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REBAMIPIDE ATTENUATES NONSTEROIDAL ANTI-INFLAMMATORY DRUGS (NSAID) INDUCED LIPID PEROXIDATION BY THE MANGANESE SUPEROXIDE DISMUTASE (MnSOD) OVEREXPRESSION IN GASTROINTESTINAL EPITHELIAL CELLS

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Nonsteroidal anti-inflammatory drugs (NSAIDs) often cause gastrointestinal complications such as gastric ulcers and erosions. Recent studies on the pathogenesis have revealed that NSAIDs induce lipid peroxidation in gastric epithelial cells by generating superoxide anion in mitochondria, independently with cyclooxygenase-inhibition and the subsequent prostaglandin deficiency. Although not clearly elucidated, the impairment of mitochondrial oxidative phosphorylation, or uncoupling, by NSAIDs is associated with the generation of superoxide anion. Physiologically, superoxide is immediately transformed into hydrogen peroxide and diatomic oxygen with manganese superoxide dismutase (MnSOD). Rebamipide is an antiulcer agent that showed protective effects against NSAID-induced lipid peroxidation in gastrointestinal tracts. We hypothesized that rebamipide may attenuate lipid peroxidation by increasing the expression of MnSOD protein in mitochondria and decreasing the leakage of superoxide anion in NSAID-treated gastric and small intestinal epithelial cells. Firstly, to examine rebamipide increases the expression of MnSOD proteins in mitochondria of gastrointestinal epithelial cells, we underwent Western blotting analysis against anti-MnSOD antibody in gastric RGM1 cells and small intestinal IEC6 cells. Secondly, to examine whether the pretreatment of rebamipide decreases NSAID-induced mitochondrial impairment and lipid peroxidation, we treated these cells with NSAIDs with or without rebamipide pretreatment, and examined with specific fluorescent indicators. Finally, to examine whether pretreatment of rebamipide attenuates NSAID-induced superoxide anion leakage from mitochondria, we examined the mitochondria from indomethacin-treated RGM1 cells with electron spin resonance (ESR) spectroscopy using a specific spin-trapping reagent, CYPMPO. Rebamipide increased the expression of MnSOD protein, and attenuated NSAID-induced mitochondrial impairment and lipid peroxidation in RGM1 and IEC6 cells. The pretreatment of rebamipide significantly decreased the signal intensity of superoxide anion from the mitochondria. We conclude that rebamipide attenuates lipid peroxidation by increasing the expression of MnSOD protein and decreasing superoxide anion leakage from mitochondria in both gastric and small intestinal epithelial cells.

Key words: *gastrointestinal epithelial cells, lipid peroxidation, nonsteroidal anti-inflammatory drugs, rebamipide, superoxide dismutase, prostaglandin*

Abbreviations: COX - cyclooxygenase; CYPMPO - 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline N-oxide; ESR - electron spin resonance; ETCs - electron transport chains; H_2Ras - histamine 2-receptor antagonists, Hfsc - hyperfine stricture constants; MitoRed - 9-[2-(4-_methycoumarin-7_-oxycarbonyl)phenyl]-3,6-bis (diethlamino) xanthylium chloride; MnSOD - manganese superoxide dismutase; NF-κB - nuclear factor-kappa B; NSAIDs - non steroidal anti- inflammatory drugs; PG - prostaglandin; PPIs - proton pump inhibitors

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and aspirin are the most commonly prescribed drugs for arthritis, inflammation, and cardiovascular protection. It is clinically important that NSAIDs often cause gastrointestinal complications such as gastric ulcers and erosions. The pathogenesis of these complications has mostly been ascribed to NSAID's action on the cyclooxygenase (COX) inhibition and the subsequent prostaglandin (PG) deficiency (1). Extensive researches have revealed how gastric defense system is maintained in the presence of PG (2-4).

However, recent studies have revealed that NSAIDs induce lipid peroxidation and cellular injury, independently with COX-inhibition and PG deficiency, by impairing mitochondrial oxidative phosphorylation and subsequent superoxide anion (O_2^-) production (3-7). In contrast to the PG dependent mechanism, the PG-independent gastric defense mechanism against NSAID is poorly understood.

