B). This concentration of cycloheximide did not affect the cell viability by itself but inhibited protein synthesis, the incorporation of [^{35}S]methionine into acid-insoluble fractions to more than 90%. Since cycloheximide itself inhibits apoptosis, we could not examine its effect on NCX 530-induced apoptosis or the cytoprotective effect of NCX 530 on apoptosis. The results, however, suggest that proteins newly synthesized in the presence of NCX 530 do not contribute to the low level of NCX 530-induced necrosis or to the cytoprotective effect of NCX 530 on celecoxib-induced necrosis.

NO stimulates guanylate cyclase, resulting in an increase in cGMP. Since an increase in cGMP in cells is known to inhibit apoptosis via the inhibition of caspase-3 (35), it is possible that activation of guanylate cyclase

by NCX 530 is responsible for its low cytotoxicity and cytoprotective effects. In order to test this possibility, the effect of an inhibitor of guanylate cyclase (ODQ) on cell death in the presence of NCX 530 was examined. Pre-treatment of cells with ODQ did not affect the cell viability after treatment with NCX 530 for 1 hr (necrotic conditions) (Figure 6A). Furthermore, pre-treatment of cells with ODQ did not alter the extent of cell death induced by celecoxib in the presence NCX 530 for 1 hr (necrotic conditions) (Figure 6B). On the other hand, when the incubation period was changed to 16 hr (apoptotic conditions), pre-treatment of cells with ODQ decreased the cell viability following treatment with NCX 530 (apoptotic conditions) (Figure 6C) and increased the level of cell death induced by celecoxib in the presence NCX 530 (apoptotic conditions) (Figure 6D). This concentration of ODQ did not affect the cell viability by itself (data not shown), however, it is enough to almost completely inhibit guanylate cyclase, based on previous papers (36, 37). These results suggest that activation of guanylate cyclase by NCX 530 may play an important role in the low cytotoxic activity and the cytoprotective effect of NCX 530 for apoptosis, but not for necrosis.

Production of Gastric Lesions by NCX 530. The low cytotoxicity of NCX 530 suggests that it is less likely to produce gastric lesions *in vivo*. As shown in Figure 7, orally administered NCX 530 (42.7 mg/kg) did not produce gastric lesions to any significant extent, whereas orally administered indomethacin (30 mg/kg) (equal molar) clearly produced gastric lesions. This finding is consistent with a previous report (17) and shows that, in relation to its effects on the gastric mucosa in vivo, NCX 530 is safe for use.

As described in the introduction section, we recently found that gastric lesions develop in a manner that depends on both intravenously administered low doses of indomethacin and orally administered cytotoxic COX-2 selective inhibitors, such as celecoxib (16). Using this model, the ability of NCX 530 and indomethacin to produce gastric lesions was tested when either of these compounds was used in combination with the oral administration of celecoxib. Here, NCX 530 and indomethacin were administered intraperitoneally. As shown in Figure 8, the oral administration of celecoxib alone or the intraperitoneal administration of a low dose (5 mg/kg) of indomethacin alone did not produce gastric lesions to any significant extent; however, simultaneous administration of both compounds clearly produced gastric lesions as previously reported (16). In contrast, gastric lesions were not produced when the oral administration of celecoxib and the intraperitoneal administration of NCX 530 were used in combination (Figure 8). Furthermore, intraperitoneally

