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Stress ulceration is a common complication in critically ill patients and can result in significant upper gastrointestinal bleeding associated with a high morbidity and mortality. At present, little is known of the molecular mechanisms underlying the incidence of this type of gastric damage. In the present study, we investigated the temporal activation of the redox-sensitive p38 signaling transduction cascade and its roles in a well-defined experimental model of cold immobilization stress-induced gastric ulceration. Exposure of Sprague-Dawley rats to 6 h of cold immobilization stress led to a rapid activation of p38 in the gastric mucosa at as early as 15 min after stress, and this activation was maximal after 1.5 h of stress and still persisted until the end of stress. Selectively blocking p38 by pretreatment with SB 239063, a potent and selective p38 inhibitor, suppressed the stress-promoted TNF-α, IL-1β, and CINC-1 production and then prevented the subsequent neutrophil infiltration, gastric mucosal epithelial necrosis and apoptosis, and the ulcerative lesions formation. Prior administration of the free radical scavengers, tempol and N-acetyl-L-cysteine, abolished the stress induction of p38 activation and the resulting mucosal inflammation and gastric injury. These results demonstrate that reactive oxygen species-mediated p38 activation plays an essential role in the pathogenesis of stress-induced gastric inflammatory damage in the rat model of cold immobilization stress. Our findings suggested that inhibition of p38 activation might be a potential strategy for the prophylaxis and treatment of stress ulceration.


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Abbreviations used in this paper: ROS, reactive oxygen species; CINC, cytokine-induced neutrophil chemoattractant; GSH, reduced glutathione; MAPKAPK-2, MAPK-activated protein kinase 2; MDA, malondialdehyde; MKP, MAPK phosphatase; NAC, N-acetyl-L-cysteine; tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; MPO, myeloperoxidase.
mediators (13–16). Recruitment and activation of neutrophils contribute greatly to the pathophysiological processes occurring in gastric mucosa at the later stage of stress (13, 14). Synthesis and release of inflammatory mediators, especially of proinflammatory cytokines such as TNF-α, IL-1β, and IL-18, of chemokines such as cytokine-induced neutrrophil chemoattractant-1 (CINC-1, a rat analog of human IL-8/Gro-α and a potent neutrophil chemoattractant), and of adhesion molecules such as ICAM-1 as well as of inducible NO synthase/NO, are recently emerged as important determinants of mucosal inflammation and gastric injury following cold immobilization stress (15–20). However, despite intense research, much is unknown about the molecular mechanism or the signal transduction mechanism by which this type of stress induces the incidence of acute gastric mucosal injury.

Overproduction of ROS results in oxidative damage, including lipid peroxidation, protein oxidation, and DNA damage, which can lead to cell death. Furthermore, ROS are known to act as second messengers to activate diverse redox-sensitive signaling transduction cascades, including the MAPK family member p38 and its associated downstream transcription factors such as NF-κB and AP-1, thereby regulating the expression of numerous proinflammatory genes and hence leading to tissue and cell to inflammatory injuries (21, 22). The p38 MAPK is a ubiquitous and highly conserved proline-directed serine/threonine protein kinase considered important in mediating stress, inflammatory and immune responses, and cell survival and apoptosis processes (23–25). In a wide variety of in vivo experimental models, p38 kinase inhibitors exhibit profound anti-inflammatory effects through inhibiting proinflammatory mediator production and preventing cell death and thus significantly reduce the ultimate degree of tissue injury (26). Given that the pathogenesis of cold immobilization-stress-induced gastric mucosal lesions is typically associated with ROS overproduction and inflammatory molecules overexpression and that the local generation of ROS is an initial event in the early phase of stress (10, 12), it is therefore plausible to speculate that ROS generated during stress in gastric mucosa may trigger the activation of p38 signaling cascade, which may then in turn contribute to the development of mucosal inflammation and gastric injury. Accordingly, the present study was performed to test this hypothesis. In this study, we examined the time course of p38 activation in gastric mucosa in rats subjected to cold immobilization stress. With the use of a potent and selective second-generation inhibitor of p38, SB 239063 (27), we determined the effects of in vivo inhibition of p38 on stress-induced TNF-α, IL-1β, and CINC-1 gene expression and the subsequent neutrophil infiltration, epithelial cell injury, and mucosal lesions formation. Using the membrane-permeable free radical scavengers, tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) and NAC (N-acetyl-L-cysteine), we investigated the potential role for ROS in the process of p38 activation during the period of stress.

Materials and Methods

Animal protocols
All animal experiments were approved by the Ethics Committee of Second Military Medical University in Shanghai, following the National Institutes of Health guidelines for the care and use of laboratory animals. All efforts were made to reduce both the suffering and number of animals used. The experimental groups were chosen by means of a completely randomized schedule. Male Sprague-Dawley rats weighing 200–210 g were starved but allowed free access to water for 24 h before the experiments. To induce gastric mucosal lesions, the conscious animals were restrained and immersed up to the depth of the siphon process in water bath at 19°C for 6 h as previously described by Senay and Levine (5). Animals were killed by decapitation under ether anesthesia 0.5, 15, 30, or 45 min or 1.5, 3, or 6 h after the beginning of the stress period (n = 4–6 animals for each time point).

