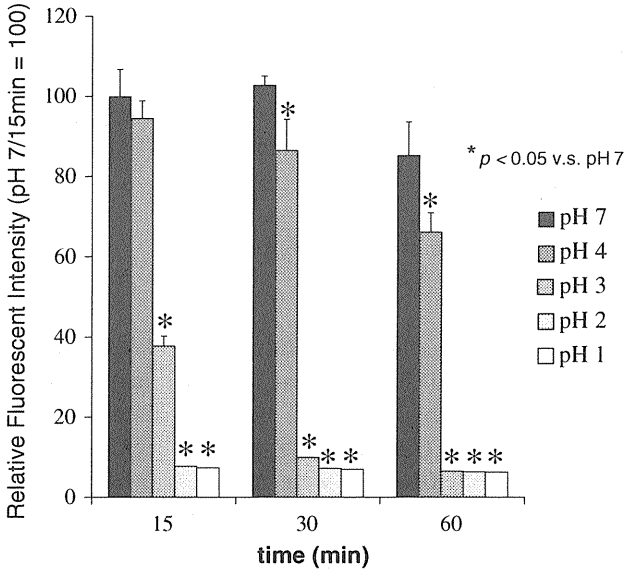

Fig. 1 Cellular injuries examined using the Tetra Color One (TC-1) assay in RGM1 cells treated with solutions of various pH values (pH 1, 2, 3, 4, and 7) for 15, 30, and 60 min. Data are expressed as percentages of pH 7 and 15 min (mean ± SD). Cellular viabilities were decreased in a time- and pH-dependent manner. Cells treated with strong acid (pH 1 and pH 2) were significantly more injured than cells treated with milder acid (pH 3 and pH 4) and the control cells (pH 7). Most of the cells treated with stronger acid (pH 1 and pH 2) were considered necrotic, and this was confirmed by microscopic analysis (data not shown). $*P < 0.05$ versus pH 7

that had a pH of less than 6 indeed underwent injury in a pH-dependent manner (Fig. 1).

Lipid peroxidation due to acid exposure

To examine whether gastric acid exposure induced lipid peroxidation in gastric epithelial cells, we treated RGM1 cells with acidic solutions and examined them for lipid peroxidation using DPPP-oxide fluorescence and using an antibody for 4-HNE-modified protein. The fluorescence analysis using DPPP-oxide, a specific marker of lipid peroxidation [15], indicated that lipid peroxidation was induced in a time-dependent manner in the cells treated with pH 3 solution, compared with the cells treated with pH 7 solution (Fig. 2a). We also evaluated, by immunohistochemical staining, the acid-exposed cells using 4-HNE-modified protein, a highly toxic aldehyde product of lipid peroxidation and a sensitive marker of lipid peroxidation [16]. The fluorescence intensity of anti-4-HNE-modified protein antibody was significantly increased in a time-dependent manner in the cells treated with pH 3 solution (Fig. 2b), indicating that lipid peroxidation was induced in the acid-treated cells. These results clearly demonstrated that lipid peroxidation was induced by the acid exposure.

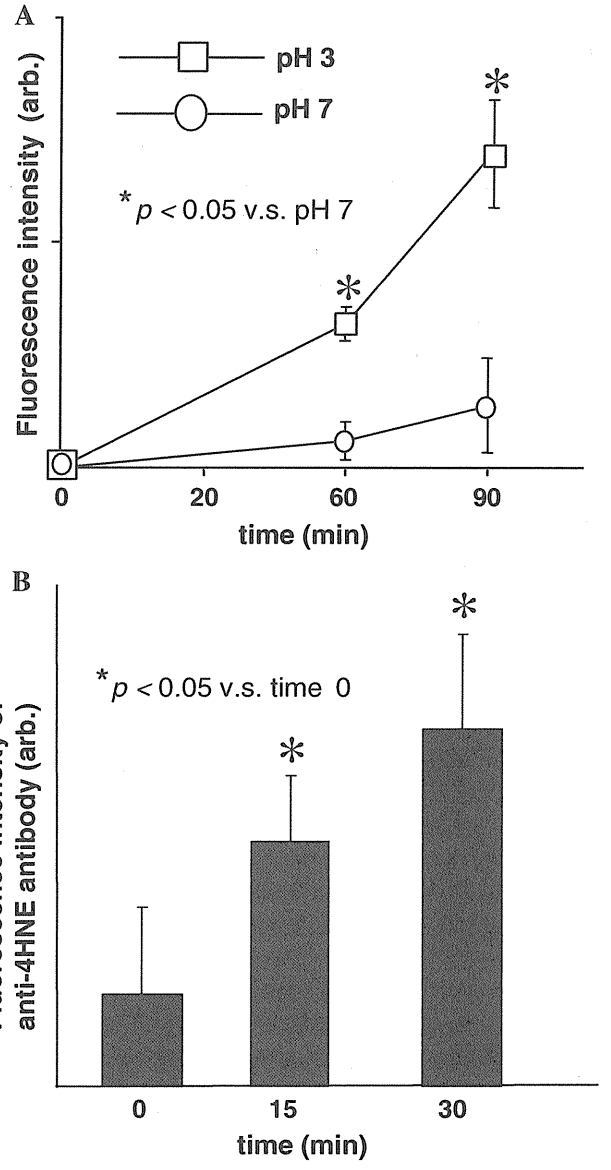

Fig. 2 Lipid peroxidation of acid-treated RGM1 cells measured with diphenyl-1-pyrenyl-phosphine (DPPP) fluorescence and 4-hydroxy-2-nonenal (*4-HNE*)-modified protein. **a** The DPPP fluorescence intensities of the cells treated with pH 3 solution were significantly higher than those of the control cells at 60 and 90 min, indicating that lipid peroxidation was induced by the acid. $*P < 0.05$ versus time 0. **b** Cells with acid-induced lipid peroxidation were immunohistochemically stained and lipid peroxidation was measured with 4-HNE-modified protein, a sensitive marker of this phenomenon. The fluorescence intensity of anti-4HNE-modified protein antibody in the acid-treated cells was significantly increased in a time-dependent manner. $*P < 0.05$ vs. time 0. *arb.* arbitrary unit

Apoptosis induced in cells treated with pH 3 and pH 4 solutions

Apoptosis was investigated using the Cell Death Detection ELISA, a photometric enzyme immunoassay for qualitative and quantitative in vitro determination of cytoplasmic

histone-associated DNA fragments of induced cell death. The results showed that DNA fragments were extensively produced in RGM1 cells exposed to pH 3 and pH 4 solutions, indicating the induction of apoptosis (Fig. 3a). Interestingly, the cells exposed to pH 1 and pH 2 solutions showed almost no DNA fragments, indicating that these cells underwent almost no apoptosis. Considering the significant damage induced in the cells exposed to pH 1 and pH 2 solutions (Fig. 1) and the morphological analysis of the microscopic images (data not shown), we concluded that these cells underwent necrosis. We also used Hoechst 33258 nuclear staining to analyze the acid-treated cells and found increased numbers of stained nuclei in the pH 3-treated cells, whereas no stained nuclei were found in the pH 7-treated cells (Fig. 3b), a result consistent with the previous finding that the cells treated with pH 3 underwent apoptosis.