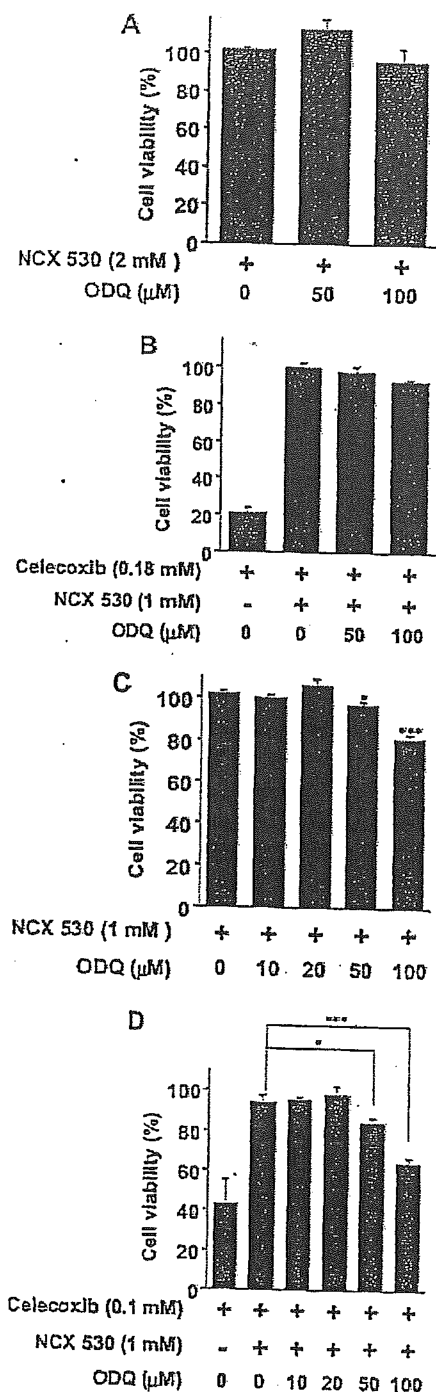

Fig 6. Effect of ODQ on cell viability in the presence of NCX 530. Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of ODQ for 1 hr. Cells were further incubated with indicated concentrations of NCX 530 and ODQ (A, C). Cells were pre-incubated with indicated concentrations of ODQ and 1 mM NCX 530 for 1 hr. Cells were further incubated indicated concentrations of ODQ, NCX 530 and celecoxib (B, D). Incubation was performed for 1 hr (A, B) (necrotic conditions) or for 16 hr (C, D) (apoptotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. ($n = 3$). ***$P < 0.001$; *$P < 0.05$.

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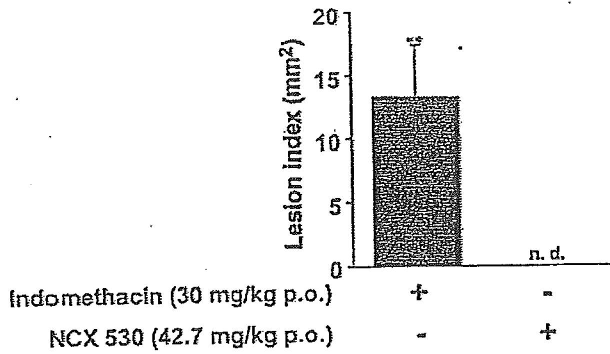


Fig 7. Production of gastric lesions by NCX 530 or indomethacin. Rats were orally administered with NCX 530 or indomethacin as indicated. After 6 hr, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n = 5–6). $^{**}P < 0.01$. n.d.; not detected.

administered NCX 530 suppressed the production of gastric lesions following the oral administration of celecoxib together with the intraperitoneal administration of indomethacin (Figure 8).

We also examined the effect of the intraperitoneal administration of NCX 530 on the production of gastric lesions by other gastric irritants. As shown in Figure 9A, NCX 530 administered in this way significantly decreased the ethanol-induced production of gastric lesions. In contrast, gastric lesions were clearly apparent when indomethacin was administered in place of NCX 530 (Figure 9A). On the other hand, the intraperitoneal administration of NCX 530 did not affect the production of gastric lesions following the oral administration of high doses (30 mg/kg)

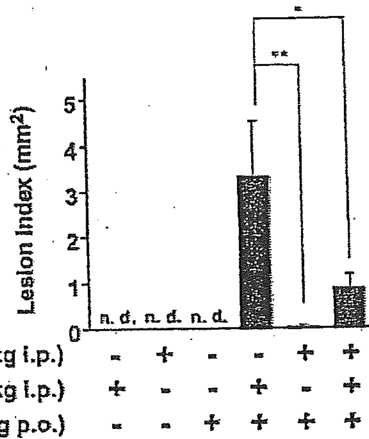
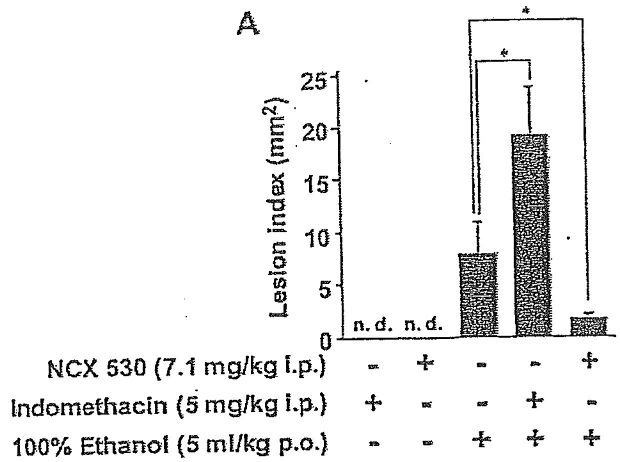


Fig 8. Production of gastric lesions by NCX 530 or indomethacin in combination with celecoxib. Rats were intraperitoneally administered with 5 mg/kg indomethacin or 7.1 mg/kg NCX 530 or vehicle. After 1 hr, animals were administered orally with 15 mg/mL celecoxib or vehicle. After 6 hr, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. ($n = 5 - 6$). $^{**}P < 0.01$; $^{*}P < 0.05$. n.d.: not detected.



