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ORIGINAL ARTICLE—ALIMENTARY TRACT

Reactive oxygen species-quenching and anti-apoptotic effect of polaprezinc on indomethacin-induced small intestinal epithelial cell injury

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Abstract
Background To protect the small intestine from mucosal injury induced by nonsteroidal anti-inflammatory drugs is one of the critical issues in the field of gastroenterology. Polaprezinc (PZ), a gastric muco-protecting agent, has been widely used for the treatment of gastric ulcer and gastritis for its unique effects, such as its strong reactive oxygen species (ROS)-quenching effect. The aim of this study was to clarify the mechanism by which indomethacin-induced small intestinal mucosal injury occurs, by using a rat intestinal epithelial cell line (RIE-1). In addition, the protective role of PZ and the possible mechanism of its effect on indomethacin-induced small intestinal injury were investigated.
Methods Cell death was evaluated by methyl thiazolyl tetrazolium (MTT) assay and a double-staining method with Hoechst33342 dye and propidium iodide. Indomethacin-induced ROS production was evaluated by detecting the oxidation of a redox-sensitive fluorogenic probe, Redox Sensor, and the oxidation of cysteine residues of proteins (protein S oxidation). The activation of cytochrome *c*, smac/DIABLO, and caspase-3 was assessed by western blotting. In some experiments, PZ or its components, L-carnosine and zinc, were used.
Results We found that indomethacin caused apoptosis in RIE-1 cells in a dose- and time-dependent manner. Indomethacin also induced ROS production and an increase in the protein S oxidation of RIE-1. Pretreatment of RIE-1 with PZ or zinc sulfate, but not L-carnosine, significantly reduced the indomethacin-induced apoptosis. PZ prevented ROS production and the increase in protein S-oxidation. PZ inhibited indomethacin-induced cytochrome *c* and smac/DIABLO release and subsequent caspase-3 activation.
Conclusions The protective effect of PZ on indomethacin-induced small intestinal injury may be dependent on its ROS-quenching effect.

Keywords Protein S oxidation · Reactive oxygen species · Apoptosis · Zinc · NSAIDs

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Introduction

In studies of the side effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on the digestive tract, stomach and duodenal symptoms have usually been emphasized. However, with the appearance of capsule endoscopes and double-balloon endoscopes, many studies have reported that NSAID-related small intestinal mucosal injury is common [1, 2] and that this injury can be caused by various mechanisms [3].

Concerning the treatment of NSAID-induced digestive tract injury, most research and development has been conducted regarding gastric mucosal injury, and no small intestine-specific agent has been developed yet. In clinical practice, however, a prostaglandin E1 (PGE_1) derivative, misoprostol, and antacids, including proton pump inhibitors

(PPIs), are routinely used for the treatment and prevention of NSAID enteropathy [4, 5]. Although we have previously reported the usefulness of PPIs in the treatment of NSAID-induced small intestinal mucosal injury in an animal experiment [6], there are no clinical data on the usefulness of PPIs in such injuries. Some studies have indicated the efficacy of misoprostol for NSAID-induced small intestinal mucosal injury [7], whereas others have reported that this agent was ineffective [8]. Moreover, it is known that misoprostol often causes diarrhea, reducing compliance. Several studies have suggested the usefulness of metronidazole [8, 9]; however, metronidazole also has a limitation: long-term combination therapy with metronidazole and NSAIDs may lead to the appearance of resistant bacteria. Therefore, the development of small intestine-specific agents that can be safely used for a long period, such as muco-protective drugs, is anticipated.

In order to develop new strategies for the treatment and prevention of NSAID-induced enteropathy, the pathogenesis of NSAID-induced small intestinal mucosal injury should be clarified. We previously reported that cyclooxygenase (COX)-dependent and COX-independent pathways were involved in this phenomenon, and we found that indomethacin significantly induced reactive oxygen species (ROS) production in small intestinal epithelial cells through a COX-independent pathway [10].

As a clinically available agent for the treatment or prevention of NSAID-induced enteropathy, we chose polaprezinc (PZ) among muco-protective drugs, as we have previously reported the protective effect of PZ on the gastric mucosa via its potent antioxidant actions [11, 12]. PZ is a chelate compound consisting of zinc (Zn) and L-carnosine (L-car). In the United States, it is commercially available as a dietary supplement. In Japan, it is commonly employed as an agent for the treatment of gastric ulcers. Several studies have indicated the protective role of PZ in gastric mucosal injury related to various stimuli in vivo [13, 14] and in vitro [15].

In this study, we investigated the pathogenesis of NSAID-induced small intestinal mucosal injury in vitro, using indomethacin as an NSAID. In addition, we examined the effects of PZ on indomethacin-induced cell injury and investigated the protective mechanism of PZ.

Materials and methods

Intestinal epithelial cell line

A rat intestinal epithelial cell line (RIE-1) [16], which was kindly provided by Professor Tsujii (Osaka University, Osaka, Japan) was used as an in vitro model of the small intestine. RIE-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and reseeded when the cell monolayer became subconfluent.

Reagents

DMEM/F12, FBS, and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA, USA). Indomethacin, prostaglandin E_2 (PGE_2), Zn, L-car, and ethanolamine were purchased from Sigma–Aldrich (St. Louis, MO, USA). L-Cysteine and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). PZ was kindly provided by Zeria Pharmaceutical (Tokyo, Japan). Indomethacin was dissolved in dimethylsulfoxide (DMSO). PGE_2 was dissolved in ethanol. Zn and L-car were dissolved in distilled water purified by Milli-Q (Millipore, Billerica, MA, USA). PZ was dissolved in 0.2 M HCl and adjusted to pH 6.5 with 0.2 M NaOH just before use. PZ, Zn, and L-car were added to the medium 6 h before indomethacin administration. Biotinylated cysteine (Bt-Cys) was synthesized as described below: 10 mM cysteine/$NaHCO_3$ buffer (0.2 M $NaHCO_3$, 0.5 M NaCl; pH 8.3) and 11 mM sulfo-NHS-LC-biotin/H_2O were mixed (5 ml each) and left to derivatize for 1 h at room temperature. After 30 min incubation with 5.48 µl of ethanolamine at room temperature, 5 µl of DTT (1 M) was added, and the product was stored at −20°C.