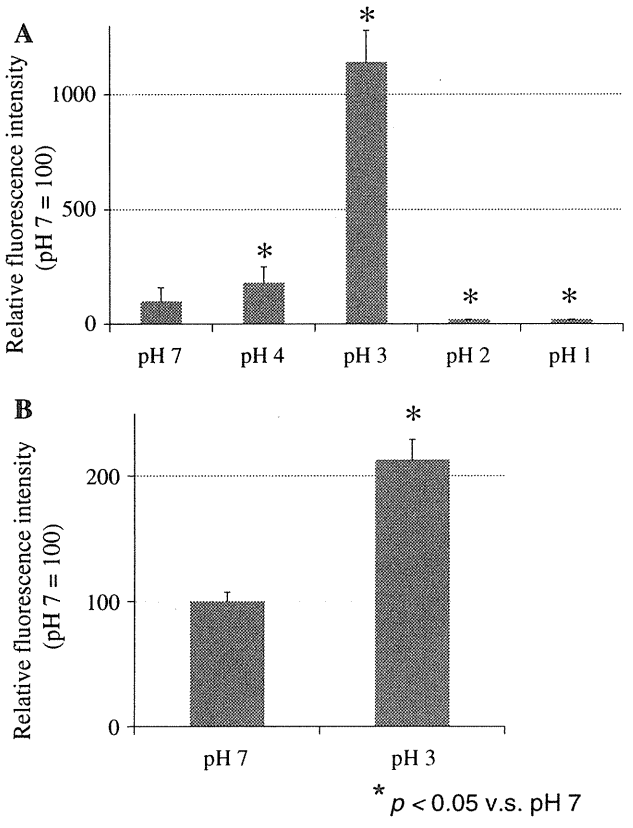

Fig. 3 Analysis of apoptosis in acid-treated cells, performed using a Cell Death Detection ELISA kit (**a**) and a fluorescence dye, Hoechst 33258 (**b**). **a** Apoptosis was investigated using the Cell Death Detection ELISA, which is a photometric enzyme immunoassay for qualitative and quantitative in vitro determination of the cytoplasmic histone-associated DNA fragments of induced cell death. The fluorescence intensity of the cells treated with milder acid, in particular, pH 3, was significantly higher than that of the cells treated with pH 7 solution, indicating that the milder acid treatment induced considerably more apoptosis. Interestingly, treatment with strong acid (pH 1, pH 2) induced no apoptosis. $*P < 0.05$ versus pH 7. **b** DNA fragmentation in RGM1 cells treated with pH 3 and pH 7 solutions was investigated using Hoechst 33258. The number of apoptotic cells, detected by the fluorescence intensity of cleaved nuclei, was significantly more increased in the cells treated with pH 3 solution than in those treated with pH 7 solution, indicating that apoptosis was induced in pH 3-treated cells. $*P < 0.05$ versus pH 7

Lipid peroxidation of acid-treated mitochondrial fractions

To confirm whether mitochondria were involved in gastric acid-induced lipid peroxidation, we fractionated RGM1 cells into mitochondrial and residual fractions. The residual fraction contained the cellular membrane and cytoplasm. Both fractions were treated with pH 3 solutions and examined by means of a DPPP fluorescence assay. The mitochondrial fraction treated with the acid showed significantly higher DPPP-oxide fluorescence intensity than did the residual fraction treated with the acid (Fig. 4). These results confirmed that the presence of mitochondria was essential for the acid-induced lipid peroxidation.

Mitochondrial transmembrane potential disruption induced by acid exposure

To examine our hypothesis that gastric acid disrupts the mitochondrial transmembrane potential that triggers

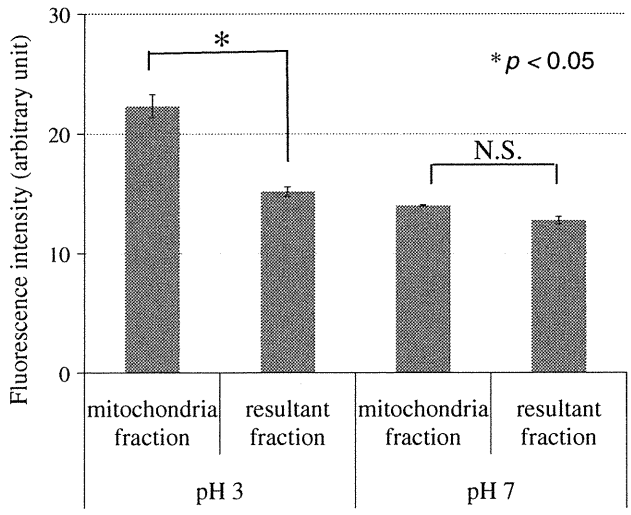

Fig. 4 Acid-induced lipid peroxidation induced in mitochondrial and residual fractions. To confirm whether mitochondria were involved in acid-induced lipid peroxidation, both the mitochondrial fraction and the residual fraction were treated with pH 3 solutions and examined using the DPPP fluorescence assay. The fluorescence intensity of the acid-exposed mitochondrial fraction was significantly higher than that of the residual fraction containing the cytosol and cellular membrane. *N.S.* not significant

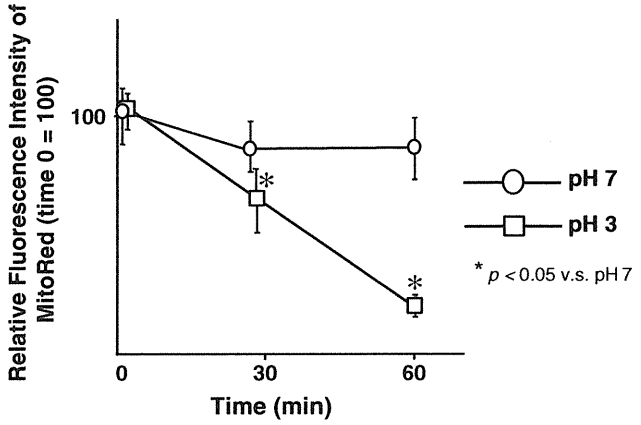

Fig. 5 Mitochondrial membrane potentials of the acid-treated RGM1 cells measured with MitoRed. Mitochondrial membrane potentials were measured with a cell-membrane-permeable rhodamine-based dye, MitoRed. The fluorescence intensity of MitoRed was significantly more reduced in the cells treated with pH 3 solution than in the control cells, and this reduction occurred in a time-dependent manner, indicating that the mitochondrial transmembrane potential was reduced by the exposure to the acid. $*P < 0.05$ versus pH 7

apoptosis, we investigated the transmembrane potentials of the acid-treated RGM1 cells by using a fluorescence indicator, MitoRed. This rhodamine-based dye attaches to mitochondria, and its fluorescence intensity depends on the mitochondrial membrane potential [18]. The fluorescence intensity of MitoRed was significantly more reduced in the cells treated with pH-3 solution than in the control cells, and the reduction occurred in a time-dependent manner (Fig. 5), indicating that the mitochondrial transmembrane potential was reduced in response to the acid exposure.

