Original Article

Bisphosphonate-induced gastrointestinal mucosal injury is mediated by mitochondrial superoxide production and lipid peroxidation

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Bisphosphonates such as alendronate and risedronate are commonly used for the treatment of postmenopausal osteoporosis. They have the gastrointestinal adverse effects such as erosions and ulcers in stomach and small intestine. However, the detailed biological mechanism remains to be elucidated. Since alendronate is suggested to increase the risk of non-steroidal anti-inflammatory drug-related gastropathy, we hypothesized that bisphosphonates and non-steroidal anti-inflammatory drugs have the same pathophysiological mechanisms in gastrointestinal mucosa: Bisphosphonates may induce cellular lipid peroxidation by inducing the production of mitochondrial superoxide. We also hypothesized that geranylgeranylacetone, an antiulcer drug, may prevent lipid peroxidation by reducing superoxide production. We treated gastric RGM1 cells and small intestinal IEC6 cells with alendronate or risedronate, and examined cellular injury, lipid peroxidation and superoxide production with specific fluorescent dyes, and underwent electron paramagnetic resonance spectroscopy to detect the production of superoxide *in vitro*. The results indicated that bisphosphonates indeed induced cellular injury, cellular lipid peroxidation, and superoxide production. We also demonstrated that the pretreatment of geranylgeranylacetone decreased superoxide production and prevented cellular lipid peroxidation. These results suggested that bisphosphonates, like non-steroidal anti-inflammatory drugs, induce lipid peroxidation by producing mitochondrial superoxide, which was prevented by geranylgeranylacetone.

***Key Words*: bisphosphonates, non-steroidal anti-inflammatory drugs (NSAIDs), geranylgeranylacetone, superoxide, lipid peroxidation**

Nitrogen-containing bisphosphonates (BPs) such as alendronate and risedronate are commonly used for the treatment of postmenopausal osteoporosis. Oral administration of BPs has been associated with gastrointestinal adverse effects such as gastritis, gastric ulcer, and erosive esophagitis.(1) Moreover, alendronate has been reported to inhibit the healing of aspirin-induced gastric damage.(2) This raise the possibility that BPs may increase the risk of non-steroidal anti-inflammatory drug (NSAID)-related gastropathy. A recent clinical study demonstrated this is the case: among healthy volunteers taking alendronate, or naproxen, a NSAID, or both, the incidence of endoscopically-confirmed gastric ulcer was significantly increased in co-administered group.(3) These results were interpreted as reflecting the inhibition of prostaglandin (PG) synthesis and the consequent impedance of the usual epithelial defense mechanisms.(1,2,4,5)

However, as demonstrated in *in vitro* studies, the intramural PG-deficiency and subsequent pathophysiological changes, including decreased mucosal blood flow and increased secretion of bicarbonate, was not involved in BP-induced gastrointestinal injury.(1,2) Moreover, alendronate did not alter the PG synthesis and its response to indomethacin treatment.(2) These and other studies thus concluded that BPs primarily act as topical irritants to induce injury to the mucosal lining of the gastrointestinal tract.(2,6,7,8) Nevertheless, the detailed biological mechanism that BPs induce mucosal injury topically and synergistically with NSAIDs remains to be elucidated.

We have recently reported lipid peroxidation is involved in NSAID-induced gastric injuries: NSAIDs, independently of the cyclooxygenase inhibition and PG deficiency, dissipated mitochondrial transmembrane potentials and produced reactive oxygen species (ROS), mainly superoxide ($O_2^{\bullet-}$), from mitochondria, thereby causing cellular lipid peroxidation and apoptosis.(4,9)

Cellular lipid peroxidation was reportedly involved in BP-induced gastric mucosal injury.(10) The treatment of alendronate induced the development of antral ulcers of rat stomach that was accompanied by an increase in myeloperoxidase (MPO) activity and lipid peroxidation as well as a decrease in superoxide dismutase (SOD) activity and Glutathione (GSH) content, indicating that the treatment of BP induced lipid peroxidation of gastric mucosa.(10) Although the authors ascribed this to the attenuation of anti-oxidative mechanisms by BPs, we hypothesized that BPs, like NSAIDs, may induce cellular lipid peroxidation and subsequent cellular injury by inducing the production of mitochondrial $O_2^{\bullet-}$ in gastric mucosal cells.

In addition to stomach, recent clinical examinations revealed the injuries of small intestinal mucosa by the administration of NSAIDs more commonly than previously expected.(4,11) Although the pathogenesis of NSAID-induced small intestinal injuries is much less well understood than that of gastric injuries, it has recently been proposed that impairment of oxidative phosphorylation in mitochondria and subsequent $O_2^{\bullet-}$ production as the main underlying mechanism.(4,12–14) We thus hypothesized that mitochondrial $O_2^{\bullet-}$ production and lipid peroxidation may also be involved in BP-induced small intestinal mucosal injury.

