Inhibition of p38 MAPK activation attenuates esophageal mucosal damage in a chronic model of reflux esophagitis

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Key Messages
• This study was designed to investigate the role of p38 MAPK in experimental chronic acid RE model of rats.
• Chronic acid RE rats were induced by fundus ligation and partial obstruction of the pylorus and treated with SB203580 (a p38 MAPK inhibitor, i.p., 1 mg/kg/day) for 14 days.
• Inhibition of p38 MAPK activation attenuated esophageal mucosal damage in chronic acid RE rats, possibly by modulating esophageal barrier function and regulating inflammatory cells recruitment, and the subsequent formation of cytokines, NO, and reactive oxygen species. These studies suggest that p38 MAPK play an important role in RE and inhibition of p38 MAPK activation may be a promising therapeutic strategy to attenuate esophageal mucosal damage in patients who suffered from RE.

Abstract
Background Reflux esophagitis (RE) is one of the common gastrointestinal diseases that are increasingly recognized as a significant health problem. This study was designed to investigate the role of p38 mitogen-activated protein kinase (MAPK) in experimental chronic RE model of rats. Methods Chronic acid RE rats were induced by fundus ligation and partial obstruction of the pylorus and treated with SB203580 (a p38 MAPK inhibitor, i.p., 1 mg/kg/day) for 14 days. Key Results Immunohistochemical staining and Western blotting results revealed the activation of p38 MAPK signaling in the esophagus mucosa 14 days post injury. Through gross and histological assessment, we found that inhibition of p38 MAPK activation by SB203580 attenuated esophageal mucosal damage in RE rats. Inhibition of p38 MAPK activation in RE rats attenuated esophageal barrier dysfunction, through enhancing the expression of tight junction proteins and reducing the expression of matrix metalloproteinases-3 and -9. Inhibition of p38 MAPK activation in RE rats reduced CD68-positive cells in esophagus mucosa and mRNA levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β in esophagus and protein levels of TNF-α, IL-6, and IL-1β in serum. In addition, we found that inhibition of p38 MAPK activation in RE rats suppressed protein expression of inducible nitric oxide synthase and reduced formation of nitric oxide (NO), 3-nitrotyrosin, and malondialdehyde in esophagus. Conclusions & Inferences Inhibition of p38 MAPK activation attenuated esophageal mucosal damage in acid RE rats, possibly by modulating esophageal barrier function and regulating inflammatory cell recruitment, and the subsequent formation of cytokines, NO, and reactive oxygen species.

Keywords acid reflux esophagitis, inflammation, p38 MAPK, rats.

INTRODUCTION
Reflux esophagitis (RE) is one of the common gastrointestinal diseases that are increasingly recognized as a
significant health problem with extensive global, social, and economic impact.\textsuperscript{1,2} Reflux esophagitis is an inflammation of the lower esophagus due to the regurgitation of gastric acid, characterized by a burning pain in the chest (so-called heartburn) and nausea following eating.\textsuperscript{3} As gastric acid plays a key role in the pathogenesis of RE, luminal pH control, including histamine type 2 antagonists and proton pump inhibitors, is considered of importance in the treatment of this disease.\textsuperscript{4,5} However, in spite of their marked therapeutic effect, a number of patients have suffered from incidences of relapse and shown incomplete mucosal healing, continued symptoms, and complications.\textsuperscript{6–8} Therefore, the exact pathophysiological mechanisms of esophageal mucosal damage during gastroesophageal reflux are not fully explained by acid reflux alone.

Differences in mitogen-activated protein kinase (MAPK) pathways activated by acid exposure were found in esophageal squamous cell lines and biopsies from patients with gastro-esophageal reflux disease (GERD) with and without Barrett’s esophagus.\textsuperscript{9} The MAPK signaling cascade comprises three principal components including the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase. The p38 MAPK pathway is activated in response to cell stress (e.g., ionizing irradiation, chemotherapeutic drugs, or osmotic stress) and inflammatory insults (e.g., Toll-like receptor ligands or cytokines).\textsuperscript{10} It was found that acidic deoxycholic acid, chenodeoxycholic acid, and bile acids induced the activation of p38 MAPK in human esophageal epithelial cells.\textsuperscript{11,12} The esophagoduodenostomy (a model of duodenoesophageal reflux) in rats led to the activation of p38 MAPK in esophageal samples.\textsuperscript{13} Recently, it was found that the activity of p38 MAPK was higher in the esophageal epithelium of patients with Barrett’s esophagus than that in normal esophageal mucosa.\textsuperscript{14}

This study was designed to investigate the role of p38 MAPK in experimental chronic RE (gastro-esophageal reflux/acid reflux), induced by fundus ligation and partial obstruction of the pylorus in rats.