Superoxide anion is physiologically produced at electron transport chains (ETCs) in mitochondria. Because of its toxicity as an oxygen radical, superoxide anion produced in physiological condition is immediately transformed into hydrogen peroxide and diatomic oxygen with manganese superoxide dismutase (MnSOD), or SOD2, located in inner membrane of mitochondria. MnSOD protein is constitutively expressed in mitochondria, and is up-regulated under various stress conditions such as ionizing radiation, interferon-γ, and proinflammatory cytokines (8-10). In addition to stomach, recent clinical examinations revealed NSAIDs induce small intestinal mucosal damage more commonly than previously expected (4, 11). Although the pathogenesis was less well understood than that of gastric injuries, it has recently been proposed that NSAIDs' impairment of oxidative phosphorylation in mitochondria and subsequent superoxide anion production as the main underlying mechanism in small intestinal injuries (4, 12-14).

Rebamipide, 2-(4-chlorobenzoylamino)-3-[2-(1H)-quinolinon-4-yl], is an antiulcer agent that showed protective effects against NSAID-induced gastric and small intestinal mucosal damages *in vitro* and *in vivo* (6, 7, 15-18). The biological mechanisms of rebamipide on gastric protection has been demonstrated as an inducer of PG and as a scavenger of hydroxyl radical and superoxide anion (15, 19-21). The latter mechanism was demonstrated by Yoshikawa *et al*. firstly in 1993 in an electron spin resonance (ESR) study that rebamipide administration significantly reduced the signal intensity of superoxide anion generated from opsonized zymosan-stimulated human neutrophils (20). Interestingly, the study also showed that rebamipide *per se* did not scavenge the superoxide anion generated by the hypoxanthine-xanthine oxidase system *in vitro.* These results suggested that rebamipide's superoxide anion scavenging property is dependent on the presence of activated neutrophils. However, other *in vitro* experiments demonstrated that rebamipide attenuated the leakage of oxygen radicals and lipid peroxidation in the absence of activated neutrophils (6, 7, 22). Therefore it is not clear how rebamipide affects gastric mucosal cells and scavenge superoxide anion, especially in the absence of activated neutrophils.

We hypothesized that rebamipide may increase the expression of MnSOD protein in mitochondria and decrease the leakage of superoxide anion both in NSAID-treated gastric and small intestinal cells. Firstly, to examine rebamipide increases the expression of MnSOD proteins in mitochondria of gastric and small intestinal epithelial cells, we underwent Western blotting analysis against anti-MnSOD antibody in mitochondria of rebamipide-treated gastric RGM1 cells and small intestinal IEC6 cells. Secondly, to examine whether the pretreatment of rebamipide decreases NSAID-induced mitochondrial impairment and lipid peroxidation in gastric and small intestinal epithelial cells, we treated these cells with indomethacin, diclofenac and aspirin with or without rebamipide pretreatment, and examined with specific fluorescent indicators. Finally, to examine whether pretreatment of rebamipide attenuates NSAID-induced mitochondrial superoxide anion leakage, we examined the mitochondria from indomethacin-treated RGM1 cells with ESR spectroscopy using a specific spin-trapping reagent, CYPMPO (23).

The results indicated that rebamipide indeed increased the expression of MnSOD protein in mitochondria and attenuated NSAID-induced mitochondrial impairment and lipid peroxidation in RGM1 and IEC6 cells. The pretreatment of rebamipide significantly decreased the signal intensity of superoxide anion from mitochondria of indomethacin-treated RGM1 cells.

Therefore we concluded that rebamipide attenuates lipid peroxidation by increasing the expression of MnSOD protein and decreasing superoxide anion leakage from mitochondria in both gastric and small intestinal epithelial cells.

MATERIALS AND METHODS

Cell culture

Rat gastric epithelial cell line RGM1 and rat small intestinal epithelial cell line IEC6 were obtained from RIKEN BioResource Center (Tsukuba, Japan) (24, 25). RGM1 cells were grown in a 1:1 mixture of both Dulbecco's modified Eagle medium and Ham's F-12 medium (DMEM/F12; Cosmo Bio, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY) and 2 mM glutamine. IEC6 cells were grown in DMEM supplemented with 5% FCS and 4 μg/mL insulin. The cells were grown at 37°C in a humidified incubator with 5% CO_2.