In pharmacological intervention experiments, polyethylene catheters were placed in the external jugular vein of animals under general anesthesia and analgesia with pentobarbital (50 mg/kg, i.p.) 3 days before exposure to stress, as previously described (28). To inhibit p38 activity, animals were initially treated with an i.v. infusion of SB 239063 (Calbiochem; solution concentrations of 0.09, 0.27, 0.9, and 2.7 mg/ml, respectively, in isotonic saline solution) at a rate of 4.5 ml/h with a calibrated infusion pump 1 h before the start of stress, followed by a constant rate infusion of 1.1 ml/h as a maintenance dose for the duration of stress. Thus a 6-h stressed rat received a total dose of 1, 3, 10, or 30 mg/kg SB 239063. The doses and regimen of SB 239063 used in this study were chosen according to previous studies (27, 29, 30). Animals in the stress alone group received an i.v. infusion of the same volume of vehicle of 11.1 ml/kg at the same rate of 4.5 ml kg−1 h−1 for 1 h then 1.1 ml kg−1 h−1 for 6 h. Animals in the SB 239063/vehicle group were given SB 239063 only. For antioxidant administration, tempol or NAC (Sigma-Aldrich; 30 and 60 mg/ml in saline, respectively) was dosed as a single i.v. bolus of 30 or 60 mg/kg 1 h before stress, followed by continuous i.v. infusion separately at 10 and 20 mg kg−1 h−1. Each rat thus received a total of 100 mg/kg tempol or 200 mg/kg NAC over a 6-h period of stress. Stress control group received the equal volume of saline. Animals in these experimental groups were killed at 30 min or 6 h after the start of stress (n = 12–16 animals for each time point and group). We chose 30 min time point because it was the threshold time for stress-induced mRNA expression of most inflammatory cytokines in our earlier study (31). In our preliminary studies, we found that vehicle or SB 239063 or tempol or NAC alone administration did not cause any pathological changes in the gastric mucosa in normal animals and that there was no difference in basal gastric acid secretion among the normal animals treated with or without tempol or NAC.

male rats were killed at 30 min or 6 h after the start of stress (n = 12–16 animals for each time point and group). We chose 30 min time point because it was the threshold time for stress-induced mRNA expression of most inflammatory cytokines in our earlier study (31). In our preliminary studies, we found that vehicle or SB 239063 or tempol or NAC alone administration did not cause any pathological changes in the gastric mucosa in normal animals and that there was no difference in basal gastric acid secretion among the normal animals treated with or without tempol or NAC.

For biochemical analyses, the stomachs of half the animals in each group were rapidly removed, placed on ice, opened along with the greater curvature, and rinsed with ice-cold normal saline, and then gastric corpus mucosa was harvested by first scraping the mucosa off the underlying muscularis mucosa and serosal layers with a microscope slide. To minimize the degradation of proteins and mRNAs, the collection of tissues was completed as soon as possible. The samples were snap-frozen in liquid nitrogen and stored at −80°C until assayed. For macroscopic assessment of gastric damage, the stomachs of the remaining half of the animals in each group were secured by ligatures at both esophagus and duodenum and then inflated with 5 ml of cold saline (to stretch and fix mucosal folds to the same extent). The removed stomachs were immersed in 10% buffered formalin for 10 min to fix both the inner and outer layers of the gastric wall and the length and width of each hemorrhagic erosive lesion in gastric corpus mucosa were measured under a stereoscopic microscope (×10) in a blind manner. The extent of gastric damage was expressed as the ulcer index, which was scored according to the criterion of Guth et al. (32). For microscopic examination, part of the tissue was fixed, embedded, sectioned (5 μm), and stained with H&E.

Preparation of whole cell protein lysates and immunoblotting
The whole cell protein extracts from frozen mucosal tissues were prepared as described previously (33). In brief, the samples were ground in liquid nitrogen into a fine powder with a porcelain mortar and pestle, and then Dounce homogenized in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 25 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO4, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mg/ml p-nitrophenolphosphate, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Cell debris and nuclei were removed by centrifugation at 12,000 × g for 10 min at 4°C, and the supernatants obtained were used as the whole cell lysates. Protein concentrations were measured using the Micro BCA protein assay reagent kit (Pierce) with BSA as standard, according to the manufacturer’s instructions. The activation of p38 was examined as its phosphorylated forms detected by Western blot analysis (33). In brief, equal amounts of proteins (50 μg) were resolved by 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking, the membrane was probed overnight at 4°C with the rabbit polyclonal Ab to phosphorylated p38 or total-p38, (Cell Signaling Technology), or the monoclonal Ab to NF-κB subunit p65 (Santa Cruz Biotechnology), followed by HRP-conjugated secondary Abs. Immunoreactive bands were detected using an ECL Western blotting kit (Pierce). Densitometry was performed using a gel documentation system (Fluor-S-MultiImager and Quantity one analysis software; Bio-Rad). The levels of GAPDH were served as an internal standard. The levels of NF-κB inhibitory proteins IκBα and NF-κB subunit p65 were also performed using specific Abs for the phosphor-IκBα (Ser32/36), respectively.
In-gel kinase assays

Direct in-gel kinase assays for p38 activity using MAPK-activated protein kinase-2 (MAPKAPK-2) as a specific substrate were performed according to the method described by Sadoshima et al. (34) with minor modifications. In brief, whole cell lysates prepared as above containing equal amounts of protein (200 μg) were resolved on 10% SDS-polyacrylamide gel, which was polymerized in the presence of 0.4 mg/ml GST-MAPKAPK-2 fusion protein (Cell Signaling Technology). After electrophoresis, SDS was removed by washing the gel with two changes of 20% 2-propanol in 50 mM Tris (pH 8.0) for 1 h and then with two changes of 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-ME for 1 h. The enzyme was denatured by incubating the gel with two changes of 6 M guanidine-HCl for 1 h and then reanimated with five changes of 50 mM Tris-HCl (pH 8.0) containing 0.04% Tween 40 and 5 mM 2-ME for 1 h (5 times for 10 min each). The gel was then incubated with 40 mM HEPES (pH 8.0) containing 2 mM DTT and 10 mM MgCl2. Phosphorylation of in-gel substrate was performed by incubating the gel at 25°C for 1 h with 40 mM HEPES (pH 8.0) containing 0.5 M EGTA, 10 mM MgCl2, 2 μM protein kinase inhibitor peptide (Sigma-Aldrich), 40 μM ATP, and 2.5 μCi/ml [γ-32P]ATP (6000 Ci/mm; Amersham Biosciences). After incubation, the gel was washed with a 5% (w/v) trichloroacetic acid solution containing 1% (w/v) sodium pyrophosphate until the radioactivity of the solution became negligible. The gel was dried, and incorporation of 32P into kinase substrates was visualized and quantified using a PhosphorImager system (STORM 820; Molecular Dynamics). Activities of the other two MAPK family members, JNK and ERK, were similarly evaluated using GST-c-Jun and GST-myelin basic protein fusion proteins (Sigma-Aldrich) as the substrates, respectively.

