Significance of ERK nitration in portal hypertensive gastropathy and its therapeutic implications

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Kinjo N, Kawanaka H, Akahoshi T, Yamaguchi S, Yoshida D, Anegawa G, Konishi K, Tomikawa M, Tanoue K, Tarnawski A, Hashizume M, Maehara Y. Significance of ERK nitration in portal hypertensive gastropathy and its therapeutic implications. Am J Physiol Gastrointest Liver Physiol 295: G1016–G1024, 2008. First published September 11, 2008; doi:10.1152/ajpgi.90329.2008.—Portal hypertensive (PHT) gastric mucosa increases susceptibility to injury and delayed mucosal healing. It is possible that nitration of ERK by peroxynitrite might alter MAPK (ERK) signaling in PHT gastric mucosa, leading to delayed mucosal healing, since excessive nitric oxide production is implicated in PHT gastric mucosa and MAPK (ERK) signaling induces cell proliferation and leads to gastric mucosal healing in response to injury. Portal hypertension was produced by staged portal vein ligation, and sham-operation (SO) rats served as controls. Lipid peroxide (LPO) and nitrotyrosine increased significantly in PHT gastric mucosa compared with SO rats. ERK activation was impaired in PHT gastric mucosa in response to ethanol injury, whereas no significant difference in the phosphorylation of MEK, an upstream molecule of ERK, was seen between the two groups. The nitration of ERK by peroxynitrite, as detected by the immunoprecipitation of ERK and nitrotyrosine, was significantly enhanced in PHT gastric mucosa. Administration of rebamipide, a gastroprotective drug that acts as an oxygen-derived free radical scavenger, significantly decreased LPO and nitrotyrosine as well as the nitration of ERK by peroxynitrite in PHT gastric mucosa, therefore normalizing ERK activation and restoring the gastric mucosal healing response to ethanol injury. Enhanced nitration of ERK by peroxynitrite is involved in the impaired MAPK (ERK) signaling in PHT gastric mucosa. These findings demonstrate a new molecular mechanism in which PHT gastric mucosa is predisposed to injury and impaired healing.

rebamipide; oxidative stress; MAP kinase

PORTAL HYPERTENSIVE GASTROPATHY (PHG) is a frequent and serious complication of liver cirrhosis. PHG is a potential cause of gastric hemorrhage in patients afflicted with liver cirrhosis (6, 22, 35). There is experimental evidence that PHG increases the susceptibility of the gastric mucosa to injury by noxious factors such as alcohol and NSAIDs and that it impairs the gastric mucosal healing response to such injury (10, 36–38). Excessive nitric oxide (NO) production via the upregulation of endothelial NO synthase (eNOS) in the endothelium (19, 28, 30) and the increased generation of reactive oxygen species (ROS) and lipid peroxide (LPO) (13, 14) have been implicated in these phenomena. However, a clear understanding of the molecular basis for these phenomena remains to be elucidated.

Accumulating evidence indicates that ROS are involved in the pathogenesis of gastrointestinal diseases (41, 44). ROS can cause extensive cellular damage through the modification of protein function, DNA damage, production of lipid peroxide by oxidation, and the nitration and nitrosation of biomolecules (2, 11, 12, 20). Peroxynitrite (ONOO−) is a powerful oxidant and cytotoxic species produced by the nearly diffusion-limited reaction between nitric oxide (NO•) and superoxide (O2••−). Peroxynitrite reacts with a diverse array of other biological target molecules, including the cysteine, tyrosine, methionine, and tryptophan residues of proteins in a posttranslational process (4, 11, 12, 32). Peroxynitrite-induced protein modification, especially the nitration of tyrosine, also contributes to the pathophysiology of a variety of diseases (4). Nitrination of the ortho position of tyrosine residues by peroxynitrite can modulate the signaling processes by inhibiting tyrosine phosphorylation (5, 8, 18).