Cell viability

Cell viability was quantified by using a methyl thiazolyl tetrazolium (MTT)-based WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay kit (Dojindo Laboratories, Kumamoto, Japan). The cells were seeded in 96-well plates, and were incubated for 0–48 h in the absence or presence of indomethacin and/or other reagents such as PGE_2 and PZ. The cells were washed twice with PBS and WST-8 solution was added. The cells were then incubated for 1 h at 37°C and the optical density of each well was read at 450 nm, using a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA).

Cell apoptosis

The effect of indomethacin on apoptosis was evaluated morphologically by fluorescence microscopy after labeling

with Hoechst33342 (HO342 dye; Sigma–Aldrich) and propidium iodide (PI) (Sigma–Aldrich) as described by Handa et al. [17]. The cells were grown in 35 mm cell culture dishes until almost confluent. After exposure to indomethacin for 24 h, the cells were incubated with 10 μg/ml HO342 dye for 15 min at 37°C and with 10 μg/ml PI for 10 min at 37°C. Dual-stained cells were examined using an inverted fluorescence microscope (IX70-23FL/DIC-SP; Olympus, Tokyo, Japan). Photographic images (JPEG format) were taken from four random fields. Live cells, viable cells, and early-stage apoptotic cells, which have cell membrane function, take up blue dye (HO342). Apoptosis was characterized morphologically by condensed chromatin. Red-stained cells (PI) were considered to be late apoptotic (condensed chromatin) or necrotic cells.

Quantitative evaluation of apoptosis was determined with a Cell Death Detection ELISA kit (Roche Diagnostics, Basel, Switzerland). This ELISA kit is designed for detecting the cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) after the induction of cell death. After incubation with indomethacin with/without PZ, Zn, and L-car for periods of up to 24 or 48 h, the supernatant was removed (exclusion of necrotic cells) and cells were lysed with lysis buffer. After centrifugation of the lysate, the supernatant was examined with the ELISA kit. The ELISA was evaluated with the use of a 20 μl sample and 80 μl immunoreagent per streptavidin-coated microplate well. Then the microplate was incubated on a plate shaker with gentle shaking for 2 h at room temperature. After removing the solution and washing each well with incubation buffer, 100 μl 2,2′-azino-di(3-ethylbenzthiazolinsulfonate) (ABTS) solution was added to each well and incubation was carried out on a plate shaker until the color development was sufficient for photometric analysis (after approximately 10–20 min). Finally, 100 μl of ABTS stop solution was added to each well. The optical density of each well was read at 405 nm, using a microplate reader (SpectraMax M2; Molecular Devices).

Separation of cell lysate into cytosolic and mitochondrial fractions

Cytosolic and mitochondrial compartments were separated by ultracentrifugation, by using a Mitochondria/Cytosol Fractionation Kit (Bio Vision, Mountain View, CA, USA) as described before [18]. Briefly, cells were collected, washed, resuspended with 1× cytosol extraction buffer, and homogenized with a grinder. The homogenates were then centrifuged at 700*g* for 10 min at 4°C. The supernatants were centrifuged at 10,000*g* for 30 min at 4°C, and then the supernatants were used as the cytosolic fraction. The resulting pellets were resuspended with 1× mitochondrial extraction buffer and used as the mitochondrial fraction.

Assessment of oxidative stress

The effect of indomethacin on ROS production in RIE-1 cells was confirmed using a laser scanning confocal microscope (FV10i; Olympus, Tokyo, Japan) after labeling live cells with a redox-sensitive fluorogenic probe, RedoxSensor Red CC-1 and a mitochondria-selective fluorogenic probe, MitoTracker Green FM (Molecular Probes, Eugene, OR, USA). Cells were grown until almost confluent and incubated in the absence or presence of 200 μM indomethacin and/or 100 μM PZ for 30 min. Then the cells were incubated with 1 μM of RedoxSensor Red CC-1 and 1 μM of MitoTracker Green FM for 10 min at 37°C before observation. Photographic images (JPEG format) were taken from four random fields.

In addition, we evaluated intracellular oxidative stress by another method detecting S-oxidized proteins. RIE-1 cells were incubated in the absence or presence of 200 μM indomethacin and/or 100 μM PZ for 3 h, and subsequently 100 μM Bt-Cys was added to the RIE-1 cells for 30 min. Then whole-cell lysate or separated lysate (prepared as described above) was prepared. The protein concentrations of the supernatants were adjusted to 1 mg/ml by dilution in 4× sodium dodecylsulfate (SDS) gel-loading buffer with or without a reducing agent and incubated at 70°C for 10 min before loading to the gel. Next, 20 μg protein was loaded onto each lane of 10% acrylamide gel and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto a nitrocellulose membrane. After transfer, the membrane was incubated for 30 min at room temperature in blocking agent (EzBlock; ATTO, Tokyo, Japan). The membrane was then incubated for 1 h at room temperature in horseradish peroxidase (HRP)-conjugated streptavidin (Invitrogen) or rabbit polyclonal anti-actin antibody (Santa-Cruz Biotechnology, Santa Cruz, CA, USA) as the internal standard. Anti-α tubulin antibody (Santa-Cruz Biotechnology) or anti-cytochrome *c* oxidase subunit IV (COX4) antibody (Santa-Cruz Biotechnology) was used to confirm that each lysate was the compartment of cytosol or mitochondria, respectively. After washing, bound HRP was detected by enhanced chemiluminescence (ECL plus western blotting detection system; GE Healthcare, Buckinghamshire, United Kingdom) and visualized with VersaDoc 5000MP (Bio-Rad Laboratories, Hercules, CA, USA).

