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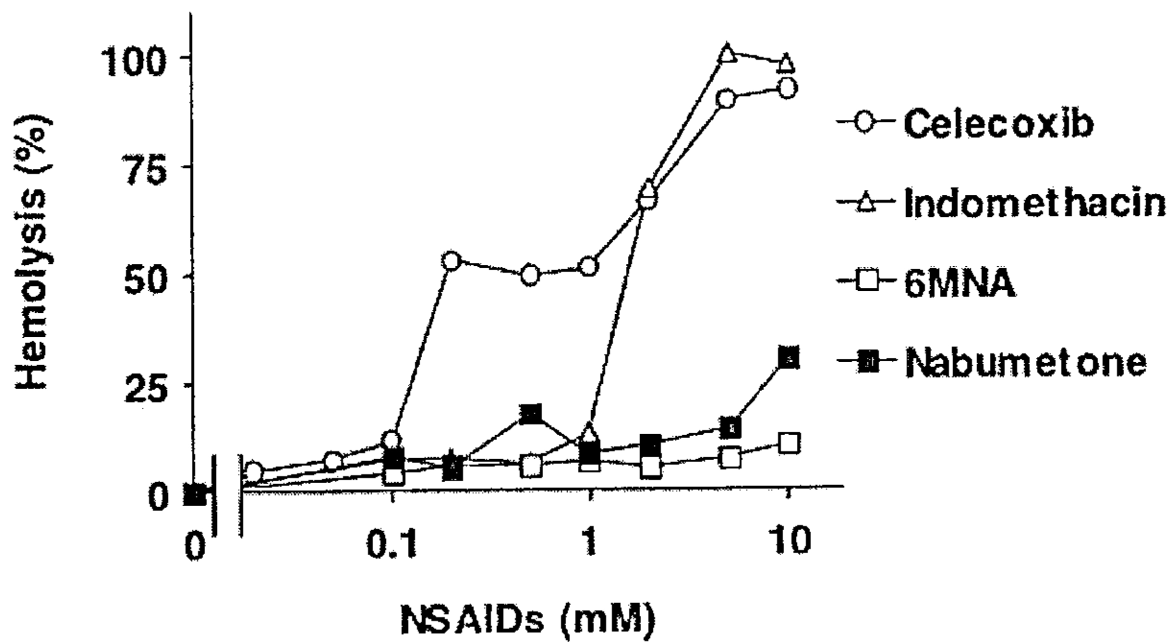