Solutions and reagents

Indomethacin and diclofenac sodium were purchased from Sigma Chemical Co. (St. Louis, USA). Acetylsalicylic acid was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rebamipide was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan). Tetra Color ONE® cell proliferation assay kit was obtained from Seikagaku (Tokyo, Japan). Diphenyl-1pyrenylphosphine (DPPP) and 9-[2-(4_-methycoumarin-7_-oxycarbonyl)phenyl]-3,6-bis (diethlamino) xanthylium chloride (MitoRed) were obtained from Dojindo (Kumamoto, Japan). 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline N-oxide (CYPMPO) was obtained from Radical Research Inc. (Tokyo, Japan). Anti-manganese SOD, rabbit-polyclonal antibody was obtained from Stressgen Bioreagents (Kampenhout, Belgium). ApoAlert® Cell Fractionation Kit (Clontech Laboratories, CA) was obtained from Takara Bio Inc (Otsu, Japan). MITOISO2® mitochondria isolation kit was obtained from Sigma. All other chemicals were reagent grade.

Western blotting analysis for the detection of manganese superoxide dismutase

RGM1 cells and IEC6 cells were incubated with 1 mM indomethacin with or without 1 mM rebamipide pretreatment and their mitochondrial fractions were prepared with previously mentioned methods. Each cellular extract containing 50 μg protein, prepared by centrifugation at 10,000×g, were used for sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, CA, USA) and were transferred onto a polyvinylidene difluoride (PVDF) membrane (Nihon Millipore Ltd., Tokyo, Japan). The blots were blocked for 1 hour with 5% skim milk in Tris-buffered saline prepared at pH 7.6 containing 0.05% Tween 20. We measured the values of MnSOD. Western blots were probed with anti-rat manganese SOD polyclonal antibody at room temperature for 1 hour. After treating with a horseradish peroxidase-conjugated secondary antibody for 1 hour, bands were visualized with the ECLplus kit.

Cellular microscopic fluorescence analysis

Cells were incubated on a Lab-Tek II slide chamber (Nalge Nunc International) at a concentration of 10^5 cells/mL per well. Cellular fluorescence images of the cells were observed, and their intensities measured using a chilled CCD camera (AxioCam color, ZEISS)-mounted epifluorescence microscope (Axiovert135M, Zeiss) connected to an image analyzing system (Axio Vision, Zeiss). The fluorescence intensities were analyzed using ImageJ 1.42q.

Measurement of lipid peroxidation

Diphenyl-1pyrenylphosphine (DPPP) is a nonfluorescent triphenylphosphine compound. When it reacts with hydroxyperoxide, a fluorescent substance, DPPP-oxide is formed. After incubated and treated with 1 mM indomethacin for 2 h with or without 1 mM rebamipide pretreatment for 18 h in mictotiter plates, cells were treated with DPPP and examined with the epifluorescence microscope with the excitation and emission wavelength at 352 and 461 nm, respectively.

Measurement of mitochondrial transmembrane potential

Mitochondrial membrane potentials were measured with a cell membrane permeable rhodamine-based dye, MitoRed. The fluorescence intensity of this dye depends on mitochondrial membrane potentials, and can be used as an indicator of mitochondrial activity. MitoRed is a cell membrane permeable rhodamine-based dye. The fluorescence intensity of this dye depends on mitochondrial membrane potentials, and can be used as an indicator of mitochondrial activity. Cells were incubated and treated indomethacin with or without rebamipide pretreatment in previously mentioned methods and examined with the epifluorescence microscope with the excitation and emission wavelengths of MitoRed are 559 and 588 nm, respectively. The fluorescence intensities were analyzed using ImageJ 1.42q.