Superoxide detection by EPR in mitochondria of acid-treated cells

To examine whether mitochondria generated superoxide ($O_2^{\cdot-}$) upon gastric acid exposure, we performed EPR spectroscopy of mitochondria isolated from RGM1 cells treated with the acidic solutions, using a spin-trapping reagent, CYPMPO [11, 20] (Fig. 6). The EPR spectra of mitochondria were observed. The parameters obtained from the mitochondria of the cells treated with pH 3 and pH 4 solutions 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 source, as well as being similar to those of CYPMPO-OOH spin adducts reported previously [21]. The hyperfine structure constants of the spectrum agreed with those of the computer-simulated spectrum of CYPMPO-OOH. Therefore, we concluded that the spectrum could be assigned to the CYPMPO-OOH spin adduct formed by the reaction of superoxide with CYPMPO. Interestingly, the mitochondria of the cells treated with pH 1 and pH 2 solutions showed no

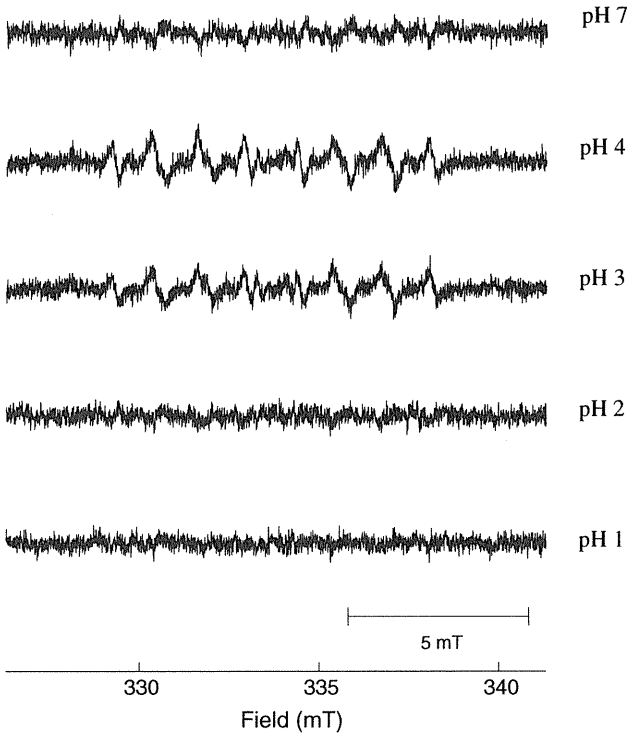

Fig. 6 Detection of superoxide production in isolated mitochondria of the acid-treated RGM1 cells. Cells were treated with pH 1, 2, 3, 4, and 7 solutions, and their mitochondria were isolated and examined by electron paramagnetic resonance (EPR) spectroscopy using a spin-trap agent, 5-(2,2-dimethyl-1,3-propoxy 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 pH 3 and pH 4 solutions 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 source, as well as being similar to those of CYPMPO–OOH spin adducts. The signal intensities of the EPR spectra of the pH 3- and 4-treated cells were obviously higher than those of the pH 1-, 2-, and 7-treated cells

specific EPR spectra, indicating an absence of detectable superoxide production in these fractions.

Prevention of acid-induced injury and superoxide production in cells overexpressing MnSOD

To examine whether acid-induced mitochondrial superoxide induced lipid peroxidation, we examined RGM1 cells that overexpressed MnSOD, which is expressed exclusively in mitochondria and removes superoxide from them [13, 14, 22] to prevent injury and lipid peroxidation. MnSOD stable clones (MnSOD6 and MnSOD8) and a vector-alone clone (vector) were constructed, and their activity was confirmed by native polyacrylamide gel electrophoresis (PAGE) analysis (Fig. 7a, b). Notably, the SOD activity of cytosolic copper/zinc superoxide dismutase (Cu/ZnSOD), which removes cytosolic superoxide, was not changed in any of the clones (Fig. 7c). The RGM1

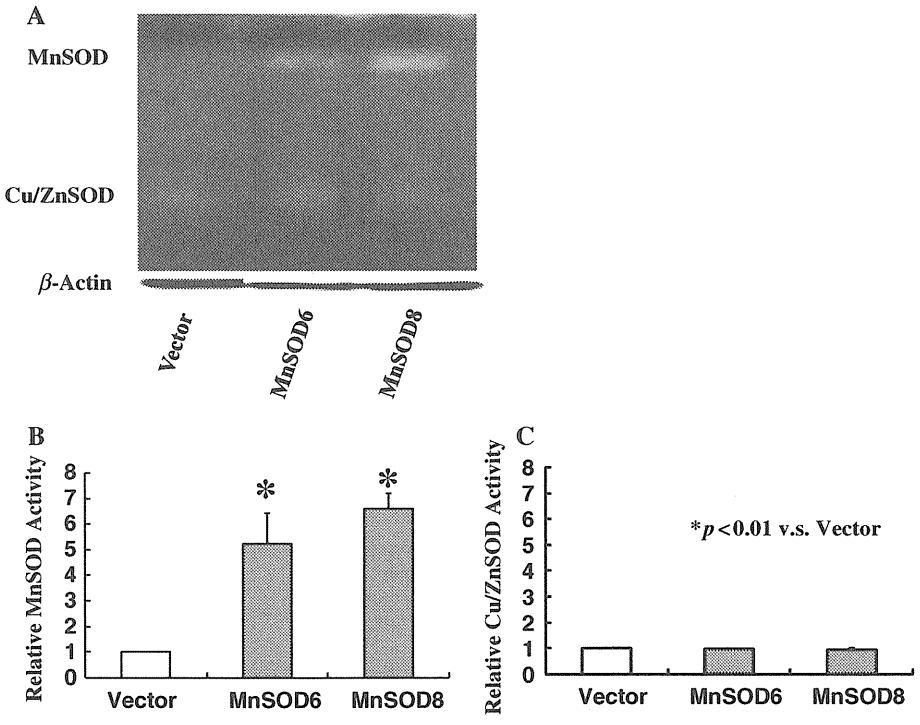

Fig. 7 Detection of manganese superoxide dismutase (*MnSOD*) activity (**a**, **b**) and Cu/ZnSOD activity (**c**). Native polyacrylamide gel was stained for SOD activity in MnSOD clones 6 and 8 and in the control plasmid transfectant (*vector*). Each *lane* was loaded with 50 mg protein and electrophoresed through a 12% polyacrylamide gel. Whereas the activity of MnSOD in the vector was very low, in the MnSOD-transfected cells (*MnSOD 6* and *MnSOD 8*), it was clearly detectable (**a**) and significantly increased (**b**). Notably, no difference in the activity of Cu/ZnSOD was detectable between the MnSOD and vector clones (**c**)

cells that overexpressed MnSOD and the control cells were treated with each acidic solution, and the resulting damage and lipid peroxidation were examined using the above-mentioned methods. The TC-1 analysis indicated that the extent of acid-induced damage in cells overexpressing MnSOD (RGM1-MnSOD6 and RGM1-MnSOD8) was significantly less than that in the control cells (RGM1 vector) (Fig. 8a). The DPPP fluorescence analysis revealed that the extent of lipid peroxidation was significantly reduced in the cells overexpressing MnSOD (Fig. 8b). These results confirmed that MnSOD had a protective effect against lipid peroxidation and the subsequent cellular injury induced by acid exposure. Considering that MnSOD scavenges mitochondrial, not cytosolic, superoxide, these results suggested that superoxide produced in mitochondria induced the lipid peroxidation and injury in acid-treated RGM1 cells.