Geranylgeranylacetone (GGA) is an antiulcer drug. It acts as a cellular membrane-stabilizer against NSAIDs,(15,16) as well as a heat shock protein (HSP)-inducer.(17) Since GGA showed cellular protective effect against oxidative stress by preserving mito-

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chondrial respiratory function,[15] we hypothesized that GGA may prevent lipid peroxidation by reducing the production of $O_2^{\bullet-}$ in mitochondria, and examined the protective effects on BP-treated gastric and small intestinal epithelial cells.

Firstly, to examine whether the treatment of BP, with or without the co-administration of NSAIDs, induces lipid peroxidation in gastrointestinal mucosal cells, we treated gastric epithelial RGM1 cells and small intestinal IEC6 cells with alendronate or risedronate with or without the co-treatment of acetylsalicylic acid (ASA), and examined cellular injury, lipid peroxidation with specific fluorescent indicators. Secondly, to examine whether mitochondria generate $O_2^{\bullet-}$ on BP treatment, we examined BP-treated cells with a mitochondrial superoxide-specific fluorescent indicator, and we also underwent electron paramagnetic resonance (EPR) spectroscopy of isolated mitochondria from the alendronate-treated gastric cells using a spin-trapping reagent, CYPMPO.[18] Finally, to examine whether the treatment of GGA attenuates BP-induced lipid peroxidation and mitochondrial $O_2^{\bullet-}$ production, we examined BP-treated gastric and small intestinal cells with the fluorescent indicators, and the mitochondria from alendronate-treated gastric cells with or without the treatment of GGA with EPR spectroscopy.

The results indicated that the exposure to BPs indeed induced cellular injury, cellular lipid peroxidation, and that ASA had a synergistic effect on the cellular injury of BP-treated gastric and small intestinal cells. The result also demonstrated the exact $O_2^{\bullet-}$ spectra in EPR spectroscopy in alendronate-treated gastric epithelial cells, and that the treatment of GGA decreased $O_2^{\bullet-}$ production and prevented cellular lipid peroxidation and injury. These results suggested that BPs, like NSAIDs, induce lipid peroxidation by producing mitochondrial $O_2^{\bullet-}$, which is prevented by GGA.

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).[19,20] 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. Alendronate and resedronate were obtained from LKT Laboratories, Inc. (MN). ASA and GGA were obtained from Sigma-Aldrich Co. LLC (MO). 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). MITOISO2® mitochondria isolation kit was obtained from Sigma. All other chemicals were reagent grade.

Cytotoxicity assay. Cellular injury was examined with the Tetra Color One assay kit according to the manufacturer's instructions. Cells at a concentration of 10^5 cells/mL were placed on a microtiter plate well. Twenty-four hours later, cells were incubated in medium containing each 0, 50, 100 and 200 μM alendronate or resedronate for another 3 days. After incubated in medium with or without 100 μM GGA or 5 mM ASA for 2 h, the cells were incubated with Hanks Buffer containing 10 μL Tetra Color One for 30 min. Each well's absorbance at 450 nm was measured with a multimode plate reader (DTX 880; Beckman Coulter, Fullerton, CA).

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. DPPP is a non-fluorescent triphenylphosphine compound. When it reacts with hydroxyperoxide, a fluorescent substance, DPPP-oxide is formed. After incubated and treated with 100 μM alendronate or 50 μM risedronate for 3 days in mictotiter plates, the 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.[21] 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 with alendronate of risedronate 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.

Measurement of mitochondrial superoxide. Superoxide leakage in mitochondria was detected using a fluorescence indicator, MitoSOX. This reagent permeates live cells where it selectively targets mitochondria, and is rapidly oxidized by $O_2^{\bullet-}$ but not by other ROS and reactive nitrogen species (RNS). Cells were incubated and treated with alendronate or risedronate in previously mentioned methods and examined with the epifluorescence microscope with the excitation and emission wavelengths of MitoSox are 510 and 580 nm, respectively. The fluorescence intensities were analyzed using ImageJ 1.42q.

Electron paramagnetic resonance (EPR) spectroscopy. RGM1 cells were incubated with 100 μM alendronate with or without 100 μM GGA co-treatment 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 × 6 × 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).[22] The EPR spectra were recorded by using a JEOL-TE X-band spectrometer (JEOL). All EPR 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 EPR 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.