Electron spin resonance spectroscopy

RGM1 cells were incubated with 1 mM indomethacin with or without 1 mM rebamipide pretreatment for 2 h and their mitochondrial fractions were prepared with MITOISO2 according to the manufacture's instruction. The pellet of mitochondria was suspended with 5 mM respiratory substrates (succinate, glutamate and malate), 5 mM NADH and 10 mM CYPMPO. The reaction mixture was immediately transferred to a quartz flat cell (RDC-60; 60 mm×6 mm×0.3 mm, Radical Research). The concentration of proteins in the final reaction mixture was 250 μg/ml as evaluated according to the method described previously (Bio-Rad Laboratories, Hercules, CA). The ESR spectra were recorded by using a JEOL-TE X-band spectrometer (JEOL). All ESR spectra were obtained under the following conditions: 10 mW incident microwave power, 100 kHz modulation frequency, 0.1 mT field modulation amplitude and 15 mT scan range. Analysis of the hyperfine splitting constants (Hfsc) and spectral computer simulation were performed using a Win-Rad Radical Analyzer System (Radical Research). All ESR spectra shown are representative of at least three independent experiments.

Statistical analysis

The statistical significance of the data was evaluated using analysis of variance (ANOVA) followed by Duncan's multiple range test. Statistical comparisons were made using Scheffe's method. A P value of <0.05 was considered significant.

RESULTS

Rebamipide increases the expression of MnSOD proteins in gastric RGM1 cells and small intestinal IEC6 cells

To examine rebamipide increases the expression of MnSOD proteins in mitochondria of gastrointestinal epithelial cells, we underwent Western blotting analysis against anti-MnSOD antibody in mitochondria of rebamipide-treated gastric RGM1 cells and small intestinal IEC6 cells. The results demonstrated that rebamipide indeed increased the expression of MnSOD proteins in both RGM1 and IEC6 cells (*Fig. 1*).

Rebamipide attenuates mitochondrial impairment and lipid peroxidation induced by indomethacin, diclofenac and aspirin in gastric RGM1 cells and small intestinal IEC6 cells

NSAIDs impair the activity of mitochondrial oxidative phosphorylation at electron transport chains (ETCs). The impairment is detected as the decrease of mitochondrial membrane potentials. To examine whether the pretreatment of rebamipide decreases NSAID-induced impairment of mitochondrial oxidative phosphorylation and lipid peroxidation in gastric and small intestinal epithelial cells, we treated gastric epithelial RGM1 cells and small intestinal epithelial IEC6 cells with indomethacin, diclofenac and aspirin with or without rebamipide pretreatment, and examined with specific fluorescent dye, MitoRed for mitochondrial transmembrane potentials and DPPP for lipid peroxidation, respectively.

The result indicated DPPP fluorescence intensities were significantly decreased in rebamipide pretreated cells, suggesting that rebamipide treatment indeed attenuated the

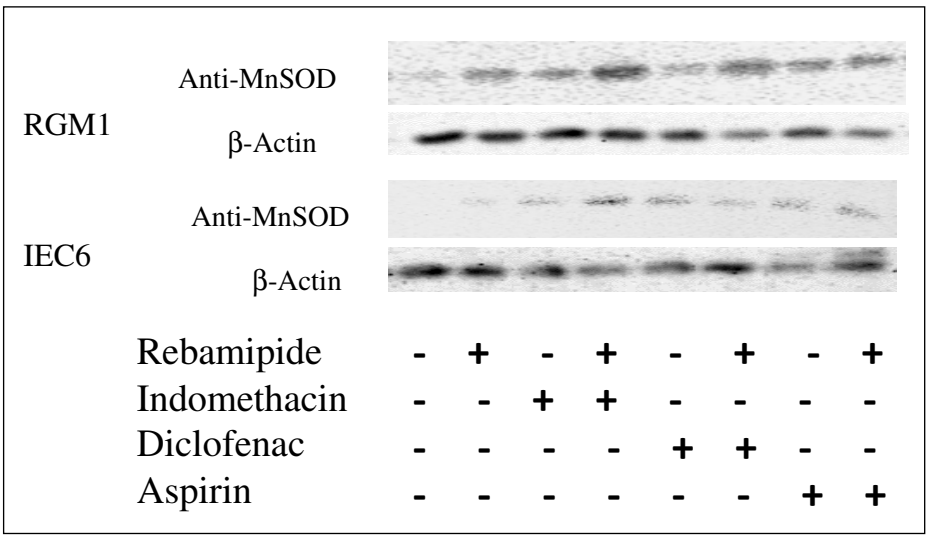

Fig. 1. Western blot analysis for MnSOD protein expression in rebamipide treated gastric RGM1 cells and small intestinal IEC6 cells. Rebamipide treatment induced the increased expression of MnSOD proteins in mitochondria of RGM1 cells and IEC6 cells. Interestingly, all NSAIDs induced the increased expression of MnSOD, which is further increased by rebamipide pretreatment.