Detection of apoptosis-related proteins by western blotting

The lysate was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After transfer, the membrane was incubated for 30 min at room temperature in blocking agent. The membrane was then incubated for 1 h at room temperature with anti-cytochrome *c* antibody (1:500;

Abcam, Cambridge, UK), or anti-smac/DIABLO antibody (1:500; Abcam), or anti-caspase-3 antibody (1:500; Santa-Cruz Biotechnology) followed by a secondary anti-rabbit IgG antibody to perform ECL development, as described above. Quantification was performed using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

Statistical analysis

The experiment was performed in triplicate, and results are expressed as means ± SEM. Statistical analyses were performed using a paired *t* test (for time-course study; Fig. 1b, Fig. 3) or Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Indomethacin induced cell death in RIE-1 cells in a concentration- and time-dependent manner

Firstly, we decided the concentration and the period of time in which indomethacin induced the cell injury. RIE-1 cells were incubated with indomethacin in concentrations ranging from 0 to 400 μM for 24 h and we found that indomethacin induced cell death in a concentration-dependent manner (Fig. 1a). The indomethacin-induced cell death also occurred in a time-dependent manner (Fig. 1b). The cell death became evident after 12 h of indomethacin treatment.

Indomethacin induced apoptosis-dominant cell death

The mode of cell death was evaluated by staining RIE-1 cells with HO342 and PI, and observing the cells under fluorescence microscopy. Indomethacin, when incubated for 24 h with RIE-1 cells, induced apoptotic changes (Fig. 2b), such as nuclear condensation and the formation of apoptotic bodies, a hallmark of apoptotic cells [19] ; such changes were less evident in the control cells (Fig. 2a). Early and late apoptotic cells were found in indomethacin-stimulated RIE-1 cells. Very few primary necrotic cells could be seen in both the control and indomethacin-stimulated groups.

We also performed a quantitative analysis of indomethacin-induced apoptosis by using an ELISA. As shown in Fig. 3, the proportion of apoptotic cells in the medium-alone group slowly increased in a time-dependent manner; however, the proportion of apoptotic cells in the indomethacin (200 μM)-stimulated group increased remarkably from 3 h ($P < 0.05$ at 3 h and 6 h, $P < 0.01$ after 9 h).

Indomethacin-induced cell death was not attenuated by PGE_2 treatment

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, have side effects on digestive organs because of their inhibitory effects on cyclooxygenase (COX), which catalyzes the formation of PGs from arachidonic acid. Therefore, PG derivatives are used clinically for the prevention of NSAID-induced gastroenteropathy and ulcer. In order to clarify the role of COX in the indomethacin-induced cell death of RIE-1 cells, we used PGE_2 and found that PGE_2 could not protect RIE-1 from indomethacin-induced cell death (Fig. 4). This result suggested that indomethacin induced cell death in RIE-1 through a COX-independent mechanism.

PZ protected RIE-1 cells from indomethacin-induced cell death

We have previously reported that indomethacin induced significant apoptosis in differentiated Caco-2 cells (an

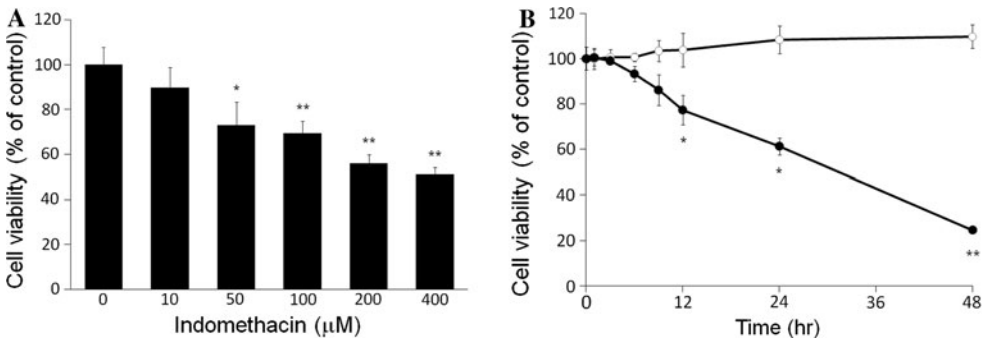

Fig. 1 Indomethacin induced cell death in RIE-1 cells in a dose- and time-dependent manner. The induction of cell death by indomethacin in RIE-1 cells was evaluated by a methyl thiazolyl tetrazolium (MTT)-based WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay. **a** RIE-1 cells were incubated with various concentrations of indomethacin for 24 h. **b** RIE-1 cells were cultured in the absence (*open circles*) and presence of 200 μM indomethacin (*closed circles*) for 0–48 h. The results are shown as percentage variation of optical density (OD) compared to (**a**) medium alone or (**b**) 0 h, and are expressed as means ± SEM of three separate experiments. $*P < 0.05$ and $**P < 0.01$ compared with (**a**) medium alone and (**b**) 0 h

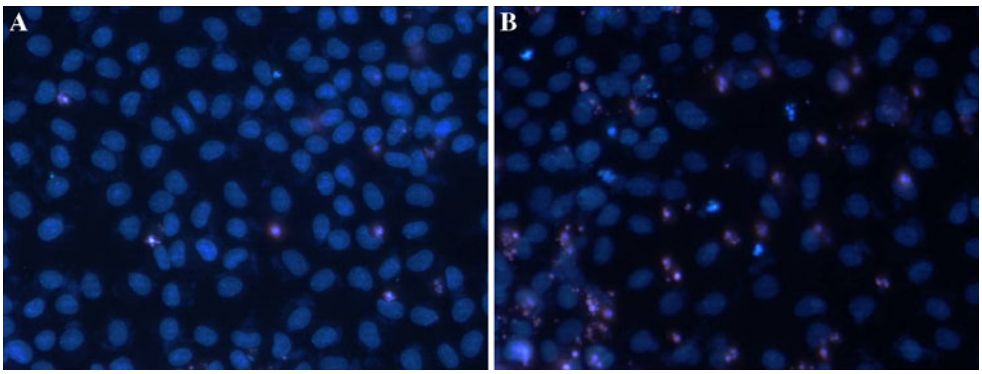

Fig. 2 Indomethacin-induced apoptosis of RIE-1 cells. Indomethacin-stimulated RIE-1 cells were stained with Hoechst33342 (HO342) and propidium iodide (PI) and observed under a fluorescence microscope. **a** Control cells. **b** Cells exposed to 200 μM indomethacin- for 24 h. *Blue condensed chromatin* indicates early apoptosis and *red* indicates late apoptosis. A representative image of three independent experiments is shown (×100)