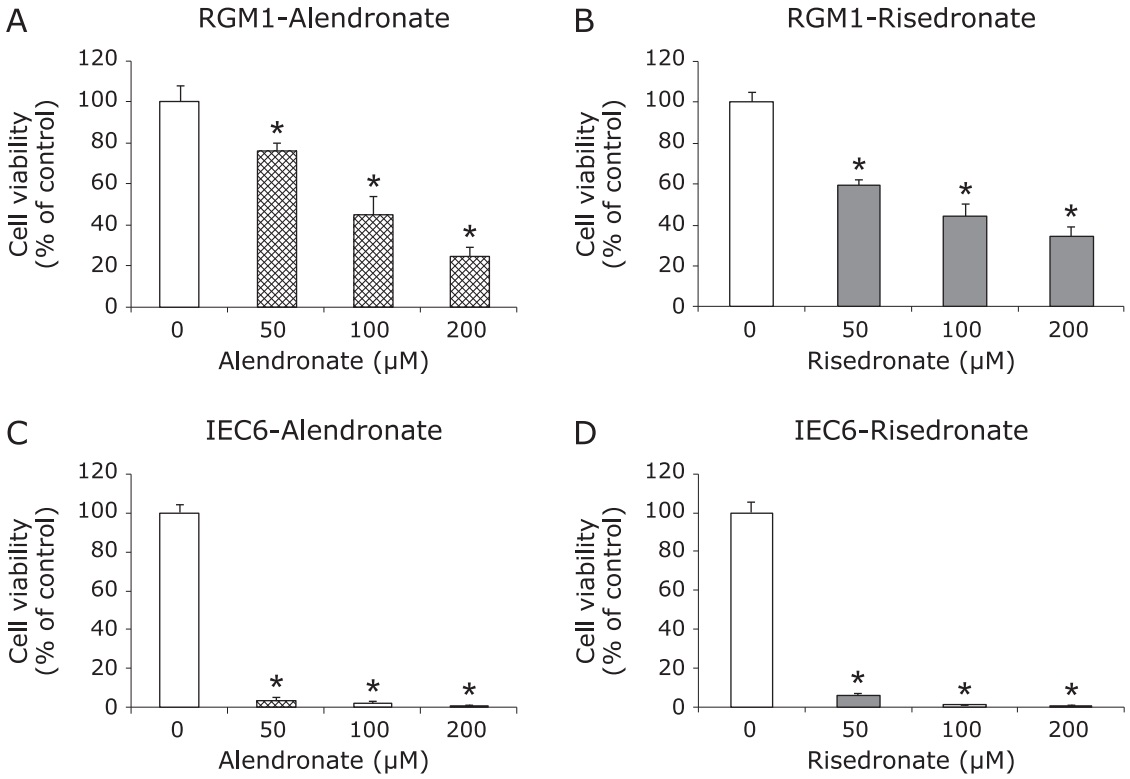

Fig. 1. Cellular injuries examined using the Tetra Color One (TC-1) assay in gastric epithelial RGM1 cells (A and B) and small intestinal epithelial IEC cells (C and D) treated with alendronate and risedronate of various doses (0, 50, 100 and 200 mM). Data are expressed as percentages of untreated cells (mean ± SD). In RGM1 cells, cellular viabilities were significantly decreased in a dose-dependent manner (A and B). Interestingly, IEC6 cells were more injured than RGM1 cell in higher dose of alendronate and risedronate. *p<0.05 vs alendronate or risedronate-alone treated cells.