RNA isolation and Northern blot analysis

Total RNA was prepared from frozen gastric mucosa using TRIzol reagent (Invitrogen) and quantified based on its absorption at 260 nm. RNA (10 μg) was electrophoresed, transferred onto a nylon membrane (Hybond-N; Amersham Biosciences). The blots were hybridized separately with [α-32P]dCTP (3000 Ci/mm; Amersham Biosciences)-labeled rat TNF-α, IL-1β, and CINC-1 cDNA probes, subsequently stripped, and reprobed with 32P-labeled rat 18S rRNA cDNA as internal control, as described previously (31). Densitometric analysis was performed using a PhosphorImager system.

ELISA

The mucosal samples were pulverized using a pestle and mortar under liquid nitrogen, and then homogenized in ice-cold lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 μg/ml aprotinin, leupeptin, and pepstatin. After centrifugation at 12,000 × g at 4°C for 10 min, the supernatant of tissue lysate was collected and aliquoted for ELISA and protein concentration measurement using the BCA assay. The level of TNF-α, IL-1, or CINC-1 protein was measured using an ELISA kit for rat TNF-α, IL-1β, and CINC-1 (Biosource International) or rat CINC-1 (R&D Systems) following the manufacturer’s instructions. Each sample was assayed in duplicate and normalized by its total protein concentration in milligrams.

Preparation of nuclear extracts and EMSA

The nuclear protein extracts from frozen mucosal tissues were prepared as described previously (35). The protein concentration was measured by the BCA assay, followed by sample dilution with 4°C nuclear extraction buffer (50 mM Tris-HCl, 0.5 mM EGTA, 10 mM MgCl2, 2% nondenaturing polyacrylamide gel at 90 V for 1.5 h. Gels were dried, autoradiographed at −70°C, and quantified using PhosphorImager analysis. The specificity of the EMSA binding reaction was determined by pre-incubation either with 100-fold molar excess of unlabeled NF-κB or AP-1 probes or with monoclonal anti-NF-κB or AP-1 Abs (anti-p50, anti-p65, anti-c-Fos, and anti-c-Jun; Santa Cruz Biotechnology).

Determination of gastric mucosal malondialdehyde (MDA) concentrations

The levels of MDA, a commonly used index of lipid peroxidation, were measured by its thiobarbituric acid reactivity in the gastric mucosa (16). Results are expressed as nanomoles per milligram of protein.
total DNA from each sample was analyzed by electrophoresis through a 1% agarose gel in parallel with a 100-bp DNA ladder. The gel was then stained with ethidium bromide and visualized with UV light for the examination of nucleosomal size fragments or ladders of 180 bp or multiples.

For determination of mucosal caspase-3 activity, frozen tissues were homogenized in lyase buffer containing 20 mM HEPES (pH 7.5), 137 mM NaCl, 0.1% Nonidet P-40, 5% glycerol, 2 mM DTT, 5 mM EDTA, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin, and 1 mM PMSF. Homogenates were centrifuged at 12,000 × g for 30 min at 4°C, and the supernatants were collected. The activity of caspase-3 was measured by the fluorometric assay with CaspACE assay system (Promega), according to manufacturer’s protocol. In brief, equal amounts of protein (50 μg) in duplicate were incubated with fluorogenic substrate Ac-DEVD-AMC at 30°C for 60 min. The specificity of the assay was determined using the caspase-3 inhibitor Ac-DEVD-CHO by adding to the sample 30 min before the substrate. The release of free AMC from substrate was quantified fluorometrically at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a fluorescence microplate reader (model FLx800; Bio-Tek Instruments). The fluorescence intensity was calibrated with standard concentrations of AMC, and the caspase-3 activity was calculated from the slope of the recorder trace and expressed in picomoles per minute per milligram of protein.

**Statistical analysis**

Data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA followed by Student-Newman-Keuls test. Difference was considered significant at *p* < 0.05.

**Results**

**Time course of p38 activation during stress**

To examine p38 activation, Western blotting analysis was performed with anti-phospho-p38 (Thr180/Tyr182) Ab to detect the dually phosphorylated form of p38. As shown in Fig. 1, phosphorylated p38 was detected at a very low level in the gastric mucosa of normal rats, but by 15–30 min after the start of stress, phosphorylation of p38 was rapidly induced, which peaked at 1.5 h, and...
declined slightly but remained elevated markedly afterward up to the end of stress. There was no significant change in total p38 at various time points during the period of stress. To determine whether this activation of p38 actually represented a functional increase in p38 activity, the direct in-gel kinase assay was performed by using an SDS-polyacrylamide gel containing MAP KAPK-2. As shown, the p38 activity was very low in the normal mucosa, but was increased at 15 min after stress, and this increase was maximal after 1.5 h of stress and still persisted until the end of stress, in accordance qualitatively with the tendency observed for the phosphorylated p38. The amount of p38 activity increased by 1.6-, 2.3-, 8.5-, and 6.1-fold at 15 min, 30 min, 1.5 h, and 6 h, respectively (n = 4, p < 0.05 for each time point). Together, these data indicated an early and sustained pattern of p38 activation in rat gastric mucosa over a 6 h period of stress.

Localization of activated p38 as well as proinflammatory cytokines TNF-α, IL-1β, and CINC-1

We next investigated the localization of phosphorylated/activated p38 induction in gastric mucosa using immunohistochemistry. As shown in Fig. 2, no cells in normal mucosa were stained positively for phosphorylated p38, whereas significant induction of phosphorylated p38 was observed in the mucosa from 6-h stressed animals. The activated p38 was mainly localized to the nuclei of the surface epithelial cells and the glandular epithelial cells. Immunohistochemical studies also showed that the stress-induced cellular expression of TNF-α, IL-1β, and CINC-1 proteins was primarily confined to epithelial cell population of the mucosa. The similar regional distribution profiles of p38 activation and proinflammatory molecules strongly suggested that p38 might be a key mediator of stress-induced proinflammatory gene expression in gastric mucosa.