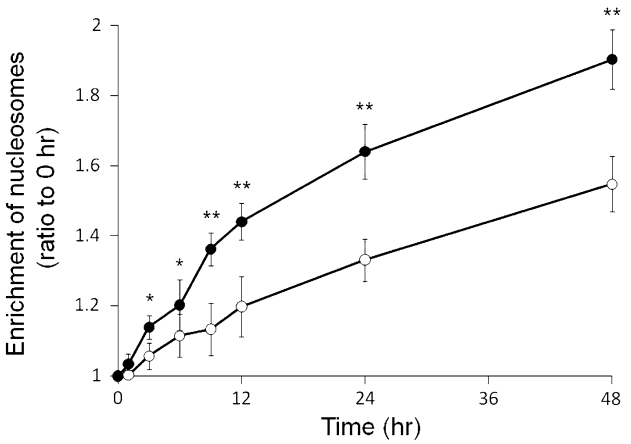

Fig. 3 Indomethacin induced apoptosis in RIE-1 cells in a time-dependent manner. Indomethacin-induced apoptosis was quantified by using an enzyme-linked immunosorbent assay (ELISA). RIE-1 cells were incubated in the absence (*open circles*) or presence (*closed circles*) of 200 μM indomethacin for 0–48 h. Values are represented by a ratio to 0 h as 1. The data are expressed as means ± SEM of three separate experiments. $*P < 0.05$ and $**P < 0.01$ compared with 0 h

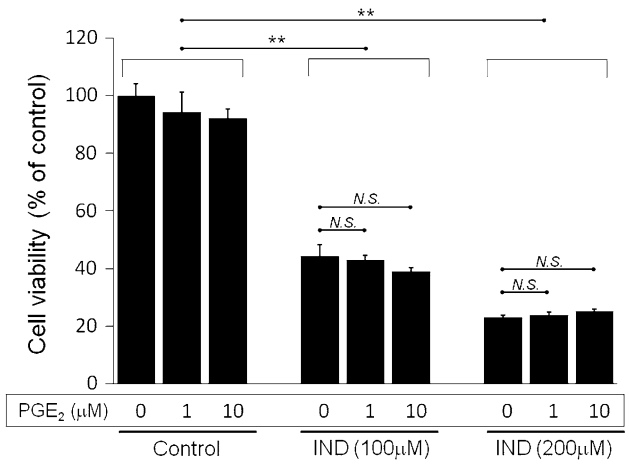

Fig. 4 Prostaglandin E_2 (*PGE_2*) could not protect RIE-1 cells from indomethacin (*IND*)-induced cell death. RIE-1 cells were incubated in the absence (control) or presence of 100 or 200 μM indomethacin with PGE_2 (0, 1, 10 μM) for 48 h, and subsequently cell viability was evaluated by MTT-based WST-8 assay. The results are shown as percentage variation of OD compared to medium alone (0 μM PGE_2 of control group), and are expressed as means ± SEM of three separate experiments. *N.S.*Not significant, compared with 0 μM PGE_2 in each indomethacin-treated group. $**P < 0.01$ compared with the same concentration of PGE_2 in the control group and indomethacin-treated group

immortalized human colonic epithelial cell line that exhibits the phenotype of the small intestine according to differentiation), and α-tocopherol significantly reduced this apoptosis through its strong antioxidant effect [20]. Given the above results, we hypothesized that indomethacin-induced apoptosis was caused through COX-independent and ROS-dependent signal transductions. We have reported that the muco-protective drug, PZ, has a strong ROS-quenching effect [11, 12], and therefore we examined the effect of PZ on the indomethacin-induced cell death of RIE-1 cells. As a result, we found that PZ at higher concentrations significantly inhibited the indomethacin-induced cell death of RIE-1 cells (Fig. 5). Indomethacin reduced the cell viability by 58.7 ± 3.5%; however, 50 and 100 μM PZ suppressed the indomethacin-induced reduction of cell viability, by 80.9 ± 4.9% and 99.8 ± 7.9%, respectively. These improvements were statistically significant compared with the effect of indomethacin alone. The difference in cell viability with 1–100 μM PZ alone compared with medium alone was not significant.

The inhibitory effect of PZ on indomethacin-induced apoptosis was not dependent on L-car but on Zn

To further evaluate the anti-apoptotic effect of PZ on indomethacin-induced apoptosis, we examined the effect of

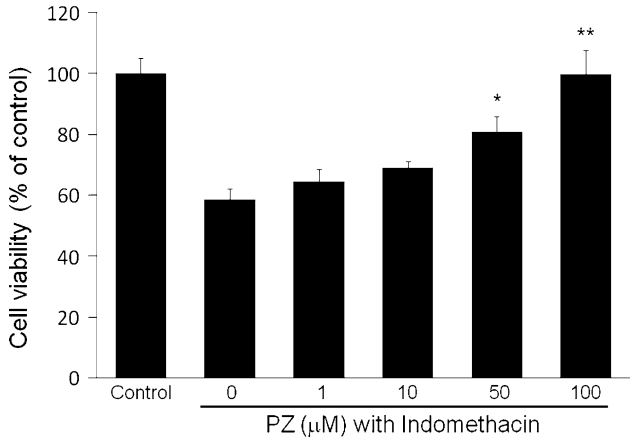

Fig. 5 Polaprezinc (*PZ*) protected RIE-1 cells from indomethacin-induced cell death. The effect of PZ on indomethacin-induced cell death was assessed by MTT-based WST-8 assay. RIE-1 cells were incubated in the absence or presence of 200 μM indomethacin for 24 h. PZ was added at a concentration range of 0–100 μM. The results are shown as percentage variation of OD compared to medium alone, and are expressed as means ± SEM of three separate experiments. $*P < 0.05$ and $**P < 0.01$ compared with indomethacin alone