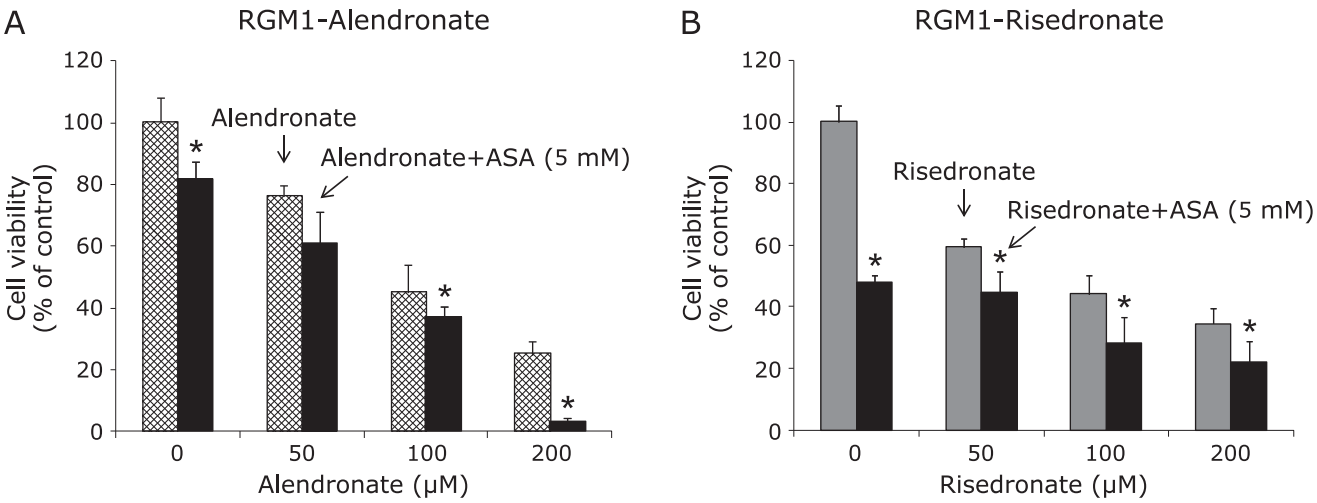

Fig. 2. Cellular injuries examined using the Tetra Color One (TC-1) assay in alendronate (A)- and risedronate (B)-treated RGM1 cells with or without acetylsalicylic acid (ASA) treatment. Data are expressed as percentages of untreated cells (mean ± SD). Cellular viabilities in ASA pretreated group were significantly decreased than alendronate- or risedronate-alone treated group. *p<0.05 vs alendronate or risedronate-alone treated cells.

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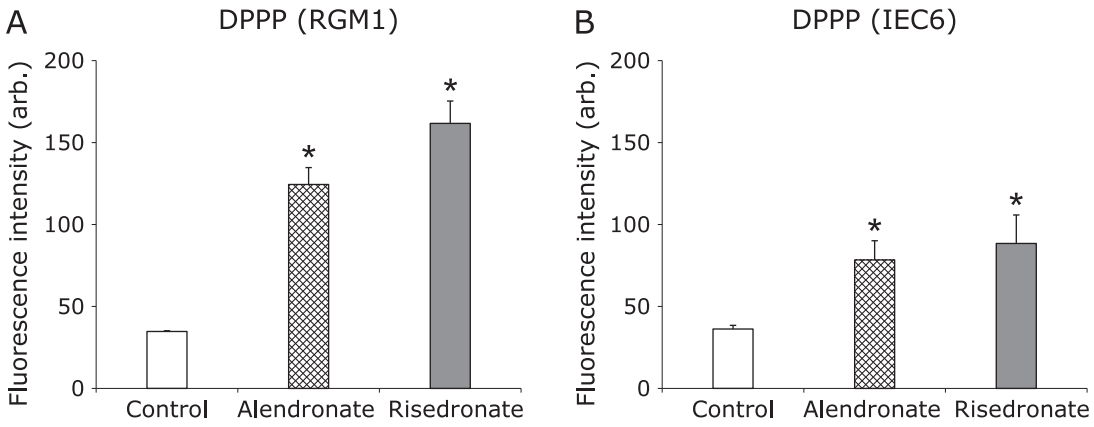

Fig. 3. Lipid peroxidation of BP-treated gastric epithelial RGM1 cells (A) and small intestinal IEC6 cells (B) measured with diphenyl-1-pyrenylphosphine (DPPP) fluorescence assay. The DPPP fluorescence intensities of both RGM1 (A) and IEC6 (B) cells treated with alendronate or risedronate were significantly higher than those of the control cells, indicating that lipid peroxidation was induced by BP treatment. *p<0.05 vs control cells.

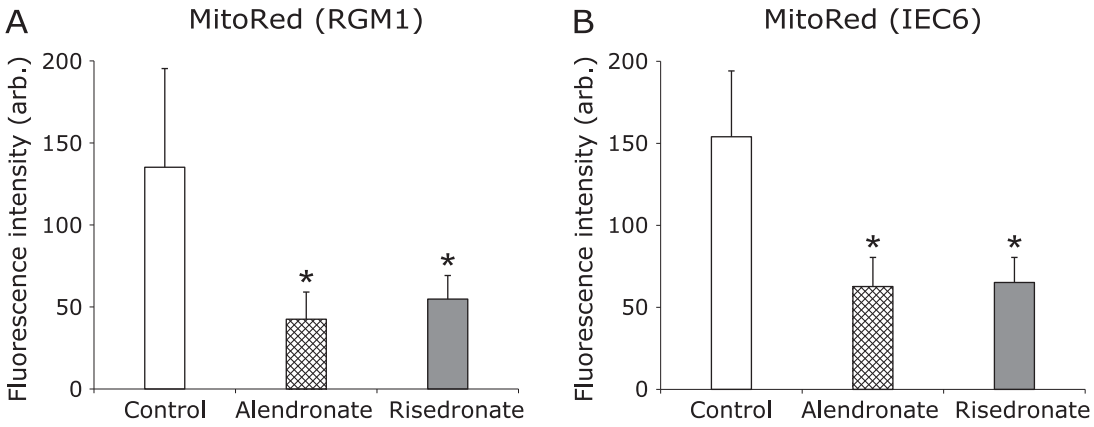

Fig. 4. Mitochondrial membrane potentials of BP-treated RGM1 cells (A) and IEC6 cells (B) measured with MitoRed. Mitochondrial membrane potentials were measured with a cell-membrane-permeable rhodamine-based dye, MitoRed. The fluorescence intensity of MitoRed was significantly more reduced in the cells treated with alendronate and risedronate than in the control cells, indicating that the mitochondrial transmembrane potential was reduced by BP treatment in these cells. *p<0.05 vs control cells.