Discussion

We previously demonstrated that the production of ROS by mitochondria, as well as lipid peroxidation and apoptosis, were induced by alkaline conditions and prevented by MnSOD, thus proving for the first time the relationship between ROS generated in mitochondria and apoptosis [22]. In the present study, we demonstrated that exposure to HCl induced the production of superoxide ($O_2^{\cdot-}$) in mitochondria and subsequently evoked lipid peroxidation and apoptosis in vitro.

We demonstrated that MnSOD prevented the lipid peroxidation and cellular injury induced by acid exposure. MnSOD appears to play an important role in low-pH cytotoxic effects, because it is mitochondrial MnSOD, not cytosolic Cu/ZnSOD, that is the dismutase superoxide in mitochondria [22] and Cu/ZnSOD activities were not changed in the cells overexpressing MnSOD or in the control cells (Fig. 7c). We concluded that superoxides generated in mitochondria induced the lipid peroxidation and injury in acid-treated RGM1 cells.

Several studies have shown that mitochondria produce superoxide, mainly in complexes I and III of the electron transport system, which is located in the inner membrane of mitochondria [23, 24]. Majima et al. [22] reported the first evidence that MnSOD could protect against apoptosis. Production of ROS in the electron transport chain may result in oxidative stress in cells and in apoptotic death. Many reports have demonstrated the biological importance of MnSOD: it scavenges superoxide located in mitochondria [25], and its expression is essential for the survival of aerobic life and the development of cellular resistance to oxygen radical-mediated toxicity [13, 14, 22]. We previously also showed that MnSOD without the mitochondrial targeting sequence could not prevent cellular injuries resulting from hypoxia-reoxygenation insults, whereas authentic MnSOD could [26]. These results suggest that only when MnSOD is located in mitochondria, after proper posttranslational import and processing, is it efficient in protecting against cellular injuries resulting from oxidative stress, and these results also indicate that mitochondria are

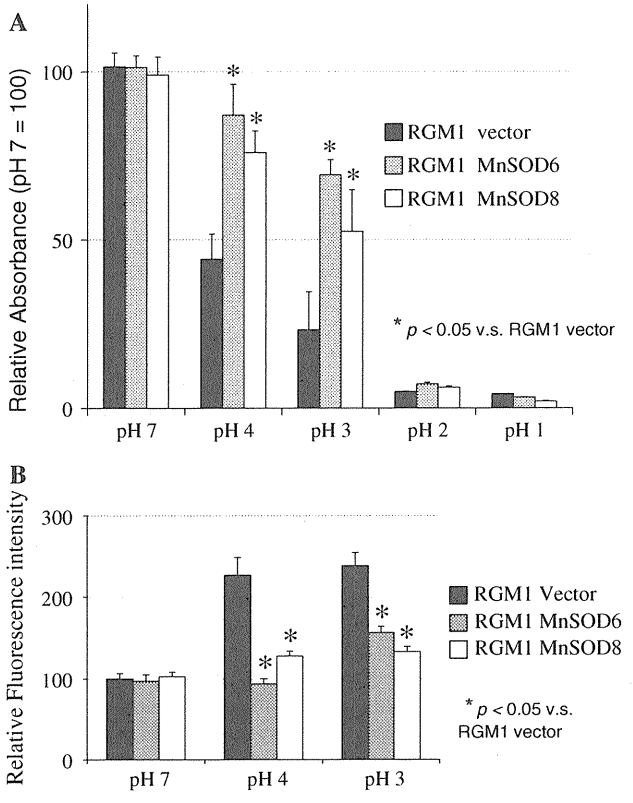

Fig. 8 Cellular injury (**a**) and lipid peroxidation (**b**) in RGM1 cells overexpressing MnSOD upon acid treatment. **a** We used a TC-1 assay to examine injury in RGM1 cells that overexpressed MnSOD. The TC-1 analysis indicated that the extent of the milder acid (pH 3 and pH 4)-induced damage in the cells overexpressing MnSOD (*RGM1-MnSOD6* and *RGM1-MnSOD8*) was significantly less than that in the control cells (*RGM1 vector*), indicating that cellular damage induced by exposure to milder acid was partly prevented by MnSOD. **b** Lipid peroxidation induced by mild acid exposure was examined using DPPP fluorescence analysis in RGM1 cells overexpressing MnSOD. The DPPP fluorescence analysis revealed that the extent of lipid peroxidation was significantly less in the cells overexpressing MnSOD than in the control cells (**b**), indicating that lipid peroxidation was partly prevented by MnSOD. $*P < 0.05$ versus RGM1 vector

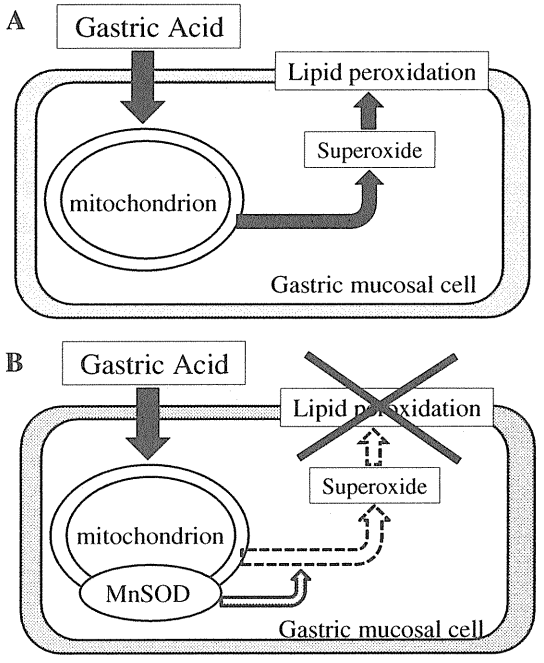

Fig. 9 A proposed working model for reactive oxygen species (ROS) formation and lipid peroxidation in gastric mucosal cells. The present study demonstrated that gastric acids cause mitochondria to release superoxide, which triggers lipid peroxidation (**a**). However, in cells overexpressing MnSOD, gastric acid-induced lipid peroxidation is partly inhibited because MnSOD scavenges superoxide (**b**)

the primary sites of oxidant-induced cellular oxidative injuries.