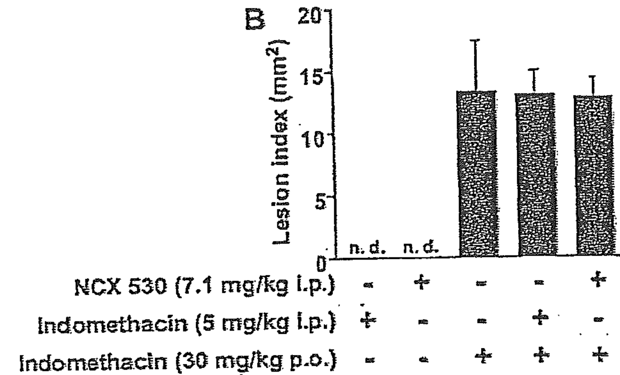


Fig 9. Effect of NCX 530 on production of gastric lesions by other gastric irritants. Rats were intraperitoneally administered with 7.1 mg/kg NCX 530 or 5 mg/kg indomethacin or vehicle. After 1 hr, animals were administered orally with ethanol (A) or 30 mg/kg indomethacin (B) or vehicle. After 6 hr, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n = 5–6). $^{*}P < 0.05$. n.d.: not detected.

of indomethacin (Figure 9B). Therefore, NCX 530 can suppress the production of gastric lesions by some but not all gastric irritants.

DISCUSSION

In this study, the cytotoxicity of NCX 530, one of the new breed of NO-NSAIDs, was assessed. NCX 530 induced both necrosis and apoptosis in gastric mucosal cells in primary culture at much lower levels than did indomethacin. These results are apparently inconsistent with recently published results (38). This may be due to the difference in species of cells and NO-indomethacin; they used colon cancer cells and another NO-indomethacin (NCX 2121) (38). The cytotoxicity of an irritant is determined by both its own toxicity and its capacity to induce cellular stress responses, which, in turn, protect cells from

the irritant. The low cytotoxicity of NCX 530, however, could not be explained by its own toxicity given that NCX 530 gave rise to a similar degree of membrane permeabilization as that seen for indomethacin. On the other hand, a cGMP-dependent cellular response could be involved in the low level induction of apoptosis by NCX 530, since an inhibitor of guanylate cyclase (ODQ) stimulated apoptosis in the presence of NCX 530.

We also found that NCX 530 protects gastric mucosal cells from celecoxib-induced necrosis and apoptosis. This cytoprotective effect of NCX 530 involved both membrane permeabilization and a cGMP-dependent cellular stress response; NCX 530 partially suppressed celecoxib-dependent membrane permeabilization and ODQ inhibited the NCX 530-dependent protection of cells from celecoxib-induced necrosis and apoptosis. However, the reason why NCX 530 protects cells from some irritants (celecoxib, ethanol), but not others (indomethacin, hydrogen peroxide) is yet to be determined. Since the suppression by ODQ of the cytoprotective effect of NCX 530 was partial, other mechanisms may be involved in this cytoprotection. In addition to membrane permeabilization, stimulation of mucus synthesis by NCX 530 may be involved in this cytoprotection as suggested previously (39).

We recently proposed that not only COX inhibition but also the direct cytotoxic effect of NSAIDs (direct cell damage at the gastric mucosa) is involved in the development of gastric lesions (16). On this basis, we proposed that NSAIDs that did not inhibit COX at the gastric mucosa or were without direct cytotoxic effects would not be capable of producing gastric lesions (16). Selective COX-2 inhibitors are NSAIDs that do not inhibit COX at the gastric mucosa, keeping in mind that the primary form of COX expressed at the gastric mucosa is COX-1. However, a recently raised issue concerning the use of selective COX-2 inhibitors is their potential risk for cardiovascular thrombotic events, which is caused by their specificity for COX-2 (40–46). As such, we proposed that NSAIDs without both specificity for COX-2 and direct cytotoxicity are safe for use from a viewpoint of the gastric mucosa and cardiovascular system and therefore have important advantages for clinical use (16). Based on results of this study, NCX 530 may belong to this category of NSAIDs.