Results

Cellular injury was induced by BP exposure with the synergic effect of ASA pretreatment in gastric and small intestinal epithelial cells. To examine whether the treatment of BPs induces cellular injury in gastric and small intestinal epithelial cells, we treated gastric epithelial RGM1 cells and small intestinal epithelial IEC6 cells with alendronate and risedronate, and examined cellular injury with Tetra-Color One (TC-1) assay. BPs indeed induced cellular injury in both gastric epithelial RGM1 cells (Fig. 1 A and B) and small intestinal epithelial IEC6 cells (Fig. 1 C and D) in a dose dependent manner.

We also examined the synergic effect of BPs and ASA on the cellular injury. Administration of alendronate or risedronate with the co-treatment of ASA indeed induced the significantly increased damage on the gastric epithelial cells than alendronate or risedronate alone-treated cells (Fig. 2).

Lipid peroxidation was induced by BP exposure in gastric and small intestinal epithelial cells. To examine whether lipid peroxidation is induced by the treatment of BPs in gastric and small intestinal epithelial cell, we treated the cells with alendronate and risedronate and examined cellular lipid peroxidation by detecting DPPP-oxide, which is a specific marker of lipid peroxidation.[23] The result indicated that lipid peroxidation of cellular membrane was induced by the exposure to BPs both in gastric and small intestinal cells (Fig. 3).

Mitochondrial transmembrane potentials were decreased by BP exposure in gastric and small intestinal epithelial cells. To examine whether the treatment of BPs disrupts mitochondrial transmembrane potential that triggers cellular lipid peroxidation, we investigated transmembrane potentials of alendronate or risedronate-treated gastric and small intestinal cells with a fluorescent indicator MitoRed. This rhodamine-based dye is attached to mitochondria, and its fluorescence intensity depends on mitochondrial membrane potentials.[21] The results demonstrated that the fluorescence intensities of MitoRed were significantly reduced in BP-treated RGM1 cells and IEC6 cells compared with controls (Fig. 4), indicating that mitochondrial transmembrane potential was reduced by the exposure to BPs in gastric and small intestinal cells.

Both fluorescence indicator and EPR analysis confirmed the mitochondrial superoxide production by BP treatment in gastric and small intestinal cells. To examine whether the treatment of BPs induces mitochondrial $O_2^{\bullet-}$ production, which

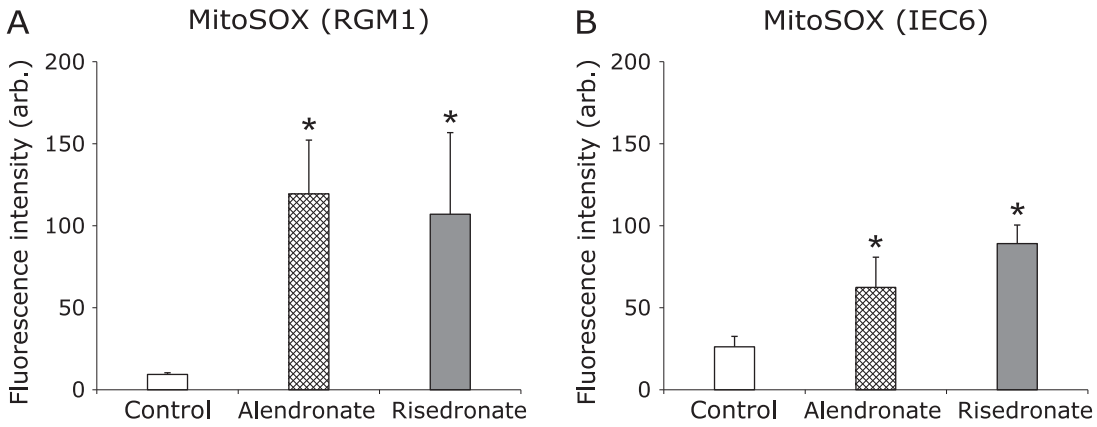

Fig. 5. Superoxide production of BP-treated RGM1 cells (A) and IEC6 cells (B) measured with a fluorescent superoxide indicator, MitoSOX. The fluorescence intensity of MitoSOX was significantly more increased in the cells treated with alendronate and risedronate than in the control cells, indicating that superoxide production in mitochondria was increased by BP treatment in these cells. *p<0.05 vs control cells.