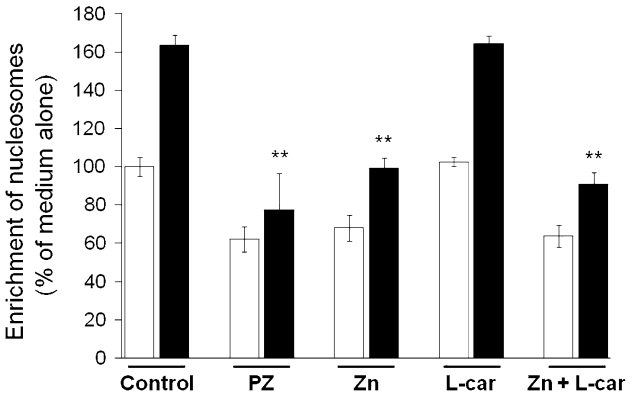

Fig. 6 PZ and its subcomponent, Zn, suppressed indomethacin-induced apoptosis. Indomethacin-induced apoptosis was quantified by using ELISA. RIE-1 cells were incubated in the absence (*white columns*) or presence (*black columns*) of 200 μM indomethacin for 24 h. PZ, Zn, and L-carnosine (*L-car*) were added at 100 μM. The results are shown as percentage variation of OD compared to medium alone, and are expressed as means ± SEM of three separate experiments. $**P < 0.01$ compared with indomethacin alone

Zn and L-car separately, because PZ is a chelate compound consisting of Zn and L-car. Apoptosis was evaluated by the ELISA that detected mono- and oligo-nucleosomes appearing after apoptosis. As shown in Fig. 6, Zn significantly reduced indomethacin-induced apoptosis to almost same extent as PZ. But L-car did not inhibit the indomethacin-induced apoptosis. The inhibitory effect of Zn plus L-car on the indomethacin-induced apoptosis was almost equivalent to that of PZ and Zn, suggesting that the protective effect of PZ on indomethacin-induced apoptosis in RIE-1 cells is Zn-dependent.

PZ reduced indomethacin-induced ROS production in RIE-1 cells

It has been reported that, in the RGM1 cell line, ROS are related to indomethacin-induced apoptosis [15]. Therefore, we examined whether or not indomethacin induced ROS production in RIE-1 cells and whether PZ inhibited the ROS production, by using a redox-sensitive fluorogenic probe, RedoxSensor Red CC-1. As shown in Fig. 7, the mitochondria of RIE-1 cells in all groups were equally stained with MitoTracker Green FM. However, the fluorescent intensity of RedoxSensor Red CC-1 was apparently increased in the cells incubated with indomethacin alone compared with cells incubated with medium alone or PZ alone. This indicated an increase of ROS ($O_2^{\cdot-}$, $HO^{\cdot}$ or H_2O_2) in indomethacin-stimulated cells. And this increase was suppressed by the addition of PZ, indicating a ROS-quenching effect of PZ. Merged images clearly indicated that mitochondria were the major source of ROS production in the indomethacin-stimulated RIE-1 cells. These results suggest that PZ might protect RIE-1 cells from indomethacin-induced apoptosis via its ROS-quenching effect.

PZ reduced indomethacin-induced oxidative stress

Because ROS have been reported to modify intracellular molecules such as proteins and lipids, we assessed the effect of indomethacin and/or PZ stimulation of RIE-1 cells on the production of ROS-modified proteins. Among amino acid residues of proteins, cysteine residues are most vulnerable to ROS attack and form S-oxidized proteins. Bt-Cys can be used as a molecular probe for the detection of S-oxidized proteins, and this oxidation effect is reversible by the application of chemical reducing agents such as DTT. As shown in Fig. 8a (left 4 lanes), indomethacin clearly increased several *S*-thiolated proteins compared to findings in the control group; however, PZ suppressed this indomethacin-induced production of *S*-thiolated proteins, suggesting the ROS-quenching effect of PZ on indomethacin-stimulated RIE-1 cells. These S-oxidized proteins were undetectable when treated with DTT (right 4 lanes), indicating that these bands were specific for and corresponded to S-oxidized proteins. In addition, S-oxidized protein production was more evident in the mitochondrial fraction of indomethacin-stimulated RIE-1 cells than in the cytosolic fraction (Fig. 8b). This finding supports our data that mitochondria were the main sites of ROS production in indomethacin-stimulated RIE-1 cells (Fig. 7).