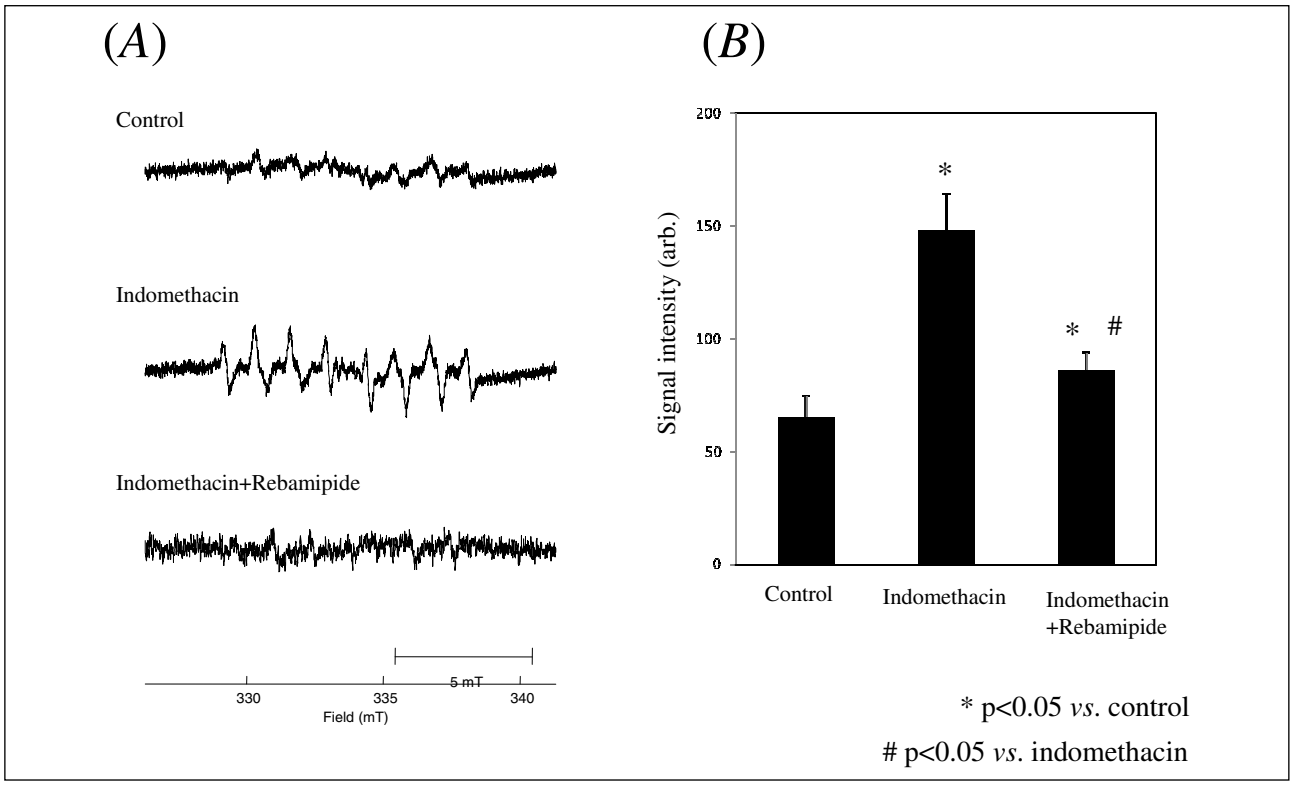

Fig. 2. Detection of superoxide anion production in isolated mitochondria of indomethacin and rebamipide-treated RGM1 cells. Cells were treated with 1 mM indomethacin with or without 1 mM repamipide, and their mitochondria were isolated and examined by EPR spectrometry using a spin-trap agent, CYPMPO. (*A*) The EPR spectra were obtained after the mitochondria were incubated in respiratory substrate and CYPMPO. The parameters obtained from the mitochondria of the cells treated with indomethacin were quite similar to those of the EPR spectrum produced by the hypoxanthine/xanthine oxidase (HX-XOD) system, which is used as a well-established superoxide anion source, and to those of CYPMPO–OOH spin adducts. (*B*) The signal intensity of this ESR spectrum of the mitochondria of the indomethacin-treated cells pretreated with rebamipide showed considerably reduced compared with those of no rebamipide pretreated cells. * $p<0.05$ *vs*. control, # $p<0.05$ *vs*. indomethacin.