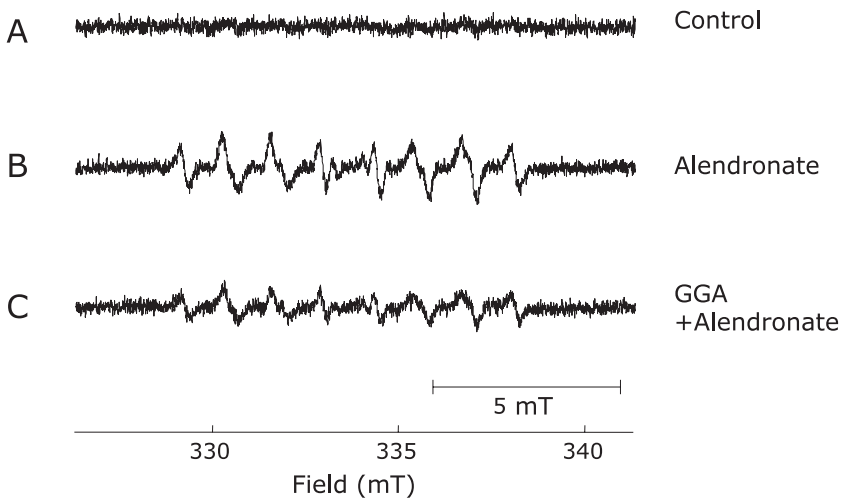

Fig. 6. Electron paramagnetic resonance (EPR) analysis of superoxide production in isolated mitochondria of alendronate-treated RGM1 cells. Cells were treated with 100 μM alendronate with or without 100 μM GGA treatment, and their mitochondria were isolated and examined by EPR spectroscopy using a spintrap agent, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO). To obtain the EPR spectra, the mitochondria were incubated in respiratory substrate and CYPMPO. The parameters obtained from the mitochondria of the cells treated with alendronate-alone (B) was 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 source (data not shown), as well as being similar to those of CYPMPO-OOH spin adducts.

subsequently triggers lipid peroxidation, we examined the detection of mitochondrial $O_2^{\bullet-}$ in alendronate or risedronate-treated gastric and small intestinal cells with a fluorescent $O_2^{\bullet-}$ indicator, MitoSox. The results demonstrated that the fluorescence intensities of MitoSox were significantly increased in alendronate- and risedronnate-treated RGM1 cells and IEC6 cells compared with controls (Fig. 5), indicating that $O_2^{\bullet-}$ was produced in mitochondria by the exposure to BPs in gastric and small intestinal cells.

We also underwent EPR spectroscopy of isolated, alendronate-treated mitochondrial fraction from gastric RGM1 cells using a spin-trapping reagent, CYPMPO.[18] Incubation with respiratory substrates, the isolated mitochondria showed EPR spectra of CYPMPO-OOH (Fig. 6). The EPR spectrum parameters obtained from the mitochondria from the cells treated with alendronate alone were quite similar to those of produced by the hypoxanthine (HX)/xanthine oxidase (XOD) system and a simulation of CYPMPO-OOH adduct. The Hfsc of the spectrum agreed with that of the computer-simulated spectrum of CYPMPO-OOH (data not shown). Therefore, we concluded that the spectrum was assigned to the CYPMPO-OOH spin adduct formed by the reaction of $O_2^{\bullet-}$ with CYPMPO (Fig. 6C).

Considering above results together, we concluded that BPs disrupted mitochondrial membrane and induced the production of $O_2^{\bullet-}$, which evoked cellular lipid peroxidation and injury in gastrointestinal mucosa.

GGA partially prevented cellular injury, lipid peroxidation, and mitochondrial superoxide production in BP-treated cells. To examine the protective effect of GGA pretreatment on BP-induced gastrointestinal mucosal injuries, we treated BP-treated gastric RGM1 and small intestinal IEC6 cells with or without GGA pretreatment, and investigated cellular injury, lipid peroxidation with the fluorescent indicators. The results demonstrated that the treatment of GGA indeed significantly prevented cellular injury (Fig. 7) and lipid peroxidation (Fig. 8)