ROS induce p38 activation during stress

ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radical are produced during stress in the gastric mucosa and are known to be crucial in the pathogenesis of stress ulceration (10–12). Because p38 is a redox-sensitive kinase (21, 22), we investigated the involvement of ROS in the mucosal activation of p38 by stress in rats with or without pretreatment with two different kinds of membrane-permeable free radical scavengers, tempol and NAC.

FIGURE 4. A, Dose-dependent effects of SB 239063 on stress-induced phosphorylation and activities of p38 as well as activities of JNK and ERK. Animals were pretreated with an i.v. infusion of 1, 3, 10, or 30 mg/kg SB 239063 1 h before stress, and animals were killed at 30 min and 6 h of stress. Results are representative of four separate experiments. The bands were quantified by densitometry and expressed as n-fold increases vs control values. B, Effects of 3 mg/kg SB 239063 on stress-induced activities of p38. Results are expressed as n-fold increases vs controls. Data show the mean ± SEM of 4 animals. **, p < 0.01 vs control. C, Dose-dependent effects of SB 239063 on stress-induced expression of TNF-α, IL-1β, and CINC-1 mRNAs. Expression of mRNAs was measured by Northern blot analysis at 6 h of stress. Results are representative of four separate experiments. The bands were quantified by densitometry and expressed as n-fold increases vs control values. D, Dose-dependent effects of SB 239063 on stress-induced gastric mucosal lesions as macroscopically assessed. Results were expressed as ulcer index. Values are mean ± SEM for 6–8 rats. *, p < 0.05 and **, p < 0.01 vs vehicle-treated animals.
Tempol is a low m.w. superoxide dismutase mimetic agent capable of scavenging directly superoxide anions as well as hydroxyl radicals, and offers an advantage over many other ROS scavengers as it is able to permeate biological membranes (36). NAC, a low m.w. thiol-based antioxidant, can increase the intracellular pool of reduced glutathione (GSH) by acting as a cysteine donor to augment GSH synthesis (37). As shown in Fig. 3, A and B, pretreatment with tempol (100 mg/kg, i.v.) or NAC (200 mg/kg, i.v.) largely blocked the stress-induced phosphorylation and activity of p38 at both the 30 min and 6 h time points of stress. To clarify whether tempol and NAC had really reduced the generation of ROS within the gastric mucosa in stressed rats, we measured the intracellular concentration of MDA by the thiobarbituric acid reaction method in the gastric mucosa from control rats and rats pretreated with vehicle (saline) or tempol or NAC for 1 h before stress for 6 h. The MDA is a classic marker of lipid peroxidation that occurs as a result of the damaging effects of ROS/oxidative stress (16). As anticipated, MDA levels in the stress group gastric mucosa were significantly higher compared with the control levels, whereas tempol and NAC dramatically reduced the stress-induced increase in MDA levels (Fig. 3, C and D), confirming that stress does provoke oxidative stress in gastric mucosa in this model and that

FIGURE 5. Effects of SB 239063 (A), tempol (B), and NAC (C) on stress-induced TNF-α, IL-1β, and CINC-1 mRNA expression. Expression of mRNAs was measured by Northern blot analysis at 30 min and 6 h of stress. Relative mRNA levels were quantified by densitometry and expressed as OD ratio with 18S served as internal standards. Results show the mean ± SEM of 3–5 animals. *, p < 0.05 and **, p < 0.01 vs normal controls; #, p < 0.05 and ##, p < 0.01 vs vehicle-treated animals. D. Effects of SB 239063, tempol, and NAC on stress-induced TNF-α, IL-1β, and CINC-1 protein production. TNF-α, IL-1β, and CINC-1 proteins in mucosal tissues were measured by ELISA at 6 h of stress. The results were normalized to total cellular protein. Data represent mean ± SEM of 6–8 animals. *, p < 0.05 vs normal controls and #, p < 0.05 vs vehicle-treated animals.
tempol and NAC can effectively prevent the stress-induced oxidative stress. Taken together, these results demonstrated a critical role for ROS in the triggering and prolonging of p38 activation in response to stress in the gastric mucosa.

**Selectivity and dose dependency of SB 239063**

SB 239063 is a novel second-generation inhibitor of p38\(\alpha\) as well as of p38\(\beta\) and has improved selectivity and activity both in vitro and in vivo over previous p38 inhibitors such as SB 203580 or SB 202190 (27). SB 239063 produces a competitive inhibition of p38 kinase at the ATP binding site, resulting in potent inhibition of its catalytic activity, with an in vitro IC\(_{50}\) value of 44 nM against p38 and >10–50 \(\mu\)M against a panel of other protein kinases including JNK, raf-MEK-ERK, IKK\(\beta\), and Akt (27, 29, 30). The dosing regimen of SB 239063 (1, 3, 10, and 30 mg/kg, i.v.) used in this study led to a plasma level of SB 239063 of \(\sim 0.43, 1.3, 4.3,\) or 13 \(\mu\)M during the period of stress, and these plasma concentrations were nearly 10-, 30-, 100-, and 300-fold higher than the IC\(_{50}\) for inhibiting p38 activity in vitro, respectively (29, 30).

Considering that most of the p38 inhibitors including SB 239063 interact with the relatively conserved ATP binding site and are therefore likely to target other protein kinases, we compared the effects of increasing doses of SB 239063 on stress-induced activity of p38, JNK, and ERK. As shown in Fig. 4A, SB 239063 (1, 3, 10, and 30 mg/kg, i.v.) suppressed stress-induced p38 activity in a dose-dependent manner (lane 1). Compared with 1 and 3 mg/kg doses, SB 239063 at high doses of 10 and 30 mg/kg significantly reduced stress-induced JNK activity (lane 2). SB 239063 had no affect on stress-induced ERK activity at all doses (lane 3). Additionally, SB 239063 at doses of 1 and 3 mg/kg did not affect stress-induced p38 phosphorylation/activation, whereas 10 and 30 mg/kg SB 239063 markedly decreased the p38 phosphorylation (lane 4). SB 239063 alone at 30 mg/kg had no marked impact on either p38 or JNK or ERK activity, or p38 activation. These data indicated the dose of 3 mg/kg as a relatively efficient and specific inhibitory dose of SB 239063 against the stress-induced mucosal p38 activity in this model in vivo. Fig. 4B further showed that the 3 mg/kg dose of SB 239063 could effectively prevent the increase of p38 activity throughout the stress.