**MATERIALS AND METHODS**

**Animals**

Male Sprague–Dawley (SD, 200–250 g) rats were purchased from the Sino-British SIPPR/BK Lab Animal Ltd (Shanghai, China). All the rats were housed in a controlled environment at 22 ± 2 °C with a 12-h light/dark cycle, and free access to food and tap water. All the antibodies used in this work were purchased from Santa Cruz Ltd (Santa Cruz, CA, USA). All the surgical and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals (NIH, revised 1996), and approval was granted by the university ethics review board.

**Chronic RE model and study design**

Sprague–Dawley rats were fasted overnight, and injected with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p) for anesthesia. The chronic RE model (gastro-esophageal reflux/acid reflux) was developed by following the methods described previously.\textsuperscript{15,16} The abdomen was opened by giving a 3–4-cm-long midline incision and a latex ring (2 mm in thickness; OD, 8–9 mm; ID, 5–6 mm, made from 18-Fr Nalaton catheter, Wincare, Yangzhou, Jiangsu, China) was placed around the pyloric sphincter so as to restrict the emptying of gastric contents. The transitional region (i.e., limiting ridge) between the fundus and the glandular portion of the stomach was ligated with 2–0 silk thread in order to restrict the compliance of the stomach, which led to the reflux of gastric contents into the esophagus. Rats were injected with Enrofloxacin (antibiotic, 2.5 mg/kg, subcutaneously) and Rimadyl (analgesic, 5 mg/kg, subcutaneously) to prevent infection and relieve pain, respectively. After surgery, the rats fasted for a further 48 h with free access of water. The rats that underwent sham operations served as the control group.

Rats were randomly divided into three groups as follows: sham-operated rats, RE rats, and RE rats treated with SB203580. SB203580 (4-[4-fluorophenyl]-2-[4-methylsulfinyl]-5-[4-pyridyl]-1H-imidazole) inhibits selectively an activation of p38 MAPK, but not JNK and p48 MAPK. The SB203580-treated group rats were then treated with SB203580 via intraperitoneal injection (1 mg/kg/day).\textsuperscript{17} An equal volume of saline was injected as a vehicle control in sham-operated rats and RE rats. Fourteen days later, the esophagus tissues were collected for histological analysis and biochemical detection. The total number of each group was 41–43. Around 10–11 samples were used for histology assessment; 10–11 samples were used for real-time quantitative polymerase chain reaction (RT-PCR); 10–11 samples were used for the assay of transepithelial electrical resistance; 10–11 samples were used for Western blotting and determination of NOx, 3-NT, and measurement of malondialdehyde (MDA).

**Gastric content pH**

The gastric contents were obtained from the stomach and centrifuged at 3000 g for 20 min. The supernatant was collected for assessment of pH (Toledo 320; Mettler, Schwerzenbach, Switzerland).

**Histology**

The rats were killed, the gross findings of the esophageal mucosa were observed. The criteria for macroscopic scoring included the presence of hyperamia, edema, erosions, ulcers, and intramural or intraluminal hemorrhage. After creating 4-μm-thick paraffin sections, hematoxylin and cosin staining was performed for histological evaluation under an optical microscope.

**Immunohistochemical assay**

Immunohistochemical methods were used to measure the protein expression levels of p38 MAPK, p-p38 MAPK, CD68, matrix
metalloproteinase 3 (MMP3), and MMP9. For these assays, the middle-lower esophagus was obtained and fixed in 10% buffered-formalin, shaped into tissue masses, dehydrated with gradient ethanol, clarified with xylene, embedded in paraffin, and sectioned into slices of 4 μm, which were then dewaxed with xylene, debenzolized with anhydrous ethanol and dehydrated with gradient ethanol, and then incubated with H2O2 (3.0%) and rinsed with water. After being restored at high pressure and high temperature, blocking agent (10% normal goat serum) was added to dissolve the slices under room temperature. After 30 min, anti-p38 MAPK, p-p38 MAPK, CD68, MMP3, or MMP9 antibody was added. The slices were incubated with primary antibody overnight at 4 °C and washed with phosphate-buffered saline (PBS) for three times. Secondary antibody was added, and the slices were incubated for 30 min under room temperature. The slices were washed with PBS four times. Streptavidin-peroxidase was added and incubated at 37 °C for 30 min. The slices were then washed again with PBS for three times. The slices were stained with 3,3-Diaminobenzidine for 5 min, rinsed with water, and then stained with hematoxylin for another 5 min. These slices of mucosa of RE were observed under the microscope, and analyzed by pathologists blinded to the treatments.