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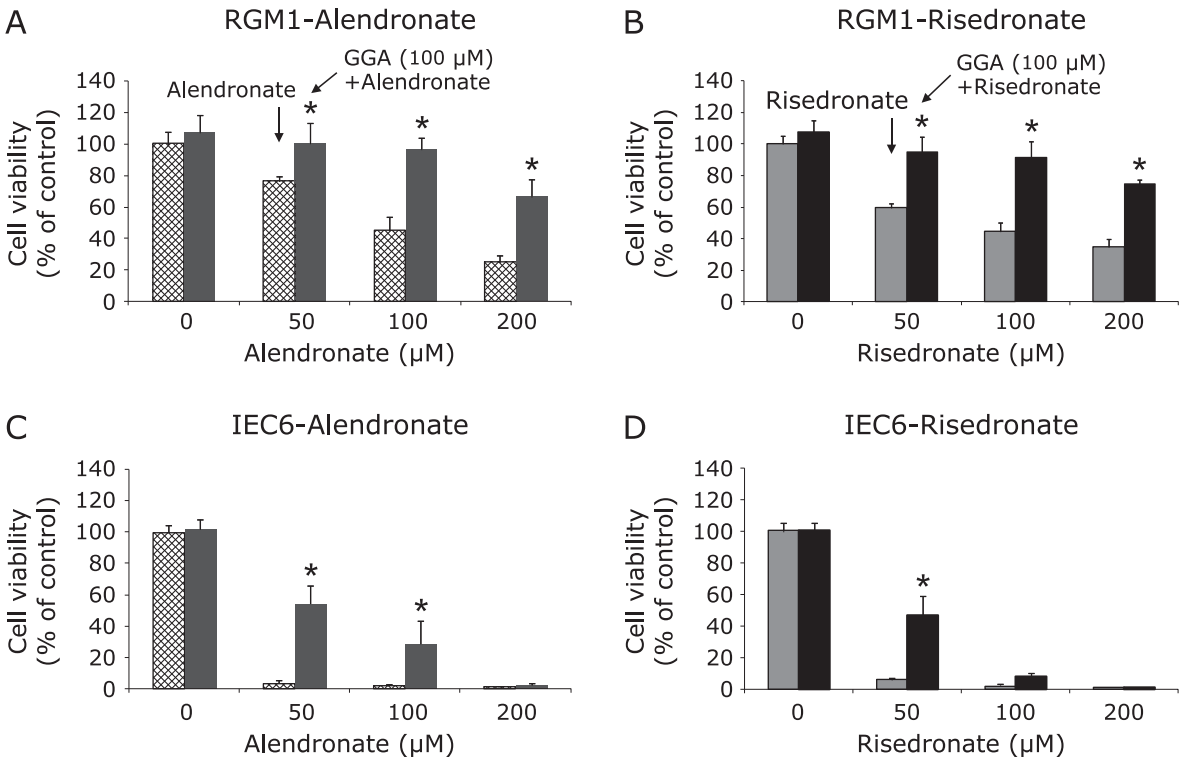

Fig. 7. TC-1 assay on the effect of GGA on BP-induced cellular injury in gastric epithelial RGM1 cells (A and B) and small intestinal IEC6 cells (C and D). Fluorescence intensities of TC-1 in GGA-pretreated cells were significantly higher than GGA-untreated cells, indicating that GGA inhibited the alendronate- and risedronate-induced cellular injury in gastric and small intestinal cells. *p<0.05 vs alendronate- or risedronate-alone treated cells.

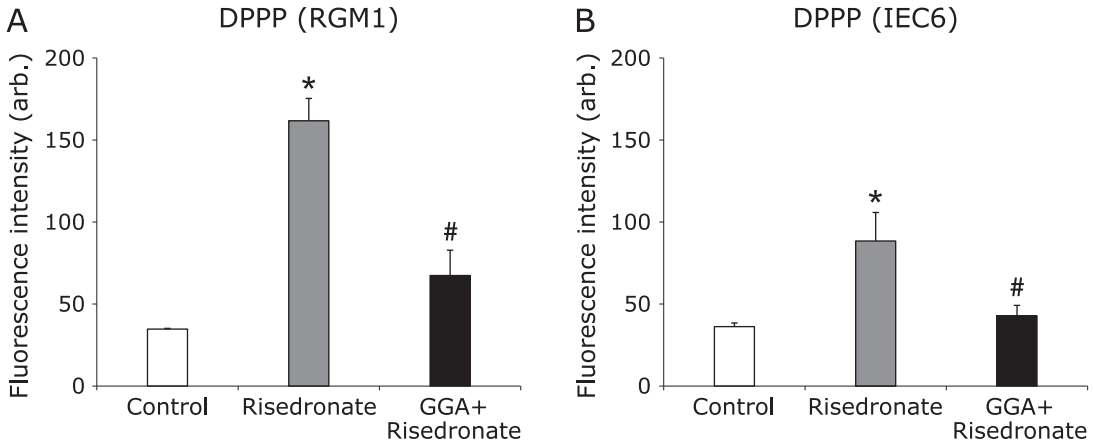

Fig. 8. DPPP assay on the effect of GGA on risedronate-induced lipid peroxidation in RGM1 cells (A) and IEC6 cells (B). The DPPP fluorescence intensities of GGA-pretreated RGM1 and IEC6 cells were significantly decreased than those of the control cells, indicating that GGA inhibited risedronate-induced lipid peroxidation. *p<0.05 vs control cells. $^{\#}p$<0.05 vs risedronate-alone treated cells.

in gastric and small intestinal cells. Furthermore, to investigate whether GGA prevented the $O_2^{\bullet-}$ production in mitochondria, we underwent ESR analysis on the mitochondria isolated from alendronate-treated RGM1 cells that were pretreated with or without GGA. EPR analysis confirmed that the signal intensity of the ESR spectrum of the mitochondria of the alendronate-treated cells pretreated with GGA was considerably reduced in comparison to that of the control cells (Fig. 6 C and D). The MitoSOX fluorescence intensities of GGA-pretreated RGM1 and IEC6 cells were significantly decreased than those of the control cells, indicating that GGA inhibited risedronate-induced superoxide production. These results strongly suggested that GGA prevented BP-induced cellular injury and lipid peroxidation by reducing the production of $O_2^{\bullet-}$ in gastrointestinal cells.