We further examined the dose dependency of SB 239063 on the expression of proinflammatory genes and the incidence of gastric injury induced by 6-h stress. As shown in Fig. 4C, SB 239063 (1, 3, 10, and 30 mg/kg) inhibited the stress-induced mRNA expression of TNF-\(\alpha\), IL-1\(\beta\), and CINC-1 in a dose-related fashion, with the 3 mg/kg dose giving the greatest reduction in all of the stress-induced cytokine mRNA levels, whereas the degree of inhibition of cytokine mRNA increases was lesser with 30 mg/kg than with 10 mg/kg, indicating a U-shaped dose-response curve. Application of SB 239063 also led to a dose-related decrease in cytokine protein levels paralleled closely the reduction in mRNA expression (data not shown). Fig. 4D showed that the most dramatic protection against stress-induced gastric injury was observed with 3 mg/kg of SB 239063, whereas 1 and 10 mg/kg SB 239063 did not provide maximal histological protection whereas SB 239063 at the highest dose of 30 mg/kg had no significant protective effect, indicating a U-shaped dose response curve, similar to the curve for its effects on cytokine expression. No mucosal lesions were observed in normal animals treated with 30 mg/kg SB 239063 alone. The U-shaped functions of SB 239063 doses further confirmed that the specific effects of low doses of SB 239063 (1 and 3 mg/kg). On the basis of above results, we chose dose of 3 mg/kg as the optimum dose of SB 239063 to study the roles of p38 in subsequent experimentations.

**Effects of SB 239063, tempol, and NAC on stress-induced proinflammatory gene expression**

To explore the functional relationship between ROS/p38 pathway and stress-induced gastric injury, we examined whether SB 239063 and antioxidants, tempol and NAC, inhibited the stress-induced proinflammatory gene production. We have shown recently that the mRNA expression of TNF-\(\alpha\), IL-1\(\beta\), and CINC-1 is linearly increased in rat gastric mucosa with the duration of cold immobilization stress up to 6 h with the threshold time at 30 min after stress for each of these genes (31). Fig. 5A showed that SB 239063 (3 mg/kg, i.v.) administration markedly suppressed IL-1\(\beta\) mRNA induction and protein phosphorylation at 30 min and 6 h for each of these genes. However, tempol (30 mg/kg, i.v.) and NAC (30 mg/kg, i.v.) administration did not suppress IL-1\(\beta\) mRNA induction and protein phosphorylation at 30 min and 6 h.
mRNA without affecting TNF-α or CINC-1 mRNA at 30 min of stress, while it notably repressed all of them at 6 h albeit with variable degree. The strongest inhibition was observed for IL-1β when compared with TNF-α and CINC-1. In comparison, tempol (100 mg/kg, i.v.) and NAC (200 mg/kg, i.v.) drastically reduced all these mRNAs at both 30 min and 6 h (Fig. 5, B and C). The ELISA results presented in Fig. 5D showed that the levels of TNF-α, IL-1β, and CINC-1 proteins failed to increase at 30 min of stress but were decreased by SB 239063, tempol and NAC in a manner similar to that observed for their mRNA inhibition at this time point. Immunohistochemical results further demonstrated that stress-induced substantial increases in TNF-α, IL-1β, and CINC-1 protein production were blocked by SB 239063, tempol or NAC (data not shown). Taken together, these data indicated that the ROS-mediated p38 activation greatly contributes to the duration of stress-induced proinflammatory genes expression but differentially regulates their early induction.

Effects of SB 239063 and tempol on stress-induced NF-κB and AP-1 activation

We recently reported that cold immobilization stress induces a rapid and persistent activation of transcription factors NF-κB and AP-1 in rat gastric mucosa, which are known as main regulators of the gene transcription of large numbers of proinflammatory molecules (31). To explore the downstream signaling mechanisms responsible for the roles of SB 239063 and antioxidants to suppress proinflammatory cytokine production, we assessed how they affected the activation of NF-κB and AP-1. Since tempol exhibited a stronger inhibitory activity against stress-induced ROS production than 200 mg/kg NAC (as indicated in Fig. 3), we selected tempol for use in this experiment. As shown in Fig. 6A, the results of the EMSA experiments illustrated that SB 239063 treatment did not influence the NF-κB binding activities at 30 min of stress while potently inhibiting them at 6 h of stress. In contrast with NF-κB, the AP-1 binding activities at 30 min and 6 h of stress were significantly blocked by SB 239063. As compared with SB 239063, tempol administration almost completely prevented both the early and late activities of NF-κB and AP-1. The specificity of NF-κB and AP-1 DNA-binding activity was confirmed by cold competition (Fig. 6A) and Ab supershift assays (Fig. 6B). The supershift
assays indicated also p50/p65 and c-Fos/c-Jun heterodimer separately as the main component of the activated NF-κB and AP-1 complex, consistent with our previous studies (32). Together, these data suggested that the stress-induced NF-κB and AP-1 binding activities are largely due to ROS while the redox-sensitive p38 is only involved in the immediate induction of AP-1 but not of NF-κB despite also participating in their late phase induction.

Since the nuclear translocation and subsequent DNA binding of NF-κB are mainly regulated by cytoplasmic IκB inhibitory proteins (38), we also examined the effects of SB 239063 and tempol on IκB phosphorylation and degradation. We have recently shown that the sustained induction of NF-κB binding activity in gastric mucosa during cold immobilization stress depends on a combined phosphorylation/degradation of IκBα and IκBβ, with the early phase activity being mediated mainly by IκBα, whereas the late phase is mediated by IκBβ. As shown in Fig. 6C, SB 239063 did not modify the early phosphorylation/degradation of IκBα at 30 min of stress but blocked the degradation of IκBβ at 6 h of stress, whereas tempol prevented either the early IκBα phosphorylation and degradation or the late degradation of IκBβ, in line with their effects on NF-κB binding activity.