A combination of the oral administration of celecoxib with the intraperitoneal administration of NCX 530 did not produce gastric lesions, which is different from the case of intraperitoneal administration of indomethacin. Furthermore, NCX 530 administered intraperitoneally suppressed the production of gastric lesions induced by ethanol or celecoxib plus indomethacin. Since many factors can affect the production of gastric lesions in vivo (mucosal blood flow and gastric motility for example), a number of interpretations for this phenomenon are possible. However, we consider that the direct cytotoxicity of NSAIDs, or direct cell damage at the gastric mucosa by NSAIDs in other words, can explain this phenomenon. In gastric lesions produced by a combination of the oral administration of celecoxib with the intraperitoneal administration of indomethacin or NCX 530, the direct cell damage at gastric mucosa should occur on account of the orally administered celecoxib. As shown in vitro, NCX 530 may suppress celecoxib-induced cell death at the gastric mucosa, meaning that NCX 530 does not actually produce gastric lesions when administered in conjunction with the celecoxib. This idea can also be used to explain the NCX 530-dependent suppression of the production of gastric lesions by ethanol or celecoxib plus indomethacin, given that, in vitro, NCX 530 protected the gastric mucosal cells not only from celecoxib but also from ethanol. Furthermore, observations that NCX 530 did not protect gastric mucosal cells from indomethacin in vitro may explain why the production of gastric lesions by the oral administration of high doses of indomethacin was not suppressed by NCX 530 in vivo. However, in Figure 8, NCX 530 almost completely inhibited the production of gastric lesions by celecoxib in vivo, whereas the effect of this drug on celecoxib-induced cell death is partial in vitro (Figures 2A and 3A). Previous papers reported that NCX 530 stimulated mucosal blood flow and mucus synthesis and did not so clearly increase gastric motility and adhesion of neutrophil as indomethacin (17, 39). We consider that these phenomenon are involved in the safety of NCX 530 on gastric mucosa in vivo.

ACKNOWLEDGMENTS

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REFERENCES

1. Smalley WE, Ray WA, Daugherty JR, Griffin MR: Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. Am J Epidemiol 141:539–545, 1995
2. Hawkey CJ: Nonsteroidal anti-inflammatory drug gastropathy. Gastroenterology 119:521–535, 2000
3. Barrier CH, Hirschowitz BI: Controversies in the detection and management of nonsteroidal antiinflammatory drug-induced side effects of the upper gastrointestinal tract. Arthritis Rheum 32:926–932, 1989
4. Gabriel SE, Jaakkimainen L, Bombardier C: Risk for serious gastrointestinal complications related to use of nonsteroidal