Discussion

In this study we demonstrated that BPs disrupted mitochondrial

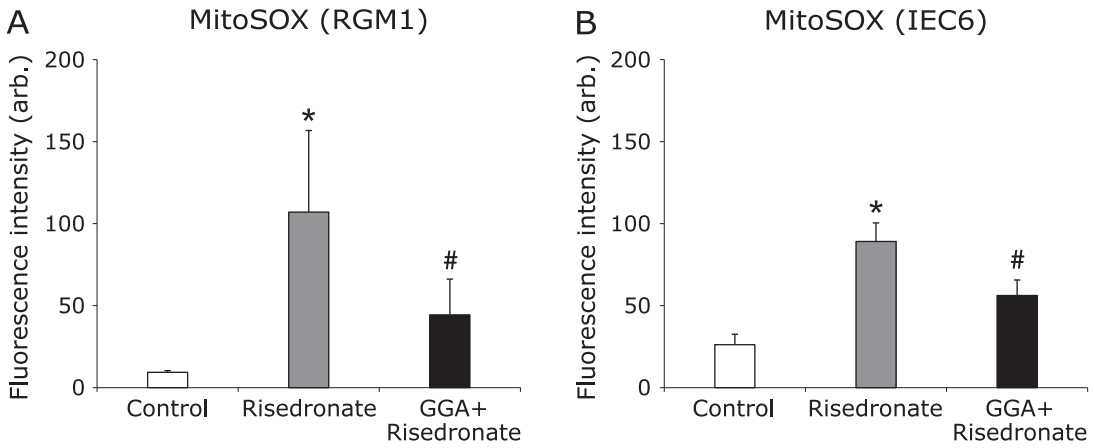

Fig. 9. MitoSOX assay on the effect of GGA on risedronate-induced superoxide production in RGM1 cells (A) and IEC6 cells (B). The MitoSOX fluorescence intensities of GGA-pretreated RGM1 and IEC6 cells were significantly decreased than those of the control cells, indicating that GGA inhibited risedronate-induced superoxide production. *$p<0.05$ vs control cells. #$p<0.05$ vs risedronate-alone treated cells.

membrane and induced the production of $O_2^{\bullet-}$, which subsequently induced lipid peroxidation and cellular injury in gastric and small intestinal epithelial cells. We concluded, for the first time to our knowledge, that mitochondria are the primary target of BP in gastrointestinal injury.

Previous studies proposed several mechanisms of the pathogenesis of BP-induced gastrointestinal injury, including cytotoxic adenosine triphosphate (ATP) action, disruption of cellular permeability barrier, and interference with the mevalonate pathway necessary for lipid synthesis.[6,24,25] However, the mechanisms by which BPs irritate the gastrointestinal mucosa, and by which BP's synergistic detrimental effect with NSAIDs were not clearly elucidated. Some studies reported the involvement of lipid peroxidation in the pathogenesis of BP-induced gastropathy.[10,26] However, these study considered that the pathogenesis was accounted for the impairment of the mucosal anti-oxidative system.[10,26]

Our study shed a new light on the pathogenesis: by considering that mitochondria are the target for BPs, we can assume the pathogenesis of gastrointestinal mucosa as a whole. More importantly, since mitochondria are the target for NSAIDs,[4,27–29] our results clearly explain the mechanism by which BPs have the synergistic effects with NSAIDs.

We also demonstrated that by reducing the production of $O_2^{\bullet-}$ in mitochondria, antiulcer drug GGA inhibited BP-induced cellular injury and lipid peroxidation. The biologic mechanism that GGA's protective effect on mitochondria remains unknown.[15] As previous reports suggested, we speculate that GGA may stabilized mitochondrial membrane by inducing the expression of HSP 72, which reportedly has the ability to protect mitochondrial respiratory function against oxidative stress.[15,29]

Our conclusion that mitochondria are the primary target of BP strongly suggested a novel therapeutic strategy for preventing BP-induced gastrointestinal mucosal injuries. At present, there is no definite choice of drug for preventing BP-associated gastrointestinal mucosal injury. However, as our study demonstrated, lipid peroxidation is involved in the pathogenesis of BP-associated gastrointestinal injury,[10,15,27] we propose agents with the membrane-stabilizing effects against lipid peroxidation can be a promising candidate.

Conflict of Interest

No potential conflicts of interest were disclosed.

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