Western blotting analysis

Rats were sacrificed and the esophageal tissues were dissected. Esophageal tissues were homogenated with RIPA buffer and then centrifuged at 20,913 g for 15 min at 4 °C. Protein samples were separated on a 12% SDS-polyacrylamide gel, and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 0.5% non-fat dry milk, the membrane was probed with primary antibodies overnight at 4 °C. The following antibodies were used in this study: p38 MAPK, phospho-p38 MAPK, and inducible nitric oxide synthase (iNOS). The membrane was treated with horseradish peroxidase-conjugated secondary antibody for 1 h at 37 °C. The Electro-Chemi-Luminescence (ECL) chemiluminescent detection system (Amersham Biosciences, San Francisco, CA, USA) was used for detection.

Assay of transepithelial electrical resistance

For chamber studies, esophageal epithelial tissues were removed and immersed in ice-cold oxygenated Ringer solution for mounting mucosal-side-up in mini-Using chambers (model MC6; Physiologic Instruments, San Diego, CA, USA), which permitted direct recording of the transmural electrical potential difference (PD) and determination of short-circuit current (Isc) by passage of current. Total electrical resistance (Rt) was calculated using Ohm’s law (Rt = PD/Isc). After equilibration for half an hour, basal electrical readings of PD and Isc were recorded every 15 min for 2 h.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from esophageal tissues using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. Reactions were performed in a real-time PCR thermocycler (iQ5, Bio-Rad, Hercules, CA, USA) using SYBR green as the fluorescence dye. Expression values were calculated relative to the housekeeping control gene, GAPDH, using the comparative threshold cycle (2−ΔΔCt) method. Real-time RT-PCR was performed to analyze the mRNA expression for occludin, zonula occludens-1 (ZO-1), TNF-x, IL-6, and IL-1β. The primers were shown in Table 1.

Measurement of TNF-x, IL-6, and IL-1β in serum

Blood was collected and serum sample was obtained by centrifugation at 220 g for 15 min. The TNF-x, IL-6, and IL-1β levels in serum were determined by using ELISA kits (R&D Systems, Shanghai, China).

Determination of nitrite/nitrate (NOx) content

The levels of NOx, the stable end products of NO, in esophageal tissues were measured by using a Total Nitrite/Nitrate Assay kit (Bevotyme Biotechnology, Shanghai, China) which employed the Griess method.

Measurement of 3-nitrotyrosine (3-NT)

Esophageal tissues were homogenized in sterile phosphate-buffered saline containing protease inhibitors and centrifuged at 12,000 g for 10 min at 4 °C, and supernatant was collected. The levels of 3-NT in esophageal tissues were determined by using ELISA kits for rats (Northwest Life Science Specialties, Vancouver, WA, USA).

Measurement of malondialdehyde

Tissue homogenates were used for the determination of MDA using a kit (Cayman, Ann Arbor, MI, USA). Esophageal tissues were homogenized in sterile PBS containing protease inhibitors and centrifuged at 12,000 g for 10 min at 4 °C, and supernatant was collected for the measurement of MDA content.

<table>
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<tr>
<th>Table 1 Sequences of oligonucleotides used as primers</th>
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<td>Target gene</td>
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<td>IL-1β</td>
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ZO-1, zona occluden-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Statistical analysis

All the data are presented as mean ± SD. One-way ANOVA with Bonferroni’s correction for multiple comparisons were applied and statistical difference was considered with \( p < 0.05 \). \( p < 0.05 \) was considered statistically significant. Statistical analysis was performed using SPSS 11.0.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Activation of p38 MAPK in RE rats

To validate p38 MAPK activation in the esophagus of RE rats, we examined expression of p38 MAPK and phosphorylated p38 MAPK by immunohistochemical staining and Western blotting analysis. Fourteen days after the operation for RE, the ratio of p-p38 MAPK/p38 MAPK was higher in the RE model group than that in the sham-operated group (Fig. 1, \( p < 0.001 \)), which indicates the activation of p38 MAPK in the esophagus of RE rats. Treatment with p38 MAPK inhibitor, SB203580, reduced phosphorylation of p38 MAPK \( (p < 0.001) \) in the esophagus of RE rats.