anti-inflammatory drugs. A meta-analysis. Ann Intern Med 115:787–796, 1991
5. Fries JF, Miller SR, Spitz PW, Williams CA, Hubert HB, Bloch DA: Toward an epidemiology of gastropathy associated with nonsteroidal antiinflammatory drug use. Gastroenterology 96:647–655, 1989
6. Kurata JH, Abbey DE: The effect of chronic aspirin use on duodenal and gastric ulcer hospitalizations. J Clin Gastroenterol 12:260–266, 1990
7. Singh G: Recent considerations in nonsteroidal anti-inflammatory drug gastropathy. Am J Med 105:31S–38S, 1998
8. Vane JR, Botting RM: Mechanism of action of anti-inflammatory drugs. Scand J Rheumatol (Suppl 102):9–21, 1996
9. Miller TA: Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. Am J Physiol 245:G601–G623, 1983
10. Ligumsky M, Golanska EM, Hansen DG, Kauffman GJ: Aspirin can inhibit gastric mucosal cyclo-oxygenase without causing lesions in rat. Gastroenterology 84:756–761, 1983
11. Ligumsky M, Sestieri M, Karmeli F, Zimmerman J, Okon E, Rachmilewitz D: Rectal administration of nonsteroidal antiinflammatory drugs. Effect on rat gastric ulcerogenicity and prostaglandin E2 synthesis. Gastroenterology 1245–1249, 1990
12. Lichtenberger LM: Where is the evidence that cyclooxygenase inhibition is the primary cause of nonsteroidal anti-inflammatory drug (NSAID)-induced gastrointestinal injury? Topical injury revisited. Biochem Pharmacol 61:631–637, 2001
13. Lichtenberger LM, Wang ZM, Romero JJ, Ulloa C, Perez JC, Giraud MN, Barreto JC: Non-steroidal anti-inflammatory drugs (NSAIDs) associate with zwitterionic phospholipids: insight into the mechanism and reversal of NSAID-induced gastrointestinal injury. Nat Med 1:154–158, 1995
14. Somasundaram S, Rafi S, Hayllar J, Sigthorsson G, Jacob M, Price AB, Macpherson A, Mahmod T, Scott D, Wrigglesworth JM, Bjarnason I: Mitochondrial damage: a possible mechanism of the "topical" phase of NSAID induced injury to the rat intestine. Gut 41:344–353, 1997
15. Tomisato W, Tsutsumi S, Rokutan K, Tsuchiya T, Mizushima T: NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. Am J Physiol Gastrointest Liver Physiol 281:G1092–G1100, 2001
16. Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, 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
17. Takeuchi K, Mizoguchi H, Araki H, Komoike Y, Suzuki K: Lack of gastric toxicity of nitric oxide-releasing indomethacin, NCX-530, in experimental animals. Dig Dis Sci 46:1805–1818, 2001
18. Hawkey CJ, Jones JI, Atherton CT, Skelly MM, Bebb JR, Fagerholm U, Jonzon B, Karlsson P, Bjarnason IT: Gastrointestinal safety of AZD3582, a cyclooxygenase inhibiting nitric oxide donator: proof of concept study in humans. Gut 52:1537–1542, 2003
19. Johnson AJ, Hsu AL, Lin HP, Song X, Chen CS: 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 366:831–837, 2002
20. Fiorucci S, Santucci L, Gresele P, Faccino RM, Del Soldato P, Morelli A: Gastrointestinal safety of NO-aspirin (NCX-4016) in healthy human volunteers: a proof of concept endoscopic study. Gastroenterology 124:600–607, 2003
21. Wallace JL, Reuter B, Cicala C, McKnight W, Grisham MB, Cirino G: Novel nonsteroidal anti-inflammatory drug derivatives with markedly reduced ulcerogenic properties in the rat. Gastroenterology 107:173–179, 1994
22. Wallace JL, McKnight W, Del Soldato P, Baydoun AR, Cirino G: Anti-thrombotic effects of a nitric oxide-releasing, gastric-sparing aspirin derivative. J Clin Invest 96:2711–2718, 1995
23. Wallace JL, Miller MJ: Nitric oxide in mucosal defense: a little goes a long way. Gastroenterology 119:512–520, 2000
24. Johal K, Hanson PJ: Opposite effects of flurbiprofen and the nitroxybutyl ester of flurbiprofen on apoptosis in cultured guinea-pig gastric mucous cells. Br J Pharmacol 130:811–818, 2000
25. Fiorucci S, Santucci L, Federici B, Antonelli E, Distrutti E, Morelli O, Renzo GD, Coata G, Cirino G, Soldato PD, Morelli A: Nitric oxide-releasing NSAIDs inhibit interleukin-1beta converting enzyme-like cysteine proteases and protect endothelial cells from apoptosis induced by TNFalpha. Aliment Pharmacol Ther 13:421–435, 1999
26. Hirakawa T, Rokutan K, Nikawa T, Kishi K: Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. Gastroenterology 111:345–357, 1996
27. Tomisato W, Takahashi N, Komoto C, Rokutan K, Tsuchiya T, Mizushima T: Geranylgeranylacetone protects cultured guinea pig gastric mucosal cells from indomethacin. Dig Dis Sci 45:1674–1679, 2000
28. Tomisato W, Hoshino T, Tsutsumi S, Tsuchiya T, Mizushima T: Maturation-associated increase in sensitivity of cultured guinea pig gastric pit cells to hydrogen peroxide. Dig Dis Sci 47:2125–2133, 2002
29. Tsutsumi S, Tomisato W, Takano T, Rokutan K, Tsuchiya T, Mizushima T: Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. Biochim Biophys Acta 1589:168–180, 2002
30. Katsu T: Application of calcein-loaded liposomes for the determination of membrane channel size. Biol Pharm Bull 22:978–980, 1999
31. New RRC: Liposomes: A Practical Approach. Oxford: IRL Press, 1990, pp 105–161.
32. 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
33. Lee BS, Chen J, Angelidis C, Jurivich DA, Morimoto RI: Pharmacological modulation of heat shock factor 1 by antiinflammatory drugs results in protection against stress-induced cellular damage. Proc Natl Acad Sci USA 92:7207–7211, 1995
34. Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ, Takenaka H, Tsuchiya T, Mori M, Mizushima T: Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. Cell Death Differ, 2004
35. Kim YM, Talanian RV, Billiar TR: Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. J Biol Chem 272:31138–31148, 1997
36. Brunner F, Stessel H, Kukovetz WR: Novel guanylyl cyclase inhibitor, ODQ reveals role of nitric oxide, but not of cyclic GMP in endothelin-1 secretion. FEBS Lett 376:262–266, 1995
37. Fiorucci S, Antonelli E, Santucci L, Morelli O, Miglietti M, Federici B, Mannucci R, Del Soldato P, Morelli A: Gastrointestinal safety of nitric oxide-derived aspirin is related to inhibition of ICE-like cysteine proteases in rats. Gastroenterology 116:1089–1106, 1999
38. Yeh RK, Chen J, Williams JL, Baluch M, Hundley TR, Rosenbaum RE, Kalala S, Traganos F, Benardini F, del Soldato P, Kashfi K, Rigas