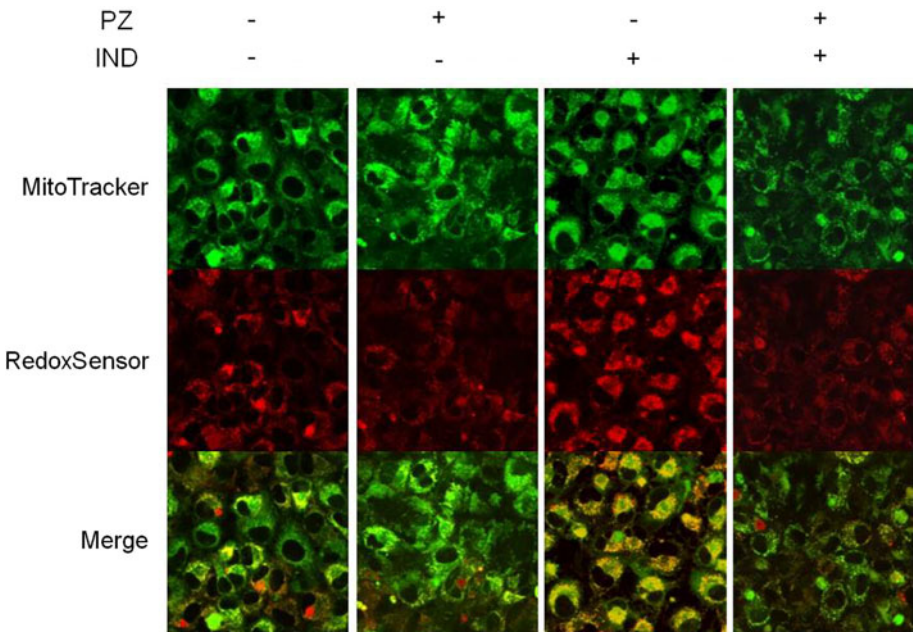

Fig. 7 PZ reduced indomethacin-induced reactive oxygen species (ROS) production in mitochondria. MitoTracker Green FM- and RedoxSensor Red CC-1-stained RIE-1 cells were observed using a laser scanning confocal microscope. RIE-1 cells were incubated in the absence or presence of 200 μM indomethacin and/or 100 μM PZ for 30 min. The *upper row* shows the MitoTracker Green FM images, the *middle row* shows the RedoxSensor Red CC-1 images, and the *bottom row* shows the merged upper and middle images. A representative image of three independent experiments is shown (×100)

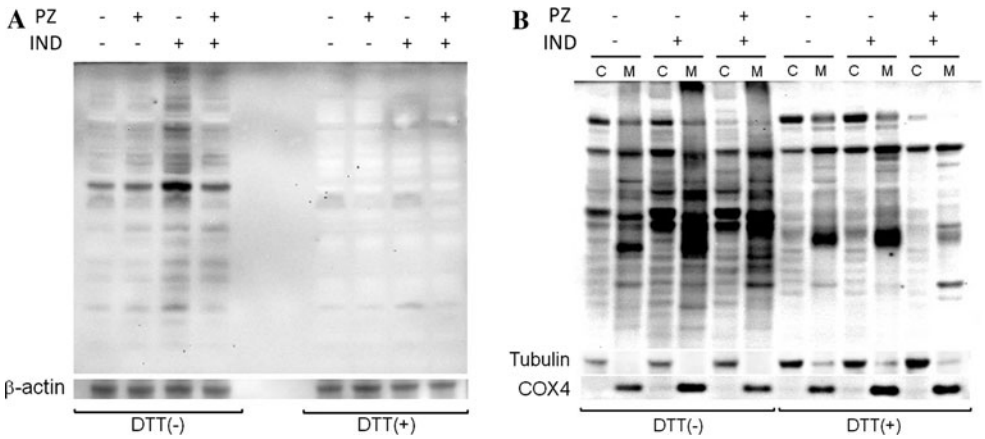

Fig. 8 PZ suppressed indomethacin-induced protein S oxidation. RIE-1 cells were incubated with medium alone, 200 μM indomethacin, and/or 100 μM PZ for 3 h and subsequently treated with 100 μM biotinylated cysteine (Bt-Cys) for 30 min. Bt-Cys-labeled proteins (*S*-thiolated proteins) were determined by western blot analysis in the absence or presence of dithiothreitol (*DTT*), which specifically reduces *S*-thiolated proteins. **a** Whole cell lysate. *β*-Actin blotting was used as an internal control. **b** Lysate separated into cytosol (indicated as *C*) and mitochondria (indicated as *M*). Tubulin and cyclooxygenase 4 (*COX4*) blotting were used as an internal control and verification of this separation

PZ inhibited indomethacin-induced cytochrome *c* and smac/DIABLO release and subsequent caspase-3 activation

Given the above results, it appeared likely that indomethacin-induced ROS produce S-oxidized proteins in mitochondria, and that these abnormal proteins lead to the activation of some apoptosis-inducing factor. Therefore we examined cytochrome *c* and smac/DIABLO, which are the main pro-apoptotic factors in mitochondria. As shown in Fig. 9a, b, PZ inhibited indomethacin-induced release of cytochrome *c* and smac/DIABLO from the mitochondria to the cytosol. Additionally, indomethacin-induced cleavage (activation) of caspase-3, a pro-apoptotic enzyme that acts downstream of cytochrome *c* and smac/DIABLO, was inhibited by PZ (Fig. 9c), suggesting that the protective effect of PZ is dependent on the ROS-quenching activity and the suppressive effects on both cytochrome *c* and smac/DIABLO release to the cytosol and subsequent caspase-3 activation.

Discussion

With the rapid maturation and aging of society, the requirement of NSAIDs for the prevention or the treatment