The pathophysiological implications of the present study are that mitochondria are susceptible to changes in hydrogen concentrations, such as those that occur in acidosis and alkalosis. Because mitochondria utilize the pH gradient as energy for generating adenosine triphosphate (ATP), any imbalance in the normally tightly regulated concentration of hydrogen may evoke serious, often life-threatening changes in any cells [8]. An in vitro study recently demonstrated that the production of superoxide in complex I of the electron transfer chain of hepatic cells was dependent on the pH gradient across the mitochondrial inner membrane [27], although the sites of superoxide production within complex I remain uncertain [8].

The results of the present study demonstrate the biological validity of a therapeutic strategy of acid-control in gastric mucosal injuries: gastric acid with a pH of less than 4 indeed enhanced cellular injuries by evoking lipid peroxidation. Although the pH may have influenced the experimental conditions, these results are consistent with those of clinical studies that concluded that it is essential to maintain the intragastric pH above 3 to promote ulcer healing [3].

Recent experimental evidence has demonstrated that mitochondria are involved in both apoptosis and necrosis [28]. The present study demonstrated that the mitochondria of cells treated with pH 3 and pH 4 solutions triggered lipid peroxidation by producing superoxide. It is possible that the mitochondria triggered necrosis in the cells treated with pH 1 and pH 2 solutions. We propose that mitochondria may be the therapeutic targets for gastric apoptosis and necrosis.

Gastric lipid peroxidation, which has been regarded as an important prime event that leads to gastric mucosal injuries, has been reported to be induced by extrinsic ROS from various sources: CagA protein of *H. pylori*, activated neutrophils, and microvascular ischemia/reperfusion [29, 30]. In contrast, several studies, including the present one and our previous study, have demonstrated that NSAIDs and gastric acid-induced intrinsic, mitochondria-derived superoxide evoked cellular peroxidation [5, 7]. We therefore propose that cellular peroxidation is triggered not only

by extrinsic ROS, but also by intrinsic ROS (Fig. 9a), and that the presence of mitochondrial, not cytoplasmic, superoxide dismutase (MnSOD) is essential for preventing mitochondria-induced lipid peroxidation (Fig. 9b).

In conclusion, this study has demonstrated the gastric acid-induced production of superoxide in mitochondria and subsequent lipid peroxidation and apoptosis in gastric epithelial cells. The results provide biological evidence for a therapeutic strategy of suppressing gastric acid secretion in gastric mucosal injuries.

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References

1. Harchelroad FP Jr, Ballester JM. Chemical burns. In: Tintinalli JE, Kelen GD, Stapczynski JS, editors. Emergency medicine. New York: McGraw-Hill; 1999. p. 1286–92.
2. Schwarz K. Ueber penetrierende magen-und jejunalgeschwure. Beitr Klin Chir. 1910;67:96–128.
3. Schubert ML, Peura DA. Control of gastric acid secretion in health and disease. Gastroenterology. 2008;134:1842–60.
4. Brzozowski T, Konturek PC, Konturek SJ, Drozdowicz D, Kwiecien S, Pajdo R, et al. Role of gastric acid secretion in progression of acute gastric erosions induced by ischemia–reperfusion into gastric ulcers. Eur J Pharmacol. 2000;398:147–58.
5. Tsutsumi S, Tomisato W, Takano T, Rokutan K, Tsuchiya T, Mizushima T. Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. Biochim Biophys Acta. 2002;1589:168–80.
6. Murphy MP, Smith RA. Drug delivery to mitochondria: the key to mitochondrial medicine. Adv Drug Deliv Rev. 2000;41: 235–50.
7. Nagano Y, Matsui H, Muramatsu M, Shimokawa O, Shibahara T, Yanaka A, et al. Rebamipide significantly inhibits indomethacin-induced mitochondrial damage, lipid peroxidation, and apoptosis in gastric epithelial RGM-1 cells. Dig Dis Sci. 2005;50(Suppl 1):S76–83.
8. Brand MD, Buckingham JA, Esteves TC, Green K, Lambert AJ, Miwa S, et al. Mitochondrial superoxide and aging: uncoupling-protein activity and superoxide production. Biochem Soc Symp. 2004;2004:203–13.
9. Mingatto FE, dos Santos AC, Rodrigues T, Pigoso AA, Uyemura SA, Curti C. Effects of nimesulide and its reduced metabolite on mitochondria. Br J Pharmacol. 2000;131:1154–60.
10. Yoshida Y, Singh I, Darby CP. Effect of salicylic acid and calcium on mitochondrial functions. Acta Neurol Scand. 1992;85:191–6.
11. Kamibayashi M, Oowada S, Kameda H, Okada T, Inanami O, Ohta S, et al. Synthesis and characterization of a practically better DEPMPO-type spin trap, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline N-oxide (CYPMPO). Free Radic Res. 2006;40:1166–72.
12. Matsui H, Murata Y, Hirano K, Sasaki T, Shiba R, Muto H, et al. Hydrogen peroxide-induced cellular injury is associated with increase in endogenous fluorescence from rat gastric mucosal epithelial cell culture: a new method for detecting oxidative cellular injury by fluorescence measurement. J Gastroenterol. 1998;33:318–25.
13. Motoori S, Majima HJ, Ebara M, Kato H, Hirai F, Kakinuma S, et al. Overexpression of mitochondrial manganese superoxide dismutase protects against radiation-induced cell death in the human hepatocellular carcinoma cell line HLE. Cancer Res. 2001;61:5382–8.
14. Indo HP, Davidson M, Yen H-C, Suenaga S, Tomita K, Nishii T, et al. Evidence of ROS generation by mitochondria in cells with impaired electron transport chain and mitochondrial DNA damage. Mitochondrion. 2007;7:106–18.
15. Okimoto Y, Watanabe A, Niki E, Yamashita T, Noguchi N. A novel fluorescent probe diphenyl-1-pyrenylphosphine to follow lipid peroxidation in cell membranes. FEBS Lett. 2000;474:137–40.
16. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med. 1991;11:81–128.
17. Duan S, Hajek P, Lin C, Shin SK, Attardi G, Chomyn A. Mitochondrial outer membrane permeability change and hypersensitivity to digitonin early in staurosporine-induced apoptosis. J Biol Chem. 2003;278:1346–53.
18. Ikeda R, Sugita T, Jacobson ES, Shinoda T. Effects of melanin upon susceptibility of Cryptococcus to antifungals. Microbiol Immunol. 2003;47:271–7.
19. Tsugawa H, Suzuki H, Satoh K, Hirata K, Matsuzaki J, Saito Y, et al. Two amino acids mutation of ferric uptake regulator determines *Helicobacter pylori* resistance to metronidazole. Antioxid Redox Signal. 2011;14(1):15–23.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
21. Ogura A, Oowada S, Kon Y, Hirayama A, Yasui H, Meike S, et al. Redox regulation in radiation-induced cytochrome c release from mitochondria of human lung carcinoma A549 cells. Cancer Lett. 2009;277:64–71.
22. Majima HJ, Oberley TD, Furukawa K, Mattson MP, Yen HC, Szweda LI, et al. Prevention of mitochondrial injury by manganese superoxide dismutase reveals a primary mechanism for alkaline-induced cell death. J Biol Chem. 1998;273:8217–24.
23. Boveris A, Cadenas E. Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. FEBS Lett. 1975;1:311–4.
24. Takeshige K, Minakami S. NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. Biochem J. 1979;180:129–35.
25. Weisiger RA, Fridovich I. Mitochondrial superoxide simutase. Site of synthesis and intramitochondrial localization. J Biol Chem. 1973;248:4793–6.
26. Hirai F, Motoori S, Kakinuma S, Tomita K, Indo HP, Kato H, et al. Mitochondrial signal lacking manganese superoxide dismutase failed to prevent cell death by reoxygenation following hypoxia in a human pancreatic cancer cell line, KP4. Antioxid Redox Signal. 2004;6:523–35.
27. Lambert AJ, Brand MD. Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. Biochem J. 2004;382:511–7.
28. Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. Trends Cell Biol. 2000;10:369–77.
29. Naito Y, Yoshikawa T. Oxidative stress involvement and gene expression in indomethacin-induced gastropathy. Redox Rep. 2006;11:243–53.
30. Handa O, Naito Y, Yoshikawa T. CagA protein of *Helicobacter pylori*: a hijacker of gastric epithelial cell signaling. Biochem Pharmacol. 2007;73:1697–702.