Since phosphorylation of RelA/p65 in many cases is necessary for the transcriptional activity of p50/p65 NF-κB heterodimer (39) and since p38 and ROS can mediate p65 phosphorylation (40), we further determined whether SB 239063 and tempol were exerting an effect on the phosphorylation status of the p65. As shown in Fig. 6C, the level of phosphorylated p65 at Ser536 residue was markedly increased at 30 min of stress and remained above basal level at 6 h after stress, whereas phosphorylation of p65 at Ser276 was not as pronounced as that of Ser536 at 30 min, but it was obvious after 6 h of stress, revealing that stress induces both Ser536 and Ser276 p65 phosphorylation but with different kinetics. SB 239063 failed to prevent stress-induced early Ser536 phosphorylation, but could block the late phosphorylation of Ser536 as well as of Ser276. Compared with SB 239063, tempol abolished not only the early and late phase of Ser536 phosphorylation but also the late phase Ser276 phosphorylation. These data suggested that p38 might be involved the duration but not the initiation of ROS-dependent NF-κB transactivation during stress, comparable with its roles in the processes of NF-κB translocation and DNA binding.

Effects of SB 239063, tempol, and NAC on stress-induced neutrophil infiltration

MPO activity, an indicator of neutrophil infiltration, was not significantly increased in the homogenized gastric tissue until the end of the 6-h stress (data not shown), consistent with previous reports (13, 14). Fig. 7 showed that the MPO activity at 6 h of stress was strongly inhibited by administration of SB 239063, tempol, or NAC.

Effects of SB 239063, tempol, and NAC on stress-induced gastric mucosal injury

No any lesion was observed in nonstressed animals, whereas large numbers of hemorrhagic necrotic lesions were observed in the corpus mucosa as well as in the antral mucosa in the 6-h stressed animals. Fig. 8 showed that application of SB 239063, tempol, or NAC prevented the stress-induced lesion formation as assessed by gross and histological examination, and caused a remarkable decrease in ulcer index as macroscopically evaluated from 49.5 ± 6.2 to 4.6 ± 0.9, 2.5 ± 0.4, and 13.4 ± 2.9, respectively (n = 8, each p < 0.01).

Effects of SB 239063, tempol, and NAC on stress-induced apoptosis of mucosal epithelial cells

As the redox-sensitive p38 pathway is known to play a critical role in the processes of cell survival and apoptosis (24, 25), we investigated the effects of SB 239063, tempol, and NAC on stress-induced apoptosis of gastric mucosal cells as assessed at 6 h of stress. A, Gastric mucosa section stained with TUNEL method (magnification, ×200). Bar graph shows the relative frequency of TUNEL-positive cells in five fields of mucosa tissue section (×400). Values represent the means ± SEM of 6–8 separate animals. **, p < 0.01 vs vehicle-treated animals. B, DNA ladder analysis for DNA fragmentation. In vehicle-treated animals, inter-nucleosomal DNA cleavage was evident after 6 h of stress. Treatment with SB 239063, tempol, or NAC abolished the internucleosomal DNA cleavage. C, Caspase-3 activity in gastric mucosa at 6 h of stress as determined by fluorometric assay. Values represent the means ± SEM of 6–8 experiments performed in duplicate. **, p < 0.01 vs vehicle-treated animals.

Discussion

Stress ulcer is a common complication in critically ill patients (3, 4). A fuller understanding of the pathophysiology of stress ulceration will shed new insights into the development of potential prophylactic or therapeutic approaches for the control of this complication. Cold immobilization stress is a commonly used and clinically relevant experimental model for stress-induced gastric mucosal lesions (5–7). This model requires at least 6 h to form profound hemorrhagic necrotic lesions in the gastric mucosa (12, 15). The finding of the current study showed that when rats were exposed to cold immobilization stress for a total of 6 h, phosphorylation/activation as well as activity of p38 kinase in gastric mucosa was remarkably up-regulated at as early as 15 min after the start of stress and still persisted until the end of stress. This pattern of activation suggested that p38 may represent a rapid and early signal mechanism for controlling gene expression during the course of stress. Because p38 is a redox-sensitive kinase (21, 22), and the excessive generation of ROS in gastric mucosa is an initial

FIGURE 9. Effects of SB 239063, tempol, and NAC on stress-induced apoptosis of gastric mucosal cells as assessed at 6 h of stress. A, Gastric mucosa section stained with TUNEL method (magnification, ×200). Bar graph shows the relative frequency of TUNEL-positive cells in five fields of mucosa tissue section (×400). Values represent the means ± SEM of 6–8 separate animals. **, p < 0.01 vs vehicle-treated animals. B, DNA ladder analysis for DNA fragmentation. In vehicle-treated animals, inter-nucleosomal DNA cleavage was evident after 6 h of stress. Treatment with SB 239063, tempol, or NAC abolished the internucleosomal DNA cleavage. C, Caspase-3 activity in gastric mucosa at 6 h of stress as determined by fluorometric assay. Values represent the means ± SEM of 6–8 experiments performed in duplicate. **, p < 0.01 vs vehicle-treated animals.
and persistent event after cold immobilization stress (10, 12), it seemed likely that ROS might be greatly involved in the process of p38 activation. This is clearly demonstrated by the effective inhibitory effects of two different kinds of antioxidants, tempol (a direct ROS scavenger) and NAC (a precursor in the biosynthesis of reductant GSH), in phosphorylation and activity of p38 as well as in the changes of the mucosal contents of MDA, the reliable and quantifiable index of ROS production and oxidative stress. Of note, the antioxidants tempol and NAC prevented both the immediate and prolonged p38 phosphorylation and activity, strongly suggesting that the activation of this signaling cascade is, at least to a great degree, independent of the additional factors such as cytokines or gastric acid. Regarding the molecular mechanism by which ROS activate p38, it has been recently known that ROS can inactivate MAPK phosphatases (MKPs), which are known to dephosphorylate activated p38, by oxidizing critical residues in their phosphatase domain, thus leading to prolonged p38 activation (41). This could be a possible mechanism for the ROS-induced sustained activation of p38 during stress in the gastric mucosa. Further studies are needed to address this issue and to define the molecular mechanisms and specific signaling pathways by which ROS trigger p38 activation in gastric mucosa following stress.