MAP kinase plays a crucial role in protecting against cellular stress and in inducing cell proliferation or differentiation (27, 33). Extracellular signal-regulated kinases, ERK1 and -2 (isoforms of MAP kinase), play key roles in initiating the proliferation of gut epithelial cells (1, 7) and in the healing process of experimental gastric ulcers (29) and ethanol-induced gastric mucosal injury (15). In portal hypertensive (PHT) gastric mucosa, the activation of MAP kinase (ERK) by oxidative stress or ethanol injury is impaired, and this is associated with increased susceptibility to injury as well as impaired gastric mucosal healing (14–16). ERK is activated by the reversible phosphorylation of both tyrosine and threonine residues (9). Because the PHT gastric mucosa produces excessive NO and increased ROS, it is possible that the nitration of the tyrosine residues of ERK by peroxynitrite might therefore increase in the PHT gastric mucosa, thereby inhibiting ERK activation in response to injury and increasing the susceptibility to injury.

Supplementation with vitamin E has been shown to correct the defective MAP kinase signaling that arises in the PHT gastric mucosa, thereby affording protection against mucosal injury (14). Consequently, the use of antioxidants may be an effective pharmacological therapy for PHT gastropathy (13,

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14). This study therefore assessed rebamipide, an antiulcer drug used in East Asian countries that protects against oxygen-derived free radical production by scavenging hydroxyl radicals and inhibiting neutrophil activation or lipid peroxidation (24–26, 34).

The aims of this study were 1) to determine whether the nitration of tyrosine residues of ERK by peroxynitrite is involved in the gastric mucosa of PHT rats and 2) if so, whether the intragastric administration of rebamipide ameliorates the defective MAP kinase (ERK) signaling and improves the delayed mucosal healing in PHT gastric mucosa by decreasing oxidative stress and the nitration of tyrosine residues of ERK by peroxynitrite.

MATERIALS AND METHODS

Experimental design. This study was approved by the Subcommittee for Animal Studies of Kyushu University. One hundred forty-four Sprague-Dawley rats (6 wk old, weighing 220–250 g) were housed in temperature- and light-controlled environmental conditions with a 12-h light/dark cycle and fed standard rat chow and water as desired. Portal hypertension was produced by staged portal vein occlusion and splenic vein ligation in 72 rats anesthetized with ether as described by Sarfeh et al. (38). The rats were then allowed to move freely in their cages with access to chow and water. Seventy-two sham-operated (SO) rats (controls) underwent similar surgery without occlusion of the portal and splenic veins.

Fourteen days after the operation, 6 PHT and 6 SO rats were fasted for 24 h, anesthetized, and then underwent a laparotomy. Portal venous pressure (PVP) was measured before stomach excision to assure the reproducibility of the method of PHT induction. After laparotomy, a 26-gauge needle was introduced into a peripheral mesenteric vein tributary. With the vena cava as the reference point, the PVP was measured from the height of the column of saline within the tube. After measurement of the PVP, the stomachs were excised and gastric mucosal tissues were frozen in liquid N2 for subsequent experiments or fixed in 10% buffered formalin for histological examination.

Fourteen days after the operation, 18 PHT and 18 SO rats were administered 2 ml of 50% ethanol intragastrically after 24-h fasting. A laparotomy was performed under anesthesia 3, 6, and 24 h after ethanol injury, and the stomachs were excised. Areas of gross hemorrhagic necrosis were photographed in a standardized manner and splenic vein ligation in 72 rats anesthetized with ether as described by Sarfeh et al. (38). The rats were then allowed to move freely in their cages with access to chow and water. Seventy-two sham-operated (SO) rats (controls) underwent similar surgery without occlusion of the portal and splenic veins.

Administration of rebamipide. Forty-eight PHT rats and 48 SO rats were administered either rebamipide (30 mg/kg per day; Ohtsuka Pharmaceutical, Tokushima, Japan) or vehicle intragastrically each day for 13 days after surgery. After fasting for 24 h, the stomachs of 12 PHT and 12 SO rats were excised, and gastric tissues were frozen in liquid N2 for subsequent experiments or fixed in 10% buffered formalin for histological examination. After fasting for 24 h, the remaining 36 PHT and 36 SO rats were administered 2 ml of 50% ethanol intragastrically, and their stomachs were excised after 3, 6, and 24 h.

Measurement of LPO. As a measure of oxidative stress, LPO was quantified by measuring lipid hydroperoxides using the Lipid Hydroperoxide LPO assay kit (Cayman Chemical, Ann Arbor, MI) as described in a previous study (23).