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NSAIDs and Acidic Environment Induce Gastric Mucosal Cellular Mitochondrial Dysfunction

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Key Words
NSAIDs · Gastric acid · Lipid peroxidation · Mitochondrial damage · Reactive oxygen species

Abstract
Non-steroidal 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 in mitochondria, independently with cyclooxygenase inhibition and the subsequent prostaglandin deficiency. More recently, gastric hydrochloric acid (HCl) has been regarded as an inciting factor of gastric mucosal injuries, and reportedly induced cellular lipid peroxidation in vitro. We hypothesized that gastric acid and NSAID treatment synergistically induce cellular injury in gastric epithelial cells. We treated gastric epithelial RGM1 cells with acidic solutions and NSAIDs, and examined cellular injury, lipid peroxidation, mitochondrial transmenbrane potential and mitochondrial superoxide. We pretreated RGM1 cells with the acidic solutions for 0.5 h and after that treated them with each NSAID for 15 h and found that the exposure to acid and NSAIDs indeed induced cellular injury. We hypothesized that gastric acid and NSAID treatment synergistically induce mitochondrial superoxide production, which induces gastric cellular injury.

Introduction

Non-steroidal 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 to know that NSAIDs often cause gastrointestinal complications such as gastric ulcers and erosions. The pathogenesis of these complications has mostly been ascribed to the action of NSAIDs on the cyclooxygenase inhibition and the subsequent prostaglandin (PG) deficiency [1]. Extensive research has revealed how the gastric defense system is maintained in the presence of PG [2–4]. NSAIDs' causative action has thus been understood how PG deficiency affects the gastric defense system.

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Recent reports have demonstrated that NSAIDs, one of the most potent inciting factors of gastric mucosal injury, dissipated mitochondrial transmembrane potentials and produced reactive oxygen species (ROS) from mitochondria, thereby causing cellular lipid peroxidation and apoptosis [5, 6]. ROS from mitochondria, especially superoxide (O_2^-), is a major cause of the cellular oxidative damage [7]. NSAIDs uncouple oxidative phosphorylation to dissipate mitochondrial transmembrane potential [8] or to open mitochondrial permeability transition pores [9], leading to the liberation of cytochrome *c* from the mitochondrial intermembranous space into cytosol and to the production of ROS from mitochondria, thereby causing cellular lipid peroxidation and apoptosis [5, 6]. More recently, we showed that gastric acid induces mitochondrial superoxide production, which induces gastric cellular injury by triggering cellular lipid peroxide and apoptosis [10].

NSAID-induced gastric mucosal injury, from being suppressed by the inhibition of H2 blocker or PPI, and cell injury by exposure to the acid environment of NSAIDs may be caused by mechanisms identical to those induced. We hypothesized that gastric acid and NSAID treatment synergistically induce lipid peroxidation and the mitochondrial transmenbrane potential change in the gastric mucosa by superoxide from mitochondria. The results suggest that gastric acid/NSAID-induced cellular injury was caused by the same mechanism and such cellular injuries synergistically increase.

Material and Methods

Cell Culture

Rat gastric epithelial cell line RGM1 [11] was obtained from RIKEN BioResource Center Tsukuba, Japan. RGM1 cells were grown in a 1:1 mixture of both Dulbecco's modified Eagle's medium and Ham's F-12 medium (Cosmo Bio, Tokyo, Japan) supplemented with 10% fetal calf serum (Gibco, Grand Island, N.Y., USA) and 2 mM glutamine. The cells were grown at 37°C in a humidified incubator with 5% CO_2.

Solutions and Reagents

Indomethacin, diclofenac sodium and acetylsalicylic acid were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Each acidic solution was prepared by adding 0.1 N HCl to deionized distilled water. To maintain the osmolarity of the solutions at a physiological level, NaCl was added to yield 154 mEq/l of chloride concentration. Hanks' balanced salt solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used as pH 7 solution. The TetraColor ONE® cell proliferation assay kit was from Seikagaku (Tokyo, Japan), DPPP and MitoRed were from Dojindo (Kumamoto, Japan), and MitoSOX was from Molecular Probes (Invitrogen, USA). All other chemicals were reagent grade.

Analysis of Cell Viability after NSAID and Acidic Solution Treatment

Cell viability was assessed using the TetraColor ONE cell proliferation assay kit according to the manufacturer's instructions. RGM1 cells were pretreated with each acidic solution for 0.5 h and after that were treated with each NSAID for 15 h. Measurements (OD 450 nm) were taken after 1 h at 37°C using a microplate ELISA reader (DTX 880; Beckman Coulter, Fullerton, Calif., USA).

Cellular Microscopic Fluorescence Analysis

RGM1 cells were incubated on a Lab-Tec II slide chamber (Nalge Nunc International, Calif., USA) at a concentration of 10^5 cells/ml per well. RGM1 cells were pretreated with each acidic solution for 0.5 h and after that treated with each NSAID for 15 h. Lipid peroxidation, mitochondrial activity, and mitochondrial superoxide were investigated with the following fluorescent dyes, respectively: DPPP, MitoRed, and MitoSOX. Cellular fluorescent images were observed and their intensities were measured with a chilled CCD camera-mounted (AxioCam Color, Zeiss, Germany) epifluorescence microscope (Axiovert135M, Zeiss) connected to an image-analyzing system (Axio Vision, Zeiss). DPPP is a non-fluorescent triphenylphosphine compound. When it reacts with hydroxyperoxide, a fluorescent substance, DPPP oxide is formed [12] with an excitation and emission wavelength of 352 and 461 nm. MitoRed is a cell membrane-permeable rhodamine-based dye [13]. The fluorescence of intensity of this dye depends on mitochondrial membrane potential, and can be used as an indicator of mitochondrial activity. MitoSOX™ Red mitochondrial superoxide indicator is a novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells [14]. Their excitation and emission wavelengths are 559 and 588 nm.