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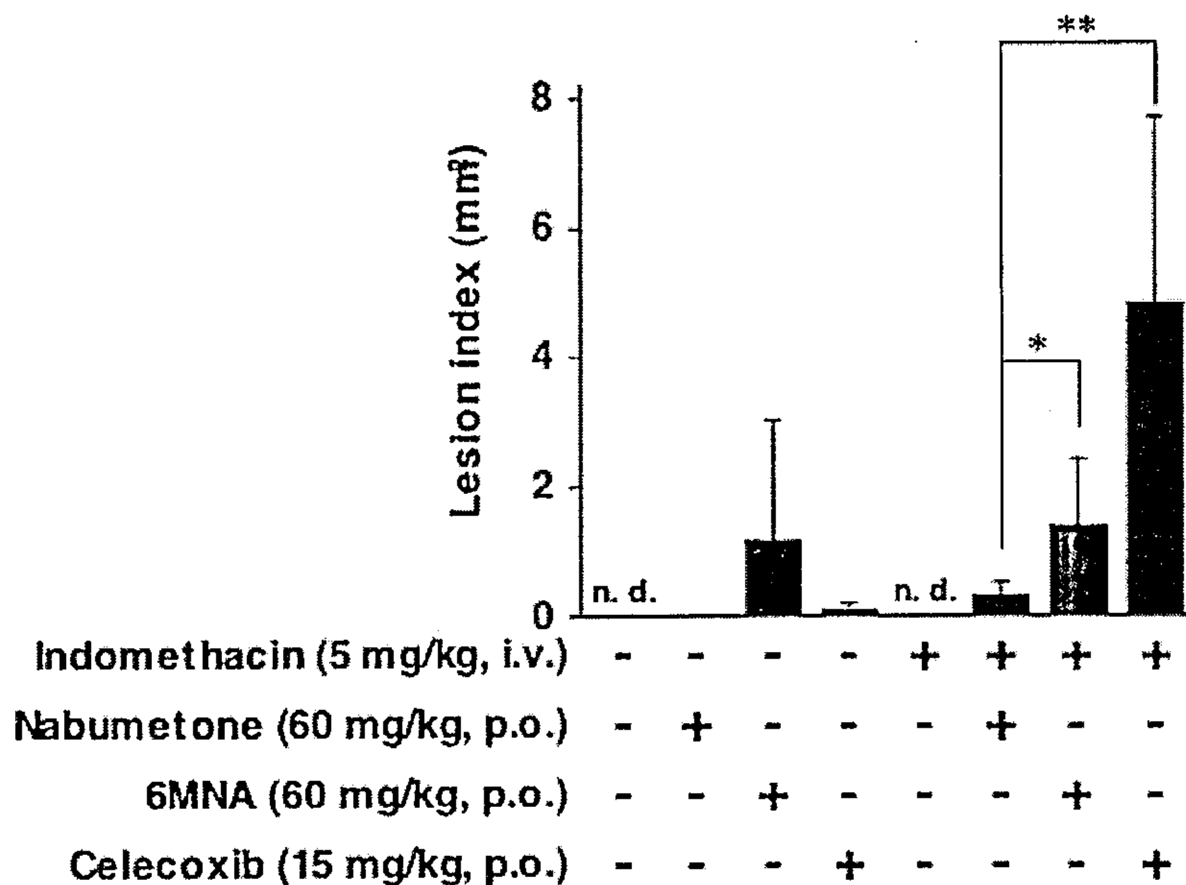

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Low Direct Cytotoxicity and Cytoprotective Effects of Nitric Oxide-Releasing Indomethacin

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Running head: Cytotoxicity of NO-NSAIDs

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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 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 h 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 (pH7.8), 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 h. 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 h 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 intraperitoneally1 h 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 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 [illegible]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 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 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 h are shown in Fig. 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 (Fig. 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 h are summarized in Fig. 1B. Again, indomethacin but not NCX 530 induced cell death. Because cell death under these conditions was accompanied by apoptotic DNA fragmentation (Fig. 1C), it is most likely to have been mediated by apoptosis. Overall, the results in Fig. 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 h (pre-incubation step) and then with one of several of gastric irritants (celecoxib, ethanol, indomethacin or hydrogen peroxide) for 16 h (apoptotic conditions) (Fig. 2) or 1 h (Fig. 3) (necrotic conditions) (incubation step). As shown in Fig. 2A, treatment of cells with 0.1 mM celecoxib for 16 h 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 h (Fig. 2B). Under these conditions (Fig. 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 Fig. 3A, B, NCX 530 partially suppressed the cell death induced by treatment of cells with 0.18 mM celecoxib or 8% ethanol for 1 h. 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 Figs. 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 (Fig. 2C, D) nor necrosis (Fig. 3C, 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 Fig. 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 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 (Fig. 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 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 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 h (necrotic conditions) (Fig. 5A). Furthermore, pre-treatment of cells with cycloheximide did not affect the protective action of NCX 530 against celecoxib-induced cell death (necrotic conditions) (Fig. 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 h (necrotic conditions) (Fig. 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 h (necrotic conditions) (Fig. 6B). On the other hand, when the incubation period was changed to 16 h (apoptotic conditions), pre-treatment of cells with ODQ decreased the cell viability following treatment with NCX 530 (apoptotic conditions) (Fig. 6C) and increased the level of cell death induced by celecoxib in the presence NCX 530 (apoptotic conditions) (Fig. 6D). This concentration of ODQ did not affect the cell viability by itself (data not shown), however, 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 Fig. 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 Fig. 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 of 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 (Fig. 8). Furthermore, intraperitoneally administered NCX 530 suppressed the production of gastric lesions following the oral administration of celecoxib together with the intraperitoneal administration of indomethacin (Fig. 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 Fig. 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 (Fig. 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) of indomethacin (Fig. 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; NCX530 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 NCX530 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