Immunohistochemical study for nitrotyrosine and ERK 1/2. Immunohistochemical studies were performed on adjacent deparaffinized sections using the peroxidase-labeled streptavidin-biotin technique with the Histofine SAB-PO kit (Nichirei Biosciences, Tokyo, Japan). The primary antibody used in this study was a specific mouse monoclonal antibody against nitrotyrosine (Cayman Chemical) and a specific rabbit monoclonal antibody against ERK1/2 (Cell Signaling Technology, Beverly, MA). Histological sections (3 μm thick) of 10% formalin-fixed, paraffin-embedded tissues were cut, mounted on glass slides coated with 3,3-aminopropyltriethoxysilane, and air dried overnight at room temperature. The sections were removed from paraffin with xylene, and hydrogen peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxidase for 15 min. After the sections were exposed to 10% nonimmunized rabbit serum for 30 min,
they were incubated overnight at 4°C with primary antibodies diluted 1:100 for nitrotyrosine and for ERK1/2. The sections were subsequently incubated with a second stage biotinylated antibody for 20 min at room temperature. After rinsing in PBS, the reaction products were visualized by immersing the section in the chromogen diaminobenzidine tetrahydrochloride. No significant staining was observed in the negative controls, which were prepared using a mouse immunoglobulin at the same concentration. Finally, the sections were counterstained with hematoxylin, dehydrated, and coverslipped.

**Gastric epithelial cell proliferation (proliferating cell nuclear antigen-labeling index).** Immunohistochemical studies were performed in the same way as shown. The primary antibody used in this study was a specific mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (Dako Cytomation Denmark, Glostrup, Denmark). A PCNA-labeled cell was regarded as labeled if the nucleus was distinctly stained brown. PCNA-labeled cells and the total number of cells in standardized rectangles were counted at 200× magnification. The PCNA-labeling index was expressed as the percentage of labeled cells per all counted cells. Measurements were made on coded slides in 10 randomly chosen areas of the fundic mucosa by two blinded investigators.

**Western blot analysis for ERK1/2 and MEK 1/2 and phosphorylation of ERK1/2 and MEK 1/2.** The tissue specimens were lysed in a lysis buffer containing 20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 1% Nonidet P-40, 50 mmol/l sodium fluoride, protease inhibitor cocktail (Sigma P8340; Sigma-Aldrich, St Louis, MO) and phosphatase inhibitor cocktail (Sigma P5726, Sigma-Aldrich). The protein concentration of the lysates was determined by a proteinassay-protein quantification kit (Daijindo Molecular Technologies, Gaithersburg, MD). Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The signals were detected with specific antibodies [rabbit monoclonal anti-phosphorylated p44/p42 MAP kinase, rabbit monoclonal anti-p44/p42 MAP kinase, rabbit monoclonal anti-MEK, and rabbit monoclonal anti-phosphorylated MEK (Cell Signaling Technology)] and visualized by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). The epitope of the phosphorylated p44/p42 antibody we used in this study was anti-phospho tyrosine (204) and anti-phospho threonine (202). Quantification of the data was performed with a video image analysis system (LAS 3000 Imaging System; Fuji Photo Film, Tokyo, Japan).

**ERK1/2 kinase activity assay.** ERK1/2 kinase activity was determined using the p44/p42 MAPK assay kit (Cell Signaling Technology). Active ERK1/2 was immunoprecipitated from tissue lysate (100 μg) using immobilized phospho-p44/p42 MAPK monoclonal antibody (Cell Signaling Technology). The conjugates were then pelleted by centrifugation and washed 4 times. Kinase reactions were performed in 40 μl of MAP kinase assay cocktail consisting of 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, 10 mM MgCl2, 200 μM ATP, and 1 μg Elk-1 fusion protein at 30°C for 30 min. The reaction was terminated by the addition of 3× SDS sample buffer. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. After being blocked with 5% milk for 1 h, the membranes were immersed with...
rabbit polyclonal phospho-Elk-1 antibody (Cell Signaling Technology) at 4°C overnight and HGP-conjugated secondary antibody (Amersham Life Science) for 1 h and then visualized by enhanced chemiluminescence. Quantification of the data was performed using a video image analysis system.