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 Scheffé's method. A p value of <0.05 was considered significant.

Results

Effects of RGM1 on the Viability of Cells Treated with NSAID and Acidic Solution

To examine whether gastric acid and NSAID treatment synergistically induce cellular injury in gastric epithelial cells, we treated RGM1 cells with acidic solutions and each NSAID, and examined cellular injury with TetraColor One assay. These data indicated that the viability of cells of acidic solutions and NSAIDs treated was increased more than in the acidic solutions (fig. 1).

Lipid Peroxidation by the Acid Solution and NSAID Exposure

To examine whether gastric acid and NSAID treatment synergistically induce lipid peroxidation in gastric

epithelial cells, we treated RGM1 cells with acidic solutions and each NSAID, and examined lipid peroxidation with a fluorescent maker DPPP oxide. The fluorescence analysis indicated that lipid peroxidation of cellular membrane was increased more than in the acidic solutions (fig. 2a).

Mitochondrial Transmembrane Potentials by Acid Solution and NSAID Exposure

To examine whether gastric acid and NSAID treatment synergistically disrupt the mitochondrial transmembrane potential in gastric epithelial cells, we treated RGM1 cells with acidic solutions and each NSAID, and examined transmembrane potentials with the fluorescence intensity of MitoRed. The results demonstrated they were significantly reduced in the cells treated with pH 3 and pH 4 solutions, indicating that mitochondrial transmembrane potential was reduced more in the acid exposure (fig. 2b).

Mitochondrial Superoxide Detection by Acid Solution and NSAID Exposure

To examine whether gastric acid and NSAID treatment synergistically induce mitochondrial superoxide leakage in gastric cells, we examined the mitochondrial superoxide from the acidic solution-treated RGM1 cells with MitoSOX. The result demonstrated that the fluorescence intensity of MitoSOX was significantly induced in the cells treated with pH 3 and pH 4 solutions, indicating that mitochondrial superoxide was induced more in the acid exposure (fig. 2c). These results suggest that acidic solutions and indomethacin synergistically induced the leakage of superoxide in mitochondria of gastric RGM1 cells.

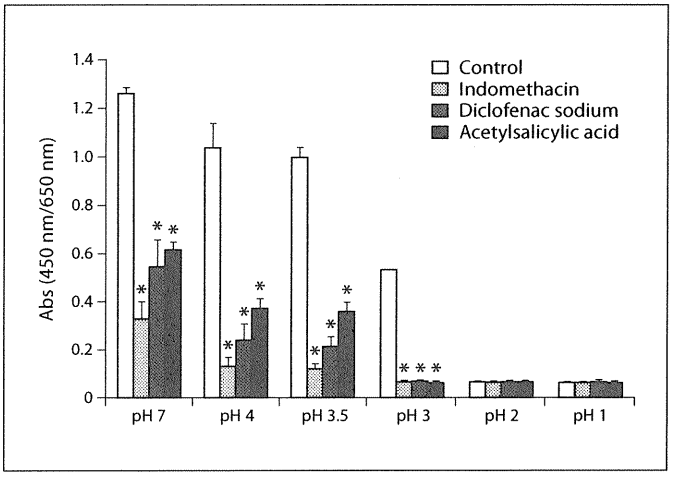

Fig. 1. Cellular injuries examined with the TetraColor ONE method in RGM1 cells treated with various pH solutions (pH 1, 2, 3, 3.5, 4, 7) for 0.5 h and after that, treated with NSAIDs (indomethacin, diclofenac sodium, acetylsalicylic acid) for 15 h. Cellular viabilities were decreased in a pH-dependent manner and NSAID-treated cells were significantly more reduced than in the acidic solution (* $p < 0.05$ vs. control).

Discussion

In this study we have demonstrated for the first time that NSAID treatment and an acidic environment can show a synergistic effect on gastric epithelial cell injuries. Both NSAIDs and acidic condition disturb mitochondrial dysfunction derived from mitochondrial ROS production via a membrane potential decrease.

Gastric acid has been reported to be the most important factor to induce peptic ulcer diseases. In 1910, Karl Schwarz [15] published a case series of 14 patients with ulcers who were treated with surgical excisions of the ulcers. His observations showed that ulcers were in the stomach, esophagus, duodenum, and jejunum – organs that he said were the only locations of acid. This led him to coin the phrase 'Ohne saueren Magensaft kein peptisches Geschwür', or 'no acid, no ulcer'.

Generally, gastric acid refers to the necrotizing factor derived from peptic ulcers which prevents a cure of these diseases. In this study, the acidic condition below pH 2 was derived from the abundant cell death with or without NSAID treatment. Recently, we investigated the role of acidic environments as an inducer of gastric epithelial cellular injuries [10]. Under the acidic condition below pH 2, the cellular membrane was immediately broken and cellular shapes were completely destroyed: one such typical necrosis occurred. In contrast, the acidic condition between pH 3 and pH 4 did not derive from cellular necrosis, but apoptosis. Under these environments, we found for the first time a superoxide anion spectrum with an electron paramagnetic resonance analyzer using a spin trap reagent CYPMPO. According to this result, we conclude that the acidic conditions from pH3 to pH 4 were oxidative stresses which induced cellular apoptosis. In this study, lipid peroxidation, mitochondrial membrane potential decrease and ROS generation were induced under the acidic conditions from pH 3 to pH 4, while almost all cells immediately died under the condition below pH 2. We propose that the acidic condition below 2 is the necrotizing factor, and the condition from pH 4 to pH 3 is an apoptosis inducer.