B: NO-donating nonsteroidal antiinflammatory drugs (NSAIDs) inhibit colon cancer cell growth more potently than traditional NSAIDs: a general pharmacological property? Biochem Pharmacol 67:2197–2205, 2004
39. Mizoguchi H, Hase S, Tanaka A, Takeuchi K: Lack of small intestinal ulcerogenecity of nitric oxide-releasing indomethacin, NCX-530, in rats. Aliment Pharmacol Ther 15:257–267, 2001
40. Mukherjee D, Nissen SE, Topol EJ: Risk of cardiovascular events associated with selective COX-2 inhibitors. JAMA 286:954–959, 2001
41. Mukherjee D: Selective cyclooxygenase-2 (COX-2) inhibitors and potential risk of cardiovascular events. Biochem Pharmacol 63:817–821, 2002
42. McAdam BF, Catella LF, Mardini IA, Kapoor S, Lawson JA, Fitzgerald GA: Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci USA 96:272–277, 1999
43. Catella LF, McAdam B, Morrison BW, Kapoor S, Kujubu D, Antes L, Lasseter KC, Quan H, Gertz BJ, Fitzgerald GA: Effects of specific inhibition of cyclooxygenase-2 on sodium balance, hemodynamics, and vasoactive eicosanoids. J Pharmacol Exp Ther 289:735–741, 1999
44. Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald DJ: Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. Circulation 102:840–845, 2000
45. Hennan JK, Huang J, Barrett TD, Driscoll EM, Willens DE, Park AM, Crofford LJ, Lucchesi BR: Effects of selective cyclooxygenase-2 inhibition on vascular responses and thrombosis in canine coronary arteries. Circulation 104:820–825, 2001
46. Dowd NP, Scully M, Adderley SR, Cunningham AJ, Fitzgerald DJ: Inhibition of cyclooxygenase-2 aggravates doxorubicin-mediated cardiac injury in vivo. J Clin Invest 108:585–590, 2001
47. Katsu T, Kobayashi H, Hirota T, Fujita Y, Sato K, Nagai U: Structure–activity relationship of gramicidin S analogues on membrane permeability. Biochim Biophys Acta 899:159–170, 1987

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Upregulation of HSP by geranylgeranylacetone protects the cochlear lateral wall from endotoxin-induced inflammation

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Abstract

We investigated whether an acyclic polyisoprenoid antiulcer drug, geranylgeranylacetone (GGA), induces the expression of HSP70 in the rat cochlea. Immunoblotting revealed upregulation of HSP70 in the cochlea at 12 h after transtympanic (local) or oral (systemic) administration of GGA, and this increased at 24 h after administration. Positive immunohistochemical staining of HSP70 was observed in the hair cells, the spiral ganglion, the stria vascularis, the spiral ligament, and the perivascular portion of modiolar vessels. We therefore subsequently studied the effects of GGA as an HSP-inducer on inner ear trauma due to inflammation. Damage to the lateral wall due to inflammation induced by lipopolysaccharide inoculation was protected against by pretreatment with GGA, as assessed physiologically by measurement of cochlear blood flow and morphologically by electron microscopy. The results of the present study suggest that GGA can protect the cochlea against other injuries including those induced by noise, ototoxic drugs, and ischemia by upregulating HSP70.
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Keywords: GGA; HSP; Inner ear; Otitis media; Cochlear trauma

1. Introduction

The stress response represents a universally conserved cellular defense program. The best example of this increased cellular protection is the phenomenon of "acquired thermotolerance" (Minowada and Welch, 1995). Transient whole-body hyperthermia has been demonstrated to protect against cerebral ischemic cell damage in the rat (Chopp et al., 1989). Molecular chaperones are produced constitutively, and play a fundamental role in several important processes under normal, unstressed conditions (Minowada and Welch, 1995; Hayes and Dice, 1996). The intracellular accumulation of abnormally folded proteins initiates the stress response by activating heat-shock factors, which rapidly trimerize in response to metabolic stress. Trimerization enables heat-shock factors to bind to heat-shock elements, resulting in a high level of transcription of heat-shock genes. Heat-shock proteins (HSPs) bind to immature proteins and prevent premature and improper binding and folding.

The cochlea contains many mechanisms that are involved in the cellular response to stress (Altschuler et al., 2002). Sound conditioning is an active process induced by exposure to low-level, nondamaging noise that provides long-term protection against noise trauma (Niu and Canlon, 2002). HSPs can be induced in the inner ear by several stresses, including acoustic overstimulation

Abbreviations: GGA, geranylgeranylacetone; HSP, heat shock protein; LPS, lipopolysaccharide; INOS, inducible nitric oxide synthase; AICA, anterior inferior cerebellar artery; LD, laser-Doppler

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(Lim et al., 1993), cisplatin (Oh et al., 2000), and hyperthermia (Fairfield et al., 2004), and sound-conditioning-induced HSP70 production was reported to protect the mouse cochlea from acoustic injury (Yoshida et al., 1999).