**Determination of nitration of ERK1/2.** The 20-μg tissue lysate of the gastric mucosa was immunoprecipitated using 5 μl of rabbit polyclonal ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 40 μl of protein G Sepharose 4 fast flow (GE Healthcare Bio-Sciences, Uppsala, Sweden). After gently rocking the reaction mixture for 2 h at 4°C, we collected the conjugates in a microcentrifuge and washed them in ice-cold PBS 5 times. The conjugates were resuspended in 2× SDS sample buffer and boiled for 5 min. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. After being blocked with 3% milk for one h, the membranes were immersed with mouse monoclonal nitrotyrosine antibody (Cayman Chemical) or rabbit monoclonal ERK2 antibody (Cell Signaling Technology) at 4°C overnight and horseradish peroxidase conjugated with secondary antibody for 1 h and visualized by enhanced chemiluminescence. Quantification of the data was performed with a video image analysis system.

**Statistical analysis.** All data are reported as the means ± SE. The Student’s t-test was used to determine the statistical significance between SO and PHT rats. Comparisons of data among multiple groups were made with an ANOVA followed by Bonferroni correction. A P value of <0.05 was considered to be statistically significant.

**RESULTS**

**PVP.** All PHT rats showed the characteristic features of splanchnic venous congestion with dilatation of mesenteric veins and thickening of the mesentery. The PVP in the PHT rats was significantly higher than in SO rats (24.9 ± 2.0 cmH2O in PHT vs. 14.6 ± 0.8 cmH2O in SO, P < 0.0001).

**Increased oxidative stress and nitrotyrosine in PHT gastric mucosa.** To evaluate oxidative state in PHT gastric mucosa, LPO was measured in the gastric mucosa as a marker for oxidative stress. The LPO content of the gastric mucosa significantly increased in the PHT rats compared with SO rats (Fig. 1A).

To determine whether protein tyrosine nitration by peroxynitrite is involved in PHT gastric mucosa, Western blot analysis and immunohistochemical analysis were conducted for nitrotyrosine-containing proteins as an indication of the nitration of protein tyrosine residues by peroxynitrite. Western blot analysis showed that nitrotyrosine-containing proteins were significantly increased in PHT gastric mucosa compared with SO rats (Fig. 1B). Immunohistochemical analysis also showed that nitrotyrosine-containing proteins were localized predominantly to the cytoplasm of the glandular base of fundic glands in both SO and PHT gastric mucosa, but the signal intensity was stronger in the PHT gastric mucosa (Fig. 1C).

**Increased susceptibility to ethanol injury and delayed mucosal healing of PHT gastric mucosa attributable to impaired ERK1/2 activation.** The gastric ulcer index significantly increased in the gastric mucosa of the PHT rats compared with the SO rats by 157% (P < 0.05) and 227% (P < 0.05), at 6 and 24 h, respectively, after ethanol injury (Fig. 2A). The phosphorylation level of ERK1/2 (phosphorylated/total protein ratio) and the activity of ERK1, as indicated by phosphorylated Elk, were more increased in the PHT rats than the SO rats at 0 h (baseline) but not significant (Fig. 2, B and C). Following ethanol injury, the phosphorylation level and activity of ERK1/2 were significantly increased in the SO rats only. The phosphorylation level was increased by 83% and 179% and the activity was increased by 109% and 127%, at 6 and 24 h, respectively, compared with the noninjured gastric mucosa (baseline) (Fig. 2, B and C). In contrast, the phosphorylation level and activity of ERK1/2 in the PHT gastric mucosa were not significantly increased from baseline. In fact, both the phosphorylation level and activity of ERK1/2 were significantly decreased in the PHT rats compared with the SO rats; the phosphorylation level was decreased by 48% and 42% and activity was decreased by 38% and 32%, at 6 and 24 h, respectively. These results indicated that impaired ERK activation in response to injury might lead to increased susceptibility to damage and delayed healing after injury in PHT gastric mucosa, which is consistent with previous studies (14, 15).

**No difference in the activation of MEK1/2 following alcohol injury between SO and PHT gastric mucosa.** To determine whether alteration of upstream participants of ERK is involved in the impaired ERK activation in the PHT gastric mucosa, the phosphorylation level of MEK1/2 (phosphorylated/total protein ratio) following alcohol injury in PHT and SO gastric mucosa was examined. The phosphorylation level of MEK1/2 increased significantly over baseline in both the PHT and SO rats at 6 and 24 h after ethanol injury, and there was no significant difference between the two groups (Fig. 2D).