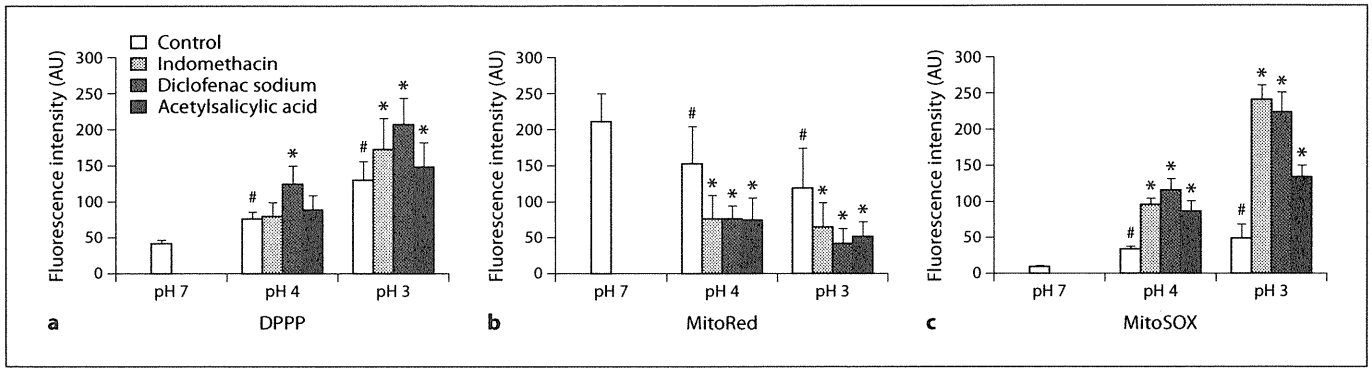

Fig. 2. Fluorescence analysis of lipid peroxidation, mitochondrial phosphorylation and mitochondrial superoxide in acidic solution (pH 3, 4) and NSAID-treated RGM1 cells. Data are expressed as the percentage of pH 7 or control (# $p < 0.05$ vs. pH 7, * $p < 0.05$ vs. control). **a** DPPP fluorescence intensities were increased in a pH-dependent manner and NSAID-treated cells were significantly more exacerbated than in the acidic solution. **b** MitoRed fluorescence intensities were decreased in a pH-dependent manner and NSAID-treated cells were significantly more reduced than in the acidic solution. **c** MitoSOX fluorescence intensities were increased in a pH-dependent manner and NSAID-treated cells were significantly more exacerbated than in the acidic solution.

NSAIDs have been reported to decrease 'defensive factors' via cyclooxygenase inhibition. Under less defensive factors, gastric acid which is the most important 'aggressive factor' involves peptic ulcers [16–18]. However, NSAID treatments themselves can involve gastric epithelial cellular injuries as we demonstrated in this study. Thirteen years before the James Vane's report, NSAID treatment was reported to decrease the oxygen consumption via the induction of mitochondrial dysfunction [19]. Moreover, recent studies revealed that NSAID treatments involved ROS generation to induce cellular apoptosis. We decided that the ROS after NSAID treatment is a superoxide anion with an electron paramagnetic resonance analyzer using a spin trap reagent CYPMPO [20]. In this study, NSAID treatment accelerated cellular injuries, lipid peroxidation, mitochondrial membrane potential decrease and ROS generation. Therefore, we also conclude that NSAID treatments are apoptosis inducers.

NSAID treatment under acidic conditions makes cellular injuries significantly worse. Not only cellular injuries, but also lipid peroxidation, membrane potential decrease and ROS generation got worse. We demonstrated that both NSAID treatments and acidic environments inhibit mitochondrial functions. These are possibly the reason why both treatments show a synergistic effect. The acid secretion inhibitor such as H2RA and PPI used to show remarkable effects in NSAID-induced peptic ulcers. These drugs may show these effects via the reduction of acid-induced mitochondrial inhibition.

In conclusion, both NSAID treatments and acidic environments synergistically involve gastric epithelial cellular injuries inducing mitochondrial dysfunction.

Disclosure Statement

The authors declare that no financial or other conflicts of interest exist in relation to the content of the article.

References

1 Laine L, Takeuchi K, Tarnawski A: Gastric mucosal defense and cytoprotection: bench to bedside. Gastroenterology 2008;135:41–60.

2 Del Valle J: Peptic Ulcer Disease and Related Disorders. New York, McGraw-Hill Professional, 2008.

3 Cryer B, Spechler SJ: Peptic Ulcer Disease. Philadelphia, Saunders, 2006 pp 1089–1110.

4 Matsui H, Shimokawa O, Kaneko T, Nagano Y, Rai K, Hyodo I: The pathophysiology of non-steroidal anti-inflammatory drug-induced mucosal injuries in stomach and small intestine. J Clin Biochem Nutr 2011;48:107–111.

5 Murphy MP, Smith RA: Drug delivery to mitochondria: the key to mitochondrial medicine. Adv Drug Deliv Rev 2000;41:235–250.

6 Nagano Y, Matsui H, Muramatsu M, Shimokawa O, Shibahara T, Yanaka A, Nakahara A, Matsuzaki Y, Tanaka N, Nakamura Y: Rebamipide significantly inhibits indomethacin-induced mitochondrial damage, lipid peroxidation, and apoptosis in gastric epithelial RGM-1 cells. Dig Dis Sci 2005;50 (suppl 1):S76–S83.

7 Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miw S, Pakay JL, Parker N: Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins (2004) Free Radic Biol Med 2004;37:755–767.
8 Mingatto FE, dos Santos AC, Rodrigues T, Pigoso AA, Uyemura SA, Curti C: Effect of nimesulide and its reduced metabolite on mitochondria. Br J Pharmacol 2000;131:1154–1160.
9 Yoshida Y, Singh I, Darby CP: Effect of salicylic acid and calcium on mitochondrial functions. Acta Neurol Scand 1992;85:191–196.
10 Matsui H, Nagano Y, Shimokawa O, Kaneko T, Rai K, Udo J, Hirayama A, Nakamura Y, Indo HP, Majima HJ, Hyodo I: Gastric acid induces mitochondrial superoxide production and lipid peroxidation in gastric epithelial cells. J Gastroenterol 2011;46:1167–1176.
11 Matsui H, Murata Y, Hirano K, Sasaki T, Shiba R, Muto H, Ohno T: Hydrogen peroxide-induced cellular injury is associated with increase in endogenous fluorescence from rat gastric mucosal epithelial cell culture: a new method for detecting oxidative cellular injury by fluorescence measurement. J Gastroenterol 1998;33:318–325.
12 Okimoto Y, Watanabe A, Niki E, Yamashita T, Noguchi N: A novel fluorescent probe diphenyl-1-pyrenylphosphine to follow lipid peroxidation in cell membranes. FEBS Lett 2000;474:137–140.
13 Ikeda R, Sugita T, Jacobson ES, Shinoda T: Effects of melanin upon susceptibility of *Cryptococcus* to antifungals. Microbiol Immunol 2003;47:271–277.
14 Zimmerman MC, Oberley LW, Flanagan SW: Mutant SOD1-induced neuronal toxicity is mediated by increased mitochondrial superoxide levels. J Neurochem 2007;102:609–618.
15 Schwarz K: Über penetrierende Magen- und Jejunalgeschwüre. Beitr Klin Chir 1910;67:96–128.
16 Sun DC, Shay H, et al: Optimal effective dose of anticholinergic drug in peptic ulcer therapy. Arch Intern Med 1956;97:442–452.
17 Vane JR: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 1971;231:232–235.
18 Carmichael HA, Nelson L, Chandra V, et al: Inhibition of aspirin and taurocholic acid-induced gastric mucosal bleeding by prostaglandin 15(R)-15-methyl-E_2 methyl ester. Gut 1975;16:822.
19 Adams SS, Cobb R: A possible basis for the anti-inflammatory activity of salicylates and other non-hormonal anti-rheumatic drugs. Nat Med 1958;15:773–774.
20 Rai K, Matsui H, Kaneko T, Nagano Y, Shimokawa O, Udo J, Hirayama A, Hyodo I, Indo HP, Majima HJ: Lansoprazole inhibits mitochondrial superoxide production and cellular lipid peroxidation induced by indomethacin in RGM1 cells. J Clin Biochem Nutr 2011;49:25–30.