The sources of ROS responsible for triggering of p38 activation in the gastric mucosa after stress have not been clearly defined. The major sources of ROS such as superoxide anion in gastric mucosa following cold immobilization stress are usually regarded to be xanthine oxidase and activated neutrophils (14, 15). However, there is no support for the involvement of xanthine oxidase and neutrophils in the early phase of p38 activation, although they may contribute to the late phase, based on the observations that xanthine oxidase activity and the neutrophil infiltration into the gastric mucosa did not increase significantly until the late stage of stress (14, 15). In fact, cold immobilization stress rapidly reduces the blood flow to gastric mucosa leading to local hypoxia and ischemia (8–10). This hypoxic-ischemic condition may increase the leakage of superoxide anion from the mitochondrial electron transport chain of the mucosal epithelial cells (10). In addition, under this hypoxic-ischemic condition the activation of nonphagocytic NADPH oxidase containing the small GTP-binding protein Rac-1 within gastric mucosal epithelial cells (42, 43) may also contribute to superoxide anion production. From these data, we postulated that the hypoxic generation of ROS by mitochondria and NADPH oxidase might be essential to initiate the early activation of p38 in the gastric mucosa in response to stress. It is of interest to point out that, as shown in this study, the degree of inhibition of p38 activation was greater with tempol than with NAC, indicating a possibility that superoxide anion but not hydroxyl radical may be greatly involved in the p38 activation since tempol as a direct free radical scavenger inactivates both superoxide anion and hydroxyl radical (36), while NAC neutralizes mainly hydroxyl radical acting by replenishing cellular GSH stores (37).

It has been recognized that proinflammatory mediators, particularly inflammatory cytokines TNF-α, IL-1β, and CINC-1, have a central role in mediating neutrophil infiltration and mucosal injury following cold immobilization stress (13, 18, 31), but it is yet unclear how expression of these proinflammatory molecules is regulated or how ROS are involved in these processes. In this study, we observed that the selective blockade of p38 activity by pretreatment with the specific p38 inhibitor SB 239063 resulted in significantly decreased TNF-α, IL-1β, and CINC-1 mRNA and protein levels. These data, together with the results showing the requirement of ROS for p38 activation and proinflammatory molecule expression, demonstrated that the persistent induction of proinflammatory genes in gastric mucosa in response to cold immobilization stress is mediated at least partially by the ROS-mediated sustained activation of p38. At present, the cellular source of stress-up-regulated proinflammatory molecules remains obscure. Here our study clearly showed that the gastric epithelial cell population is the main site of production of TNF-α, IL-1β, and CINC-1. It is worth noting that these molecules display the same regional distribution as that of phosphorylated p38. The coexistence of p38 activation and proinflammatory molecule expression within the gastric epithelial cells further demonstrated that they are correlated causally. Our findings also revealed that the gastric epithelial cell is not only a passive cell reacting to inflammatory mediators but also an active participant in the local inflammatory response of gastric mucosa during the sustained period of stress.

The redox-sensitive transcription factors NF-κB and AP-1 have been shown to play critical roles in regulating the transcriptions of large numbers of genes encoding cytokines, chemokines, and other mediators involved in inflammatory responses (44, 45). In an attempt to determine whether the p38 kinase transcriptionally or posttranscriptionally impacted the mRNA expression of proinflammatory cytokines in gastric mucosa in response to cold immobilization stress, we assessed the impact of SB 239063 on NF-κB and AP-1 pathways in this model. In a recent study (31), we demonstrated that NF-κB and AP-1 are rapidly and persistently activated through a ROS-dependent mechanism in the gastric mucosa of cold immobilization-stressed-rats and that the ROS/NF-κB pathway is necessary for the transcription activation of multiple inflammatory genes including TNF-α, IL-1β, and CINC-1, while AP-1 and other transcription factors such as Egr-1, C/EBPβ or Stat3 contribute to gene transcriptions. Accordingly, in this study we confirmed that both the early and late activation of NF-κB and AP-1 and TNF-α, IL-1β and CINC-1 genes were dramatically blocked by treatment with the antioxidant tempol. It has been shown that NF-κB and AP-1 activation are under the control of p38 (45, 46). As we demonstrated here, however, p38 does not seem to have an important role in leading to ROS-induced early activation of NF-κB while greatly participating in its late activation, because p38 inhibition repressed only the late but not the early p65 translocation and phosphorylation. This is further supported by comparing their temporal activation patterns showing that p38 activation was practically simultaneous with NF-κB activation, which showed a threshold time of 15 min after stress (31) identical with that of p38. Nevertheless, the exact role for p38 in stress-induced early activation of NF-κB remains to be defined, because NF-κB regulation can be mediated at many steps besides those we explored. In contrast to NF-κB, the ROS-induced early and late activation of AP-1 during stress were consistently dependent on p38 activation. In the current study, we found that p38 differentially regulates the stress-induced proinflammatory gene expression, based on the observations that p38 inhibition reduced IL-1β mRNA but not of TNF-α or CINC-1 mRNA at 30 min of stress and that IL-1β mRNA and protein at 6 h of stress were present at lower levels relative to those of TNF-α and CINC-1 following p38 inhibition. Considering the fact above that p38 appears to have no effect on stress-mediated initiation of NF-κB activation necessary for the transcription activation of these proinflammatory genes, this differential regulation of NF-κB-dependent gene expression by p38 may be attributed at least partially to the difference in the p38-mediated stabilization of mRNAs of different genes. Collectively, our study suggested a real possibility that stress-induced p38 activation is critically involved in the propagation of expression of the NF-κB-dependent proinflammatory molecules through transcriptional and posttranscriptional mechanisms, but may not mediate the transcription initiation of these genes despite promoting their mRNA levels by enhancing mRNA stability. Since p38 and...
its inhibitor can exert effects at various levels of gene regulation, and the induced cytokine expression can also be regulated at multiple steps, further studies are required to better understand the molecular mechanisms for p38 modulation of stress-induced inflammatory gene expression in the gastric mucosa.