An acyclic polyisoprenoid antiulcer drug, geranylgeranylacetone (GGA), has been previously used to induce transcriptional activation of HSP70 genes, and reported to increase gastric mucosal defense systems (Hirakawa et al., 1996). The effects of GGA on HSP induction have been reported in other organs, including heart (Ooie et al., 2001), liver (Oda et al., 2002), and brain (Fujiki et al., 2003). Antiviral effects of GGA have also been reported (Unoshima et al., 2003). HSP70 families stabilize unfolded proteins prior to their assembly into multimolecular complexes, and associated genes are found in most cellular compartments (Becker and Craig, 1994).

The clinical implications of stress response have been indicated in infections, and HSP induction was also reported to inhibit inducible nitric oxide synthase (iNOS) and attenuate hypotension in endotoxin challenged rats (Hauser et al., 1996). We have previously reported that otitis media induces damage to the cochlear lateral wall, and the therapeutic effects of intratympanic administration of steroid and iNOS inhibitor (Sone et al., 2003, 2004). In this study, we investigated whether GGA induces the expression of HSP70 in the cochlea, and hence could prevent the damage to the cochlear lateral wall induced by inflammation.

2. Materials and methods

2.1. Animals and administration of GGA

We used 40 female Sprague–Dawley rats weighing 150–225 g. During surgery and when they were killed, the animals were anesthetized by the intramuscular injection of a mixture of ketamine hydrochloride (40 mg/kg), xylazine hydrochloride (8 mg/kg), and acepromazine maleate (1 mg/kg). All animals were free of middle ear infection prior to experiments. GGA was obtained from Eisai (Tokyo, Japan), and administered in two ways: systemically (oral) and locally (transtympanic).

2.2. Systemic administration

GGA, as an emulsion with 5% gum arabic and 0.008% tocopherol, was given orally at a dose of 800 mg/kg (2.4×10^{-3} M/kg) (systemic GGA group). As a control, rats were given the same dose of vehicle (systemic control group).

2.2.1. Local administration

Thirty microliters of GGA (5.0×10^{-4} M/kg) dissolved in absolute ethanol (final concentration of 1%) was given transtympanically into the middle ear cavity under a microscope (local GGA group). Ethanol is known to induce of HSP, and hence control rats were given 30 μl of 1% ethanol (local control group).

The treated animals were examined at 12 or 24 h after administration. After deep anesthesia, the inner ears of the rats were resected and examined. Experimental protocols were approved by the Nagoya University Committee on the Use and Care of Animals.

2.3. Immunoblotting

Cochleas (four ears in each group) were dissected and diluted in dissociation buffer, and then centrifuged. The total protein concentration of the cochleas was quantified by the Bradford method. An equal amount of total protein in each fraction was subjected to 8% SDS–PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane. The membrane was incubated with antibodies against HSP70 (mouse monoclonal IgG; Stressgen Biotechnologies, Victoria, Canada) or actin (goat polyclonal IgG; Santa Cruz Biotechnology, CA, USA) overnight at 4 °C, and exposed to secondary antibodies of goat antimouse IgG-HRP (Amersham Biosciences, Buckinghamshire, UK) or mouse antigoat IgG-HRP (Santa Cruz Biotechnology) for 1 h at room temperature. The proteins were detected by enhanced chemiluminescence, and quantified using NIH imaging software.

2.4. Immunohistochemistry

Rat cochleas (four ears in each group) were removed and fixed with 4% paraformaldehyde. The tissues were then decalcified in 10% EDTA and embedded in paraffin. Blocks were sectioned at a thickness of 3 μm, and the sections were incubated with anti HSP70 antibody (mouse monoclonal IgG1; Stressgen Biotechnologies) as the primary antibody and biotinylated rabbit anti-mouse IgG (Dako, CA, USA) as the secondary antibody. Staining was performed with a Catalyzed Signal Amplification kit (Dako), which is an extremely sensitive immunohistochemical staining procedure incorporating signal-amplification methods based on the peroxidase-catalyzed deposition of a biotinylated phenoic compound. After incubation with streptavidin-peroxidase, sections were developed with diaminobenzidine tetrahydrochloride and hydrogen peroxide, and counterstained with hematoxylin. Sections without primary antibody treatment served as negative controls.

2.5. Pretreatment by GGA in rats with endotoxin-induced inflammation

To investigate the effects of GGA (as an inducer of HSP) on inner ear trauma caused by endotoxin-induced

inflammation in the middle ear, 800 mg/kg GGA was given orally 24 h before inflammation was induced. The preparation of the animal model with inflammation in the middle ear is described elsewhere (Sone et al., 2003, 2004). Briefly, endotoxin inoculation was performed by instilling lipopolysaccharide (LPS; 5 mg/ml) from *Escherichia coli* (Sigma, St. Louis, MO, USA) into the middle ear cavity of each animal. Control rats were given the same dose of vehicle 24 h before LPS-inoculation. Treated animals were examined at 24 h after LPS-inoculation. Each group consisted of four ears.