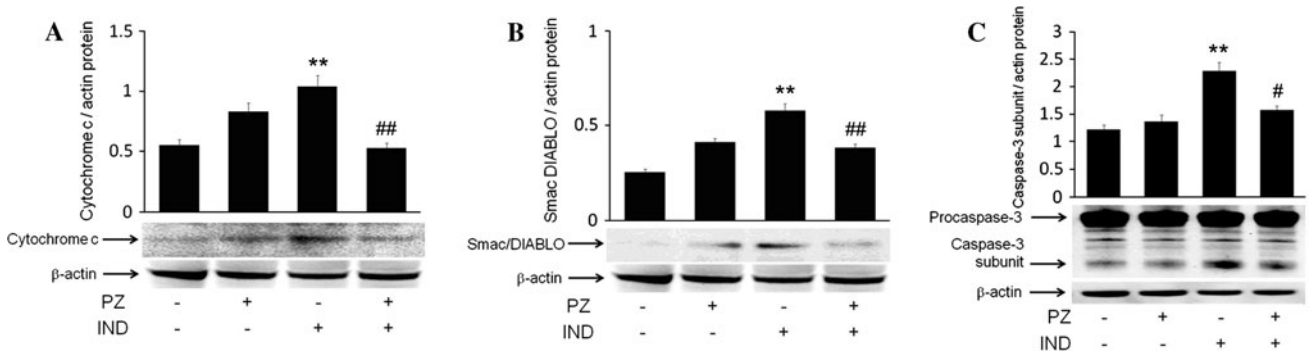

Fig. 9 PZ inhibited indomethacin-induced cytochrome *c* and smac/DIABLO efflux and caspase-3 activation. Apoptosis-related proteins were detected by western blotting. RIE-1 cells were incubated with medium alone, 200 μM indomethacin, and/or 100 μM PZ for 5 h (**a**, **b**) or 24 h (**c**). Cytosolic fractions were used for the detection of the efflux of **a** cytochrome *c* and **b** smac/DIABLO from mitochondria. **c** Activation of caspase-3 was detected as an increase of the subunit to which the precursor of caspase-3 is cleaved. *β*-Actin blotting was used as an internal control. Data are expressed as normalized ratios to *β*-actin. $^{**}P < 0.01$ compared with medium alone. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with indomethacin alone

of various diseases such as cardiovascular diseases and orthopedic diseases is getting higher and higher. Because many patients use NSAIDs for a long time, the side effects of NSAIDs are not negligible. Among various side effects of NSAIDs, gastrointestinal mucosal injury is the most serious one and many researchers have investigated the pathogenesis of NSAID-induced gastric mucosal injury. As a result, there are several useful ways to prevent or treat such injury now. However, with regard to NSAID-induced small intestinal mucosal damage, there has not been enough investigation into its pathogenesis and effective treatments. Now that capsule endoscopy and balloon endoscopy has enabled us to know the relatively high incidence of NSAID-induced mucosal damage of the small intestine, it is an urgent issue to know the mechanism by which NSAIDs induce mucosal damage in the small intestine.

In the present study, firstly, we examined the dose- and time-dependent effects of indomethacin, a representative NSAID, on the cellular viability of RIE-1 cells and found that even a low dose of indomethacin could reduce cellular viability (Fig. 1a). Although the concentration of indomethacin in human plasma is around 1.4 to 8.4 μM [21], the mucosal concentration of indomethacin might be much higher, and therefore a higher concentration of indomethacin could damage the small intestinal mucosa directly.

It has been reported that indomethacin induced small intestinal mucosal injury via a COX-dependent decrease in PG production [22] and by direct, COX-independent, cytotoxic actions of NSAIDs in the gastrointestinal tract. Tomisato et al. [23] also reported the direct, COX-independent cytotoxicity of indomethacin (around 100 μM), using pig gastric mucosal cells. In the present study, 100 or 200 μM indomethacin reduced cellular activity, and exogenously added PGE_2 did not inhibit this cytotoxicity (Fig. 4), suggesting that even in small intestinal epithelial cells this concentration of indomethacin induces a direct cellular injury that does not depend on COX inhibition. COX-independent pro-apoptotic effects of NSAIDs on the inhibition of carcinogenesis were reported in some studies [24–26]. Adebayo and Bjarnason [3], in a review, noted that the pathogenesis of NSAID-induced gastrointestinal damage could be viewed as a combination of COX inhibition and a topical, direct effect of NSAIDs. Concerning this direct effect of NSAIDs, Bjarnason et al. have reported the etiology of NSAID-induced mitochondrial dysfunction and mucosal injury; NSAIDs induce the uncoupling of mitochondrial oxidative phosphorylation, and this leads to reduced adenosine 5-triphosphate (ATP) levels, which in turn results in loss of control of the integrity of intercellular junctions, with increased intestinal permeability, and subsequently bacteria, bile acid, or proteases infiltrate into the mucosa [27]. Taken together, the above findings suggest that the mechanisms of NSAID-induced small intestinal mucosal injury occur not only via a COX-dependent PG decrease but also via a COX-independent direct effect of NSAIDs by which mainly mitochondria are damaged and subsequently cell membranes are embrittled.

Several studies have indicated the relationship between indomethacin-related apoptosis and ROS production [15, 28]. In the present study, the qualitative analysis of cellular death showed that indomethacin predominantly induced apoptosis in RIE-1 cells (Fig. 2) and the proportion of apoptotic cells was significantly increased around 3–12 h (Fig. 3). In addition, indomethacin significantly increased intracellular ROS production in 30 min (Fig. 7). Because PZ administration in RIE-1 cells reduced ROS production (Fig. 7) and intracellular oxidative stress (Fig. 8) and inhibited apoptosis (Fig. 6) in RIE-1 cells, it seems that the initial ROS production may have affected intracellular protein function, resulting in the indomethacin-induced

apoptosis of the RIE-1 cells. Although most gastrointestinal mucosal injuries such as gastritis or ulcer involve oxidative stress, the mechanisms of this stress differ with its causes. *Helicobacter pylori* leads to oxidative damage by its chemotactic activity for neutrophils. Suzuki and Hibi and Suzuki et al. reported that activated neutrophils were the main source of oxygen radicals in *H. pylori*-infected gastric mucosa. In neutrophils, myeloperoxidase (MPO) results in the formation of a potent oxidant, the hypochlorous anion (OCl^-), from H_2O_2 in the presence of Cl^-. This hypochlorous anion reacts with ammonia, derived from urea by *H. pylori*-associated urease, and yields a cytotoxic oxidant, monochloramine (NH_2Cl), whose lipophilic properties allow it to freely penetrate biological membranes to oxidize intracellular components [29, 30]. By contrast, the source of NSAID-induced ROS production is considered to be the mucosal epithelium. It has been speculated that activated neutrophils [31] and vascular endothelium-derived xanthine oxidase [32] might play a primary role in NSAID-induced ROS production. However, currently, ROS production in epithelial cells [33, 34] is emphasized, for the following reasons: the appearance of gastric mucosal injury after the administration of NSAIDs is earlier than the appearance of such injury after neutrophil infiltration [35]; and indomethacin administration increases ROS production via gastric peroxidase inhibition [36].