In the present study, we further found that corresponding with the decrements in expression of proinflammatory genes by treatment with SB 239063, tempol, or NAC, neutrophil sequestration, epithelial necrosis, and mucosal lesions were markedly repressed. Of interest in this study is the finding that SB 239063, tempol, and NAC also prevented stress-induced apoptosis of gastric mucosal epithelial cells. In this context, apoptosis has recently been suggested as an alternative mechanism for stress-induced epithelial cell death (47), although the necrosis of the epithelial cells has long been recognized as the pathological hallmark of this type of gastric injury. In this study, we confirmed that a remarkably increased apoptosis of gastric epithelial cells accompanies the extensive necrosis in the mucosal tissues from the stressed animals, as evidenced by TUNEL staining, DNA laddering analysis, and caspase-3 activity assays. Our study indicated that the ROS-induced p38 activation, besides being one of the main mechanisms for the necrosis of mucosal epithelial cells following stress, is also critically involved in the apoptotic death of gastric epithelial cells. The precise mechanisms whereby the ROS-dependent p38 activation promotes apoptosis in the gastric mucosa of stressed rats are unclear, but could be in great part related to its induction of production of proinflammatory molecules such as IL-1β, because the blockade of IL-1β production has been shown to markedly reduce the degrees of apoptosis and mucosal injury (48) in the same rat stress model as we used here. In addition to the indirect mechanism, the ROS-induced p38 activation may also cause apoptosis directly through the mitochondrial pathway and the death signal pathway (49). Overall, the findings of this study suggested that ROS generated during stress in the gastric mucosa, through a functional p38 signaling pathway, may induce the expression of proinflammatory genes, through which they may critically contribute to mucosal inflammation, epithelial necrosis as well as apoptosis, and the development of gastric ulceration. These findings suggested a molecular mechanism by which ROS contribute to the development of stress ulceration. Accordingly, our study therefore established a signaling transduction cascade linking mucosal hypoxia-ischemia to inflammatory response that leads to gastric damage in the rat model of stress-induced gastric ulceration.

It should be mentioned that, in this study, we used only the chemical inhibitor of p38 to determine the role for this kinase in stress-induced mucosal inflammation and gastric injury. Although using more specific genetic or molecular approaches will definitively define the role of p38, the use of pharmacological inhibitors selective for p38 is unavoidable in the present study because mice deficient in p38er, the major isofrom of p38, are embryonic lethal (50), and there is currently a void of efficacious methods to introduce vectors and constructs into the mucosal tissues of the whole stomach of an animal for technical and practical reasons. As most of p38 inhibitors interact with the relatively conserved ATP binding site and are therefore likely to target other protein kinases in a given dose, we carefully chose the dosing regimen of the more selective p38 inhibitor SB 239063 according to previously published preclinical pharmacokinetics and pharmacodynamics of SB 239063 (27, 29, 30) to exclude the potential inhibition of non-p38 protein kinases confounding our results. We confirmed that 3 mg/kg dose of SB 239063 can effectively block the stress-induced increase of p38 activity without obviously influencing the JNK or ERK activity, implying that this dose is a relatively specific and efficient inhibitory dose of SB 239063 for the stress-induced gastric mucosal p38 activity in our model in vivo. It is of interest that 1 and 3 mg/kg doses of SB 239063 had no substantial impact on p38 phosphorylation/activation, while 10 and 30 mg/kg SB 239063 remarkably reduced p38 activation, similar to their individual effects on stress-induced JNK activity. Nevertheless, this phenomenon cannot be explained simply as a consequence of the greater selectivity of low doses of SB 239063 compared with high doses relative to some kinases such as the p38 upstream MAPK kinases 3 or 6. In this respect, it has been suggested that inhibition of p38 activity can concomitantly prevent p38 activation because it has previously been reported that p38 can also be activated through an alternative pathway that is dependent on p38 autophosphorylation (51). In contrast, p38 activity inhibition has been recently reported to enhance p38 activation by acting as a negative modulator of protein expression of p38 as well as JNK relative to ERK (52). Therefore, the unchanged state of p38 activation following administration of low doses of SB 239063 may be due to a balance between the negative and positive feedback modulation of p38 phosphorylation by p38 inhibition, and the reduced p38 activation, as seen with higher doses of SB 239063, can be explained by an imbalance of the negative and positive feedback modulation and a greater negative effect of p38 inhibition. In the present study, most of data on the effects of p38 inhibition are obtained with 3 mg/kg dose of SB 239063. However, we cannot rule out the possibility that inhibition of other protein kinases by SB 239063 might also contribute to the beneficial effects of p38 inhibition. This slight difference, however, does not affect the conclusion that p38 is involved in the stress-induced gastric inflammatory injury when further considering the U-shaped (biphasic) dose-response curves of SB 239063 (i.e., 3 mg/kg SB 239063 was more effective than the other doses of SB 239063 in inhibiting cytokine expression and ameliorating gastric injury). The U-functions of increasing doses of SB 239063 plausibly suggested that the beneficial effects of lower doses of SB 2390263 (1 and 3 mg/kg) are due primarily to the specific inhibition of p38, while SB 239063 at the higher doses (10 and 30 mg/kg) may act on additional targets to counter its protective effects. Another potential concern with the time course used to harvest the mucosa in the present study is that the collecting would take an average time of 30–40 s and this delay may have caused the degradation of proteins and mRNAs. Further studies are needed to address this concern, but our experiments including immunohistochemical studies suggested that the results, at least in large part, actually reflect p38 activation and cytokine expression in gastric mucosal epithelial cells in vivo. Thus, the time taken from sacrifice of the animals to freezing of the isolated gastric corpus most likely was the same in all experimental animals.

In summary, the present study demonstrates that sustained activation of p38 by ROS is an important in vivo mechanism mediating the stress-induced gastric mucosal inflammation and injury. Current therapy with acid suppression drugs such as histamine-2 receptor antagonists and proton pump inhibitors can effectively reduce the frequency of stress ulcer but has questionable efficacy in a number of critically ill patients (3, 4). Our findings suggest that a combination of acid suppression drug and p38 pathway inhibitor could be more effective than acid suppression treatment alone for medical prophylaxis or treatments of stress ulcer. The specific roles of p38 in stress-induced gastric injury remain to be fully defined.

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Disclosures
The authors have no financial conflict of interest.