Inhibition of p38 MAPK activation attenuated esophageal mucosal damage in RE rats

Firstly, the gastric content pH was lower \( (p < 0.001) \) in RE rats when compared to the sham-operated rats, and treatment with SB203580 did not affect the gastric content pH in RE rats [Fig. 2A]. As shown in Fig. 2B, rats in the RE model group had esophageal mucosal conges-

dition and erosion on gross examination, while there were no visible changes in sham-operated group. However, the RE group treated with SB203580 (inhibitor of p38 MAPK) had less damage than the RE group.

As shown in Fig. 2C, the sham-operated esophagus exhibited a thin epithelial layer with squamous cells and few inflammatory cells in the submucosal layer, while the RE esophagus exhibited basal layer thickening, vascular congestion, and eosinophil infiltration. However, the RE group treated with SB203580 had less damage than the RE group.

Inhibition of p38 MAPK activation attenuated esophageal barrier dysfunction in RE rats

A significant decrease in transepithelial electrical resistance (TER; Fig. 3A, \( p < 0.001 \)) was observed in the esophageal epithelium in the RE group at 14 days after surgery. Furthermore, we found that mRNA levels of occludin (Fig. 3B, \( p < 0.001 \)) and ZO-1 [Fig. 3C, \( p < 0.001 \)], two marker proteins of tight junction, were lower in the RE group than that in the sham-operated group. Treatment of the RE group with SB203580 enhanced mRNA levels of occludin \( (p < 0.001) \) and ZO-1 \( (p < 0.001) \) in the esophagus, and increased TER \( (p = 0.003) \), which indicate that inhibition of p38 MAPK activation up-regulated...
mRNA expression of tight junction proteins and attenuated esophageal barrier dysfunction in RE rats.

Inhibition of p38 MAPK activation suppressed MMP expression in RE rats

Immunohistochemical staining (Fig. 4) result revealed that a significant increase in MMP-3-positive and MMP-9-positive cells was observed in the esophageal epithelium, which was reversed by treatment with SB203580.

Inhibition of p38 MAPK activation suppressed inflammation in RE rats

Immunohistochemical staining (Fig. 5A) result revealed that a significant increase in CD68-positive cells was observed in the esophagus mucosa, which indicated that more inflammatory cells infiltrated into the esophageal mucosa. In addition, we found that mRNA levels of TNF-α (Fig. 5B, p < 0.001), IL-6 (Fig. 5C, p < 0.001), and IL-1β (Fig. 5D, p < 0.001) in the esophagus and protein levels of TNF-α (Fig. 5E, p < 0.001), IL-6 (Fig. 5F, p < 0.001), and IL-1β (Fig. 5G, p < 0.001) in serum were higher in the RE group than that in the sham-operated group. Treatment of the RE group with SB203580 reduced CD68-positive cells in the esophagus mucosa and mRNA levels of TNF-α (p < 0.001), IL-6 (p < 0.001), and IL-1β (p < 0.001) in the esophagus and protein levels of TNF-α (p = 0.006), IL-6 (p = 0.002), and IL-1β (p < 0.001) in serum.

Inhibition of p38 MAPK activation suppressed iNOS/NO in RE rats

The protein expression of iNOS (Fig. 6A, p < 0.001), contents of NOx (Fig. 6B, p < 0.001), and 3-NT (Fig. 6C, p < 0.001) levels were higher in the esophagus of the RE group than that in the sham-operated group. Treatment of the RE group with SB203580 suppressed protein expression of iNOS (p < 0.001) and reduced the levels of NOx (p = 0.009) and 3-NT (p < 0.001) in esophagus.
Inhibition of p38 MAPK activation abated oxidative stress in RE rats

Measurement of malondialdehyde concentration is a presumptive marker of oxidant-mediated lipid peroxidation. When compared to sham-operated rats, MDA levels were higher in the esophagus of RE rats (Fig. 6D, \( p < 0.001 \)). Treatment of the RE group with SB203580 reduced MDA levels (\( p = 0.003 \)) in the esophagus, indicating that the inhibition of p38 MAPK activation abated oxidative stress in RE rats.