In the present study, we showed that the main source of ROS production was the mitochondria of indomethacin-stimulated RIE-1 cells (Fig. 7). Increased ROS production in the mitochondria of the indomethacin-stimulated RIE-1 cells resulted in the marked production of S-oxidized protein in mitochondria compared to that in the cytosol (Fig. 8b). We consider that superoxide ($O_2^{\cdot -}$) could be the first candidate of these ROS, because superoxide is the major ROS produced in mitochondria [37], and we have reported a superoxide-scavenging effect of PZ examined by electron spin resonance [12]. Regarding the mechanism of indomethacin-induced ROS production in mitochondria, it is reported that indomethacin induced the inactivation of aconitase and the intramitochondrial accumulation of free iron from the iron–sulfur cluster of aconitase, and subsequent hydroxyl radical ($HO^{\cdot}$) generation to activate the mitochondrial pathway of apoptosis [38]. Therefore, the initial ROS production in mitochondria might be the first step of indomethacin-induced small intestinal mucosal injury, and a mitochondrially targeted ROS scavenger may be effective to prevent NSAID-induced gastroenteropathy.

Because PZ has been shown to exhibit potent ROS-quenching effects [11, 12], we employed PZ, a gastric muco-protective drug, as a candidate drug for the prevention of indomethacin-induced small intestinal mucosal damage. Besides its ROS-quenching effect, PZ has various other pharmacological actions, such as induction of the expression of heat shock protein 27 (HSP-27), HSP-72 [39], and heme oxygenase-1 (HO-1) [40], molecules which exhibit cytoprotective effects or an anti-inflammatory effect via the inhibition of adhesion molecules on polymorphonuclear leukocytes or the inhibition of cytokine production by gastric epithelial cells [41]. In the present study, we found that PZ significantly inhibited the indomethacin-induced apoptosis of RIE-1 cells (Figs. 5, 6) and that the Zn component, rather than L-car, contributed to the inhibition of the indomethacin-induced apoptosis (Fig. 6). This finding is supported by a study in which Zn inhibited apoptosis via ROS reduction in an ethanol-induced HepG2 cell (a human hepatocellular liver carcinoma cell line) injury model [42]. We have reported that PZ and Zn, but not L-car, inhibited the generation of superoxide (PZ scavenged strongly and Zn not so strongly) [12]. This result is compatible with the result of the present study (Fig. 6). Regarding the mechanisms by which Zn blocks oxidative stress, Truong-Tran et al. [43] have reported that Zn protects cellular membranes and macromolecules against oxidative damage by its stabilizing effect for lipids and proteins, and it also protects sulfhydryl groups in proteins from oxidation by forming strong, yet readily reversible, thiolate complexes. On the other hand, L-car has been shown to have wound-healing [44] and cytoprotective [45] effects. Suzuki et al. [46] reported that PZ and L-car, but not Zn, protected gastric cells from NH_2Cl-induced DNA damage. The strong cytoprotective effect of PZ might be due to the synergistic effect of Zn and L-car.

To further investigate the effect of PZ on indomethacin-induced RIE-1 apoptosis, we examined the release of cytochrome *c* and smac/DIABLO from mitochondria into the cytosol and the activation of caspase-3. Cytochrome *c* interacts with apoptosis activating factor 1 (Apaf-1) and caspase-9 to activate caspase-3 proteolytically [47]. Smac/DIABLO is released into the cytosol, triggered by apoptotic stimuli, and inhibits the inhibitors of apoptosis proteins (IAPs), an action which inhibits active caspases-3, -7, and -9 [48, 49]. In the present study, indomethacin induced the release of both cytochrome *c* and smac/DIABLO, and the subsequent activation of caspase-3 (Fig. 9). These pro-apoptotic signals were inhibited by PZ, which has a strong ROS-quenching effect. Therefore, the indomethacin-induced apoptosis of RIE-1 cells seems to depend on an increase of mitochondrial protein S-oxidation, which leads to mitochondrial pro-apoptotic factors such as cytochrome *c* and smac/DIABLO being released into the cytosol. In the present study, we found, for the first time, the involvement of smac/DIABLO in indomethacin-induced apoptosis in RIE-1 cells. It was also reported that smac/DIABLO was essential for the apoptosis induced by NSAIDs in colon cancer cells [50]. The suppressive effects of PZ on cytochrome *c* release and caspase-3 activation were consistent

with previous reports that Zn suppresses apoptosis by inhibiting the release of cytochrome *c* [51], and the activation of caspase-3 [43]. However, Fujii et al. [15] reported that PZ did not influence cytochrome *c* efflux during indomethacin-induced apoptosis in rat gastric epithelial cells. The difference between their study results and ours might be dependent on the type of cells used. The detailed effect of PZ on apoptosis-related molecules is under investigation in our laboratory.

Concerning the protective effect of PZ in an in vivo study, we and another group have reported the protective effect of PZ on colitis induced by trinitrobenzene sulfonic acid [14] and dextran sulfate sodium [52], respectively. Suzuki et al. [53] reported that PZ inhibited *H. pylori*-associated gastric mucosal oxidative inflammation in Mongolian gerbils. Focusing on the small intestine, Matsuu-Matsuyama et al. [54] reported the protective effect of PZ on apoptosis induced by radiation in jejunal crypt cells. Further study should be performed, using an in vivo model, to clarify whether PZ attenuates NSAID-induced small intestinal injury.

In conclusion, indomethacin induced small intestinal epithelial apoptosis through ROS production and the subsequent release of cytochrome *c* and smac/DIABLO and the activation of caspase-3. PZ protected RIE-1 cells by suppressing the ROS production and subsequent mitochondrial protein S oxidation, which had led to the release of cytochrome *c* and smac/DIABLO from mitochondria and the activation of caspase-3. PZ may be useful for preventing NSAID-related small intestinal mucosal injury.

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