extent of lipid peroxidation induced by indomethacin, diclofenac, and aspirin in both gastric and small intestinal cells (*Table 1A*, *1B*). The fluorescence intensities of MitoRed were significantly increased in rebamipide-pretreated cells than NSAID-alone treated cells (*Table 1C*, *1D*), suggesting that rebamipide attenuated the NSAID-induced impairment of mitochondrial oxidative phosphorylation in these cells.

Electron spin resonance spectroscopic analysis in rebamipide-induced reduction of the leakage of superoxide anion in indomethacin-treated gastric RGM1 cells

To examine whether the pretreatment of rebamipide attenuates NSAID-induced mitochondrial superoxide anion leakage in gastric cells and, we examined isolated mitochondria from the indomethacin-treated gastric cells with electron spin resonance (ESR) spectroscopy using a spin-trapping reagent, CYPMPO. The ESR spectra of mitochondria were observed (*Fig. 2A*). The parameters obtained from the mitochondria from the cells treated with indomethacin was quite similar to those of the ESR spectrum produced by the hypoxanthine (HX)/xanthine oxidase (XOD) system used as a well established superoxide anion source and those of CYPMPO–OOH spin adducts as reported previously (13). The hyperfine stricture constants (Hfsc) of the spectrum were agreed with that of computer-simulated spectrum of CYPMPO–OOH. Therefore, we concluded that the spectrum was assigned to the CYPMPO–OOH spin adduct formed by the reaction of superoxide anion with CYPMPO. The signal intensity of indomethacin-treated cells was significantly increased, and partly reduced in rebamipide-pretreated cells (*Fig. 2B*). We, therefore, concluded that indomethacin induced the leakage of superoxide anion in mitochondria of gastric RGM1 cells, and that rebamipide partly inhibited the leakage of superoxide anion.

DISCUSSION

In this presenting study, we demonstrated that rebamipide increased the expression of MnSOD protein in mitochondria, and decreased the extent of both, mitochondrial damage and lipid peroxidation by NSAIDs in both stomach and small intestinal cells. We also demonstrated the pretreatment of rebamipide attenuated the indomethacin-induced superoxide anion leakage from mitochondria in gastric cells. We thus conclude that rebamipide treatment increases the expression of MnSOD proteins, which attenuate NSAID-induced mitochondrial superoxide anion leakage and lipid peroxidation in gastric and small intestinal cells.

The characteristic mechanism of rebamipide on gastric protection as a superoxide anion radical scavenger has been extensively studied especially on its property of modulating neutrophil activation since Yoshikawa's report (20). *In vitro* studies demonstrated that rebamipide not only inhibits the activation and mobilization of neutrophils but also inhibits superoxide anion production in activated neutrophils stimulated by formyl-methionyl-leucyl-phenylalanine (fMLP) and water extract of *Helicobactor pylori* (26-28). However, recent studies

Table 1. Fluorescence analysis of lipid peroxidation and mitochondrial phosphorylation in NSAID-treated RGM1 cells and IEC cells. To examine whether rebamipide treatment attenuate NSAID-induced decrease of lipid peroxidation and mitochondrial transmembrane potential, we treated gastric epithelial RGM1 cells and small intestinal epithelial IEC6 cells with indomethacin, diclofenac and aspirin with or without rebamipide pretreatment, and examined with MitoRed for mitochondrial transmembrane potentials and DPPP for lipid peroxidation, respectively. (*A*, *B*) The NSAID-induced increase of DPPP fluorescence intensities were significantly decreased in rebamipide pretreated cells, suggesting that rebamipide treatment attenuated the extent of lipid peroxidation induced by NSAIDs in both RGM1 and IEC6 cells. (*C*, *D*) The NSAID-induced decrease of MitoRed fluorescence intensities were significantly decreased in rebamipide-pretreated cells than NSAID-alone treated cells, suggesting that rebamipide attenuated the NSAID-induced impairment of mitochondrial oxidative phosphorylation in these cells. * $p<0.05$ *vs*.control.