![Image](http://ajpgi.physiology.org/)

**Fig. 3.** Increased nitration of tyrosine residues of ERK1/2 in PHT gastric mucosa. A: gastric tissue lysates obtained from PHT and SO gastric mucosa were immunoprecipitated (IP) with anti-ERK2 antibody. The immunoprecipitates were probed with anti-nitrotyrosine antibody and subsequently reprobed with anti-ERK antibody (42 kDa). The ERK immunoprecipitates in the gastric mucosa of the PHT rats had higher levels of nitrotyrosine than the SO rats. B: colocalization of nitrotyrosine and ERK1/2 protein expression in the PHT gastric mucosa. Both ERK1/2 (left) and nitrotyrosine (right) protein expression were localized predominantly in the cytoplasm of the glandular cells in the same site in the PHT gastric mucosa.

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Increased nitration of tyrosine residues of ERK1/2 in PHT gastric mucosa. Nitration of tyrosine residues by peroxynitrite can modulate the signaling processes by inhibiting tyrosine phosphorylation. To determine whether the nitration of tyrosine residues of ERK by peroxynitrite is involved in the PHT gastric mucosa, the coimmunoprecipitation of nitrotyrosine and ERK in the gastric mucosa was examined. Nitrotyrosine was detected in the immunoprecipitates of ERK1/2, indicating the direct interaction between ERK1/2 and nitrotyrosine in both the PHT and SO gastric mucosa (Fig. 3A). In addition, nitration of ERK1/2, as indicated by the direct interaction between ERK1/2 and nitrotyrosine, was significantly enhanced in PHT gastric mucosa compared with SO rats. An immunohistochemical analysis also showed colocalization of ERK1/2 and nitrotyrosine in the glandular base of the fundic glands in the PHT gastric mucosa (Fig. 3B).

Rebamipide (Mucosta) normalized LPO and nitration of ERK1/2 in PHT gastric mucosa. The use of an antioxidant may be an effective pharmacological therapy for PHT gastropathy (13, 14). In this study, rebamipide (Mucosta; Ohtsuka Pharmaceutical, Tokushima, Japan), which is an antiulcer drug that acts as a free radical scavenger, was used. Intragastric administration of rebamipide significantly decreased both the LPO and nitrotyrosine in the PHT gastric mucosa (Fig. 4, A and B). Rebamipide also normalized the nitration of ERK1/2 in the PHT gastric mucosa (Fig. 4C). As observed in a previous study (31), gastric epithelial proliferation in the noninjured gastric mucosa of the PHT rats, as indicated by the PCNA labeling index, was significantly diminished in PHT rats treated with rebamipide compared with those treated with vehicle. As observed in a previous study (31), gastric epithelial proliferation in the noninjured gastric mucosa of the PHT rats, as indicated by the PCNA labeling index, was significantly diminished in PHT rats treated with rebamipide compared with those treated with vehicle.

Fig. 4. Normalization of LPO and nitration of tyrosine residues of ERK1/2 in PHT gastric mucosa. Gastric tissues were obtained from PHT and SO rats treated either with rebamipide (Reb; 30 mg/kg per day, intragastrically daily) or with vehicle (Veh) for 13 days after the operation. A: as a measure of oxidative stress, LPO was quantified by measuring lipid hydroperoxide with a LPO assay kit. B: nitrotyrosine was determined by immunohistochemical staining with anti-nitrotyrosine antibody. Nitrotyrosine was localized predominantly in the cytoplasm of the glandular cells in both the SO and PHT gastric mucosa treated either with rebamipide or with vehicle, but the signal intensity was weaker in the PHT rats treated with rebamipide than in those treated with vehicle. C: gastric tissue lysates obtained from PHT and SO gastric mucosa treated either with rebamipide or with vehicle were immunoprecipitated with anti-ERK2 antibody. The immunoprecipitates were probed with anti-nitrotyrosine antibody and subsequently reprobed with anti-ERK antibody. Quantitative data of nitrotyrosine in immunoprecipitates of ERK were obtained using a video image analysis system. Values are expressed as intensity units and represent the means ± SE (n = 6 animals per group for each time point). D: localization of proliferating cell nuclear antigen (PCNA) in PHT and SO gastric mucosa either with rebamipide or with vehicle at baseline. E: PCNA labeling index (LI) was expressed as the percentage of labeled cells per all counted cells. #P < 0.05.
index, decreased significantly compared with SO rats, whereas rebamipide reversed the PCNA labeling index in the PHT gastric mucosa to the normal levels of the SO rats (Fig. 4, D and E).