2.5.1. Measurement of cochlear blood flow and clamping of the anterior inferior cerebellar artery

The measurement technique used are described elsewhere (Yamamoto et al., 2003). Briefly, each bulla was opened carefully through a ventrolateral approach under general anesthesia. The 1.0-mm probe of a laser-Doppler (LD) flowmeter (ALF21, Advance, Tokyo, Japan) was placed over the basal turn of the cochlea, where maximum output of the device was obtained. The basilar artery and the anterior inferior cerebellar artery (AICA) were exposed by opening the dura, after which the AICA was occluded for 5 min by pressure from a metal stick with a 0.6-mm-diameter spherical tip. The cochlear blood flow and blood pressure were monitored continuously, using a computer-based chart recorder.

2.5.2. Electron microscopy

After deep anesthesia, the inner ears of the rats were resected and the lateral walls of cochlear basal turns were examined since, in a previous study, we found that the pathological changes were most prominent in the cochlear basal turn of rats after inoculation of LPS into the middle ear cavity. The inner ears were fixed by perilymphatic perfusion with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde, postfixed in 1% osmium, dehydrated, and embedded in Epon. Ultrathin sections of the cochlear lateral walls were stained with uranyl acetate and lead citrate, and examined using a Hitachi H-7100 transmission electron microscope.

2.6. Statistical analysis

Data were analyzed using the Kruskal–Wallis test and the Mann–Whitney test.

3. Results

3.1. Expression of HSP70 in the cochlea following administration of GGA

3.1.1. Immunoblotting

At 12 h after administration of GGA, upregulation of HSP70 was observed in the cochleas of both local (the density was 151% that of untreated rats) and systemic (relative density of 198%) GGA groups compared to normal rats, with a higher level of expression in the systemic group (Fig. 1). Upregulation was not observed in the cochleas 12 h after the local application of ethanol (local control group) (102%). HSP70 expression was increased in the cochleas 24 h after the local application of ethanol (relative density of 230%), but the expression was stronger in the cochleas of both local (relative density of 271%) and systemic (relative density of 254%) GGA groups. In each group, HSP expression was higher 24 h after administration than at 12 h. There was a tendency for the relative density of HSP70 to be more varied in rats of local groups than in those of systemic groups.

3.1.2. Immunohistochemistry

Compared with normal rats, stronger positive staining of HSP70 was observed in the cochleas of all GGA-treated groups, and at 24 h after the local application of ethanol (local control group). Positive staining was found in the cytoplasm of both inner and outer hair cells, supporting cells, the spiral limbus, the spiral ganglion cells, the stria vascularis, cells and connective tissue in the spiral ligament, and the perivascular portion of modiolar vessels (Fig. 2). Similar to the results obtained by immunoblotting, there was a tendency for the positive staining of HSP70 in the cochlea to be more varied in rats of local groups than in those of systemic groups. The expression of HSP70 in the lateral wall of animals of local groups was more prominent in the basal turn than in the apical turns.

Fig. 3 shows the expression of HSP70 in the lateral wall of animals in each group. Compared with normal rats, rats at 12 h after the local administration of ethanol or GGA showed stronger positive staining in the lateral

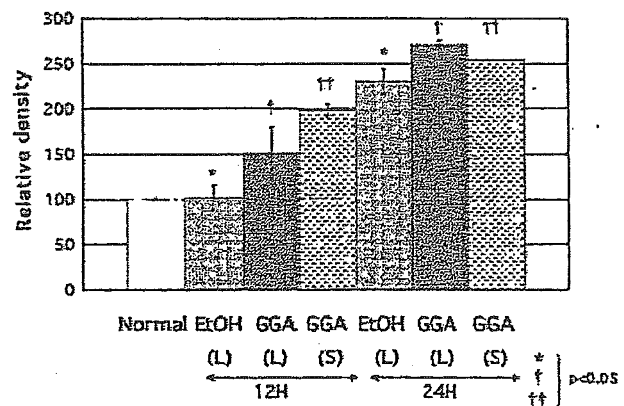

Fig. 1. Expression of HSP70 after local (L) treatment with ethanol (EtOH) or GGA, or systemic (S) treatment with GGA, as revealed by immunoblotting. Cochleas were investigated 12 h (12H) or 24 h (24H) after application. Data are the density relative to that of untreated rats (normal) (mean ± SE, $n = 4$ in each group).