DISCUSSION

As RE is a multifactorial disease, it would be plausible to use multitarget drugs for its treatment, and the objective of its treatment should be to restore the...
balance between aggressive and mucosal protecting factors, rather than simple acid suppression. In this study, we found that the inhibition of p38 MAPK by SB203580 attenuated esophageal barrier dysfunction in RE rats. Endoscopic esophageal biopsy specimens of GERD patients are characterized by the dilated intercellular spaces between esophageal epithelial cells and higher permeability to hydrogen ions than tissue from healthy subjects.19,20 The esophageal epithelium from GERD patients had lower electrical resistance and higher epithelial currents than controls.21 During gastro-esophageal reflux, luminal acid caused injury of the esophageal epithelium by altering the apical junction complexes and caused an early increase in paracellular permeability and dilated intercellular space.22 The acid then penetrated the intercellular spaces to initiate a cascade that results in cell necrosis, inflammation, and erosion.23 Gastro-esophageal epithelial barrier function is mainly maintained by tight junction proteins, such as claudins and occludin.24 Recently, it was reported that inhibiting p38 MAPK attenuated aspirin induced gastric epithelial barrier dysfunction.25 In this work, occludin, and ZO-1 was found down-regulated in esophageal epithelium and inhibition of p38 MAPK up-regulated these tight junction proteins and restored TER in RE rats, indicating that p38 MAPK mediated esophageal epithelial barrier dysfunction in this model of RE rats, through modulating tight junctions.

In addition, MMP-3 and MMP-9 were overexpressed in the esophagus of RE rats. It was reported that ectopic expression of MMP-3 in epithelial cells led to down-regulation of catenins and E-cadherin, which are critical adherens junction proteins important for the barrier function of epithelium.26 Up-regulation of MMP-9 in neutrophils also resulted in increased permeability of pigment epithelium in retina by down-regulating tight junction proteins.27 Inhibition of p38 MAPK suppressed expression of MMP3 and MMP9 in the esophagus of RE rats, which contributed to the beneficial effect of inhibition of p38 MAPK on esophageal epithelial barrier dysfunction in RE rats.

In the chronic phase of RE, continuous exposure to acid induces persistent infiltration of inflammatory cells.28 Increased TNF-α, IL-1β, and IL-6 concentrations have been demonstrated in the esophageal tissues of rats in several models of RE.29–31 These inflammatory cytokines mislocalized and down-regulated tight junction proteins, subsequently induced dysfunction of epithelial barrier,32 which further aggravated epithelial inflammation, finally evolving into a vicious cycle. The p38 MAPK pathway was identified as a regulator of pro-inflammatory cytokine biosynthesis in several chronic cytokine dependent inflammatory diseases, including rheumatoid arthritis, Crohn’s disease, psoriasis, and asthma.33 In this work, inhibition of p38 MAPK reduced the infiltration of inflammatory cells and cytokine secretion in the esophagus of RE rats and normalized the serum cytokine levels.

Clinical report revealed that the accumulation of NO, produced by iNOS, was considered to be related to the exacerbation of RE.34,35 It was reported that exogenous luminal NO exacerbates tissue damage in a RE model of rats.36 In esophageal cells, iNOS was up-regulated by common reflux constituents, leading to NO production.37 Diffusion of the luminal NO into
the adjacent superoxide-enriched inflamed tissue of the esophagus could lead to the production of the highly toxic agent peroxynitrite, which was well known by its impact on proteins through tyrosine nitration and leaving its footprint as 3-NT. It was reported that p38 MAPK mediated the iNOS activation in neutrophils and in peripheral blood mononuclear cells stimulated with rhIL-15 and rhIL-18. In this study, inhibition of p38 MAPK suppressed iNOS expression and reduced formation of NO and 3-NT. Therefore, p38 MAPK/iNOS pathway might also mediate the role of p38 MAPK in RE.

Reflux esophagitis caused considerable levels of oxidative stress in the esophageal mucosa, which is more important than acid in the pathogenesis of RE. In this work, inhibition of p38 MAPK activation by SB203580 could abate oxidative stress in the esophageal mucosa of RE rats. Significant numbers of neutrophils and/or macrophages infiltrated the esophageal mucosa during inflammation, generating large amounts of reactive oxygen species. In this work, inhibition of p38 MAPK activation reduced infiltration of inflammatory cells in esophagus of RE rats, which might interpret its antioxidant property, at least in part.

In conclusion, the inhibition of p38 MAPK activation attenuated esophageal mucosal damage in chronic acid RE rats, possibly by modulating the esophageal barrier function and regulating inflammatory cell recruitment, and the subsequent formation of cytokines, NO, and reactive oxygen species. These studies suggest that p38 MAPK play an important role in RE and the inhibition of p38 MAPK activation may be a promising therapeutic strategy to attenuate esophageal mucosal damage in patients who suffered from RE.

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CONFLICTS OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTION

LZ, GL, DWZ, ZSL contributed to the design of the experiments; LZ, GL, XH, JL, GXL performed the research; LZ, GL, XH, GXL, LZ performed the preparation of the manuscript. The authors have no competing interests.

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