Rebamipide (Mucosta) improved the susceptibility to ethanol injury and delayed mucosal healing of PHT gastric mucosa by reversal of the impaired ERK1/2 activation. The effects of rebamipide on gastric mucosal injury in response to ethanol were examined. The PHT gastric mucosa treated with vehicle had a higher gastric ulcer index at 6 and 24 h after ethanol injury compared with the SO rats treated with vehicle, whereas rebamipide administration reduced the extent of ethanol-induced gastric mucosal injury in the PHT rats to the same level found in SO rats (Fig. 5A).

Next, the effects of rebamipide on ERK signaling and gastric epithelial proliferation were examined following ethanol injury. In the PHT gastric mucosa, rebamipide treatment significantly restored the phosphorylation and activity of ERK1/2 at 6 h after ethanol injury to the level found in the SO rats (Fig. 5, B and C). In the PHT gastric mucosa, rebamipide significantly increased the PCNA labeling index at 6 h after ethanol injury to the level found in the SO rats (Fig. 5D). In the SO gastric mucosa, rebamipide treatment did not significantly affect either the ERK activation or gastric epithelial proliferation following ethanol injury.

DISCUSSION

The present study demonstrated that 1) LPO, nitrotyrosine, and the nitration of ERK were all significantly increased in the PHT gastric mucosa; 2) ERK activation and gastric epithelial proliferation in response to ethanol injury were impaired in the PHT gastric mucosa; 3) these impairments were likely to be associated with the increased nitration of ERK; and 4) rebamipide, a gastroprotective drug that acts as an oxygen-derived free radical scavenger, normalized LPO, nitrotyrosine, and the nitration of ERK ameliorated ERK activation and epithelial proliferation in response to ethanol injury and thereby completely reversed the increased susceptibility to ethanol injury and the delayed mucosal healing of the PHT gastric mucosa.

Accumulating evidence has implicated increased oxygen free radicals and LPO in the pathogenesis of PHT gastric
mucosa (13, 14). The present study showed that not only LPO but also nitrotyrosine increased significantly in the PHT gastric mucosa and that nitrotyrosine was predominantly localized in the cytoplasm of the granular base of the gastric mucosa. These results are supported by the findings of our previous study showing that the PHT gastric mucosa contains excessive levels of NO, and its regulating enzyme, eNOS, has been shown to be localized in the mucosal and submucosal veins of the PHT gastric mucosa (28).

Consistent with our previous studies, the present study has shown that ERK1/2 activity was slightly increased in the PHT rats compared with the SO rats at the baseline (noninjured gastric mucosa), whereas the increase in ERK1/2 activity could not lead to proliferation of epithelial cells in the PHT gastric mucosa (15, 16). The slight ERK1/2 activation is likely to be due to underlying oxidative stress in the PHT gastric mucosa (15, 16). Our previous study showed the negative regulation of MAP kinase due to an overexpression of MKP-1 to be one of the molecular mechanisms for the impairment of ERK activation in the noninjured PHT gastric mucosa. It has been shown that peroxynitrite nitrates tyrosine residues of various enzymes and interferes with their phosphorylation, thereby inactivating these enzymes (5, 18, 21). Since ERK1/2 is activated by the phosphorylation of both tyrosine and threonine residues and is inactivated by the dephosphorylation of either tyrosine or threonine residues, tyrosine nitration by peroxynitrite could dephosphorylate ERK1/2 and thereby inactivate it. In addition to the overexpression of MKP-1, the increased nitration of ERK might diminish the ERK activation due to the underlying oxidative stress including nitrotyrosine, finally leading to the impaired proliferation of epithelial cells in the noninjured PHT gastric mucosa in this study (Fig. 6).