A) DPPP in RGM1

		+ Rebamipide (1 mM)	
Control	12.328 ± 2.185	15.059 ± 5.763	
Indomethacin	91.723 ± 29.279	31.690 ± 3.880	*
Diclofenac	81.319 ± 11.364	29.716 ± 4.390	*
Aspirin	76.530 ± 16.223	26.502 ± 6.275	*

B) DPPP in IEC6

		+ Rebamipide (1mM)	
Control	28.942 ± 6.226	25.335 ± 9.299	
Indomethacin	110.159 ± 30.472	48.659 ± 12.415	*
Diclofenac	103.897 ± 29.512	54.445 ± 12.920	*
Aspirin	108.297 ± 22.644	54.951 ± 17.629	*

C) MitoRed in RGM1

		+ Rebamipide (1mM)	
Control	77.130 ± 18.885	79.510 ± 15.430	
Indomethacin	24.818 ± 3.638	41.205 ± 14.388	*
Diclofenac	23.704 ± 2.750	56.184 ± 16.738	*
Aspirin	21.387 ± 1.668	54.378 ± 14.352	*

D) MitoRed in IEC6

		+ Rebamipide (1mM)	
Control	71.946 ± 13.903	73.620 ± 9.109	
Indomethacin	20.624 ± 3.125	62.727 ± 9.674	*
Diclofenac	27.040 ± 5.435	63.818 ± 9.145	*
Aspirin	23.821 ± 3.779	58.037 ± 10.265	*

demonstrated that the inhibitory effect of rebamipide on oxygen radical production and lipid peroxidation is independent of neutrophil activation (6, 7, 28). Nonetheless; the mechanism of the neutrophil-independent inhibitory action of rebamipide on oxygen radical production has not been elucidated. Our present study, for the first time to our knowledge, shows rebamipide, independently with neutrophils, increased the expression of MnSOD protein and inhibits superoxide anion leakage and lipid peroxidation in gastric and small intestinal epithelial cells. Our results suggests that rebamipide may prevent other ROS-mediated lipid peroxidation and cellular injury, such as bisphosphonate-induced gastrointestinal injury (29).

The biologic mechanism that rebamipide increases the expression of MnSOD protein remains to be elucidated. Under oxidative stresses, MnSOD gene is induced *via* activation of transcription factor nuclear factor-kappa B (NF-κB) (9, 30), whereas rebamipide inhibits activation of NF-κB (22). This may contradict our result that rebamipide increases the expression of MnSOD protein. However, we speculated that rebamipide may induce MnSOD *via* other pathways than NF-κB, such as *via* cAMP-responsive element-binding protein-1 (CREB-1)/activating transcription factor (ATF-1) like factor (30).

Unlike stomach, whose defense mechanisms have been extensively studied (31, 32), the pathophysiology of small intestinal mucosa have been little elucidated (4). Therefore the treatment of choice for NSAID-induced small intestinal mucosal injury is controversial, unlike acid-reducing agents such as histamine $_2$ receptor agonists (H_2RA) and proton pump inhibitors (PPIs) for NSAID-induced gastric mucosal injury: some authors support misoprostol, a PG analogue, and others PPIs or rebamipide (4, 12, 33). Our study not only demonstrated rebamipide, a mucosal protective agent, had cellular protective effects on small intestinal epithelial cells *in vitro*, but also demonstrated the pathophysiological mechanism that yields the biological validity of the therapeutic strategy of rebamipide on NSAID-induced small intestinal injury as well as on gastric injury.

In conclusion, we have demonstrated antiulcer drug rebamipide treatment increased the expression of MnSOD proteins, and attenuated NSAID-induced mitochondria-derived superoxide anion leakage and lipid peroxidation in gastric and small intestinal epithelial cells.

Conflict of interests: None declared.

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