There could be many factors responsible for the attenuation of ERK activation in response to injury in PHT gastric mucosa. In the present study, we also showed that MEK activation in response to injury was similar both in PHT and SO gastric mucosa, thus indicating that the reduced ERK activation in the PHT gastric mucosa is due to inhibited activation of ERK itself, not upstream activators, involving receptor tyrosine kinase and Ras-dependent or -independent Raf activation. Furthermore, we showed that tyrosine nitration of ERK1/2 was significantly enhanced in PHT gastric mucosa. In fact, tyrosine nitration of ERKs has been shown to negatively regulate ERK activation in fibroblasts and kidney cells of β-sickle cell mice (17, 31). Therefore, the increased tyrosine nitration of ERK in PHT gastric mucosa is likely to prevent the activation of ERK in response to injury and thereby to impair gastric epithelial proliferation in PHT gastric mucosa (Fig. 6). This is confirmed by our finding that rebamipide administration ameliorated ERK activation following ethanol injury in PHT gastric mucosa and that this amelioration coincided with the normalization of nitrotyrosine and ERK nitration.

There could be two mechanisms for the possible effects of peroxynitrite on the ERK signaling pathway. First, a number of studies have shown that peroxynitrite, like various stressful stimuli such as oxidative stress and UV radiation, activates ERK in numerous cell types including rat liver epithelial cells (39), rat fibroblasts (3), mouse endothelial cells (40), and human neutrophils (45). The activation of ERK by peroxynitrite has been shown to be mediated via the EGF receptor and downstream targets (45). The second possible mechanism is that exposure of cells to peroxynitrite could directly affect ERK activation by tyrosine nitration of ERK (17, 31). How do these two mechanisms contribute to the modulation of the ERK signaling pathway by peroxynitrite? The activation of ERK has been shown to be rapid and to peak immediately or 15 min after exposure of cells to peroxynitrite, returning to a basal level 30–60 min after the exposure (3, 39, 40, 45). Epicathechin, a selective inhibitor of tyrosine nitration by peroxynitrite, did not diminish the immediate activation of ERK by peroxynitrite in murine aortic endothelial cells, indicating that the peroxynitrite-induced ERK activation is not due to tyrosine nitration of ERK (40). In addition, the exposure of fibroblasts to peroxynitrite for 1 h decreased ERK activity, and this inactivation of ERK by peroxynitrite was rescued by flavanone hesperetin, which can reduce tyrosine nitration by peroxynitrite.
(31). This suggests that induction of the tyrosine nitration of ERK following exposure of cells to peroxynitrite may be too late to inactivate the immediate activation of ERK. Therefore, in PHT gastric mucosa and kidneys of β-sickle cell mice with increased expression of peroxynitrite, preexisting, but not newly induced, tyrosine nitration of ERK might be able to dephosphorylate and consequently inactivate ERK (Fig. 6).

Rebamipide, a gastroprotective drug, is clinically used in East Asian countries for the treatment of peptic ulcer diseases. It accelerates gastric ulcer healing and reduces the rate of ulcer recurrence (24, 26). These protective effects of rebamipide are thought to be mediated by the production of prostaglandin after the induction of cyclooxygenase, the induction of growth factors such as hepatocyte growth factor, vascular endothelial growth factor, and fibroblast growth factor, and its antioxidant properties, which involve the scavenging of hydroxyl radicals and the suppression of superoxide production by activated neutrophils (25, 34, 42, 43). In addition, the present experiments showed that rebamipide administration reduced the levels of LPO, nitrotyrosine, and the tyrosine nitration of ERK in PHT gastric mucosa to normal. The various protective effects of rebamipide may contribute to the improvement of the delayed mucosal healing response to ethanol injury in PHT gastric mucosa, but the protective action of rebamipide was much more pronounced in PHT gastric mucosa than in SO gastric mucosa (Fig. 5A). Although it would be difficult to discriminate the inhibitory effects of rebamipide on tyrosine nitration of ERK from its other antioxidant properties, the normalization of oxidative stress including the tyrosine nitration of ERK by rebamipide would thus induce a protective effect by ameliorating the ERK activation in the healing process of PHT gastric mucosa and be a new insight into therapeutic strategies for PHT gastropathy.

In conclusion, nitration of tyrosine residues of ERK is likely to be involved in the defective MAP kinase (ERK) signaling that results in the increased susceptibility to damage and the delayed mucosal healing of PHT gastropathy. Rebamipide normalizes the oxidative state and tyrosine nitration of ERK in PHT gastric mucosa, thereby completely reversing the impaired mucosal healing. These findings provide a novel molecular mechanism and scientific basis for PHT gastropathy. The use of rebamipide to correct the MAP kinase signaling abnormalities warrants further clinical trials to elucidate its efficacy in the treatment of PHT gastropathy.

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