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Absence of NF-κB subunit p50 ameliorates cold immobilization stress-induced gastric ulcers

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ABSTRACT

Stress ulcers are a common complication in critically ill patients, but the underlying mechanism is little known. This study characterized the function of the p50 subunit of NF-κB in an experimental model of cold immobilization stress-induced gastric ulcers. Stress-induced gastric mucosal inflammation and gastric injury were examined in wild-type and NF-κB p50-deficient mice. When subjected to cold immobilization stress, NF-κB was rapidly activated in the gastric mucosa in WT mice whereas the majority of κB DNA-binding activity was abrogated from $p50^{-/-}$ mice. Deficiency of p50 ameliorated stress-induced expression of TNF-α, MIP-2, and ICAM-1, resulting in reduced mucosal accumulation of neutrophils and gastric injury. These data indicated a critical role for the p50 in the gastric mucosal inflammatory response to cold restraint stress.

1. Introduction

Stress ulcers occur commonly in patients in the intensive care unit and develop as a result of severe stressful events such as burns, trauma, surgery, shock, sepsis, and multiple organ failure [1]. Stress ulcers are usually diffuse, superficial mucosal lesions of the stomach and can result in significant upper gastrointestinal bleeding associated with significant morbidity and mortality in critical illness despite aggressive prophylaxis with acid-suppressive agents. The pathogenesis of stress ulcers is still obscure but mucosal ischemia is considered the most important pathophysiological factor, which damages gastric mucosal barrier and renders the stomach more vulnerable to offensive factors, particularly gastric acid [2].

The rodent model of cold immobilization stress is a well defined and clinically relevant experimental model for stress ulceration [3]. This powerful stress stimulus rapidly activates the sympathoadrenomedullary system and reduces the blood flow to the gastric mucosa leading to local hypoxia and ischemia. Increasing evidence indicates that this hypoxic-ischemic condition is accompanied by a pronounced inflammatory response of the gastric mucosa, which contributes substantially to epithelial necrosis and the formation of hemorrhagic erosion [4]. Consistent with experimental findings, a recent clinical study also revealed that the development of stress ulcers in traumatic brain injury patients is associated with enhanced expression of proinflammatory gene products including MIP-1α and iNOS and accumulation of inflammatory cells in the gastric mucosa [5]. These results indicate an important inflammatory mechanism in the pathophysiology of stress ulceration, however, the understanding is lacking.

Nuclear factor κB (NF-κB) is a ubiquitous transcription factor that plays a central role in regulating many inflammatory processes [6]. We have recently demonstrated that cold immobilization stress can induce a rapid and persistent activation of NF-κB in the gastric mucosa and that pharmacological inhibition of NF-κB abolishes the stress-induced inflammation and gastric injury [7]. Despite this, the exact role of NF-κB remains to be clarified, and the functional relevance of specific NF-κB subunits in stress-induced mucosal inflammatory response is unknown. In this study, we used genetic approach to characterize the function of the p50 subunit of NF-κB in stress-induced gastric mucosal inflammation and injury.

2. Materials and methods

2.1. Stress ulcer model

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Second Military Medical University in Shanghai (IACUC-2012-XuSG) in accordance with NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize both the suffering and number of animals used.

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NF-κB p50 knockout mice (B6;129P2-*Nfkb1*^{tm1Bal}/J) and wild-type mice (C57BL/6 × 129) were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.). Animals were bred under specific-pathogen-free conditions. Male p50 KO mice and littermate WT controls aged 8–10 weeks were used for experimentation. To induce acute stress ulcers, mice were restrained and immersed up to the depth of the xiphoid process in water at 23 °C for 3.5 h, as described previously [8]. Immediately after stress, animals were killed by decapitation while under ether anesthesia. For biochemical analyses, the stomachs were rapidly removed and gastric corpus mucosa was harvested by gentle scraping of the mucosa off the underlying muscularis mucosae layer. For macroscopic assessment of gastric damage, the stomachs were immersed in formalin for 10 min and the length and width of each hemorrhagic erosive lesion in the 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. [9]. For microscopic examination, part of the tissue was fixed, embedded, and stained with H&E.

2.2. Electrophoretic mobility shift assays

Nuclear extract isolation and electrophoretic mobility shift assay (EMSA) were performed as previously described [7]. Protein concentrations were determined with a Bio-Rad protein assay kit according to the manufacturer's instructions. EMSAs were performed using 3 μg of protein extract and 5×10^4 cpm of ^{32}P-labeled probe. The specificity of the binding reaction was determined by preincubation either with 100-fold molar excess of unlabeled NF-κB or AP-1 probes or with monoclonal anti-p50 antibody. DNA–protein complexes were separated in 6% nondenaturing polyacrylamide gel at 90 V for 2–3 h.

2.3. Northern blot analysis

Total RNA was prepared from gastric mucosa using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.) 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-^{32}P]dCTP (3000 Ci/mmol, Amersham Biosciences)-labeled murine TNF-α, MIP-2, and ICAM-1 cDNA probes, subsequently stripped, and reprobed with ^{32}P-labeled murine GAPDH as an internal control, as described previously [7].

2.4. Measurement of gastric mucosal myeloperoxidase activity

Myeloperoxidase (MPO) activity in gastric mucosa was assayed to evaluate neutrophil accumulation. Tissue samples were extracted by homogenization and sonication in phosphate buffer

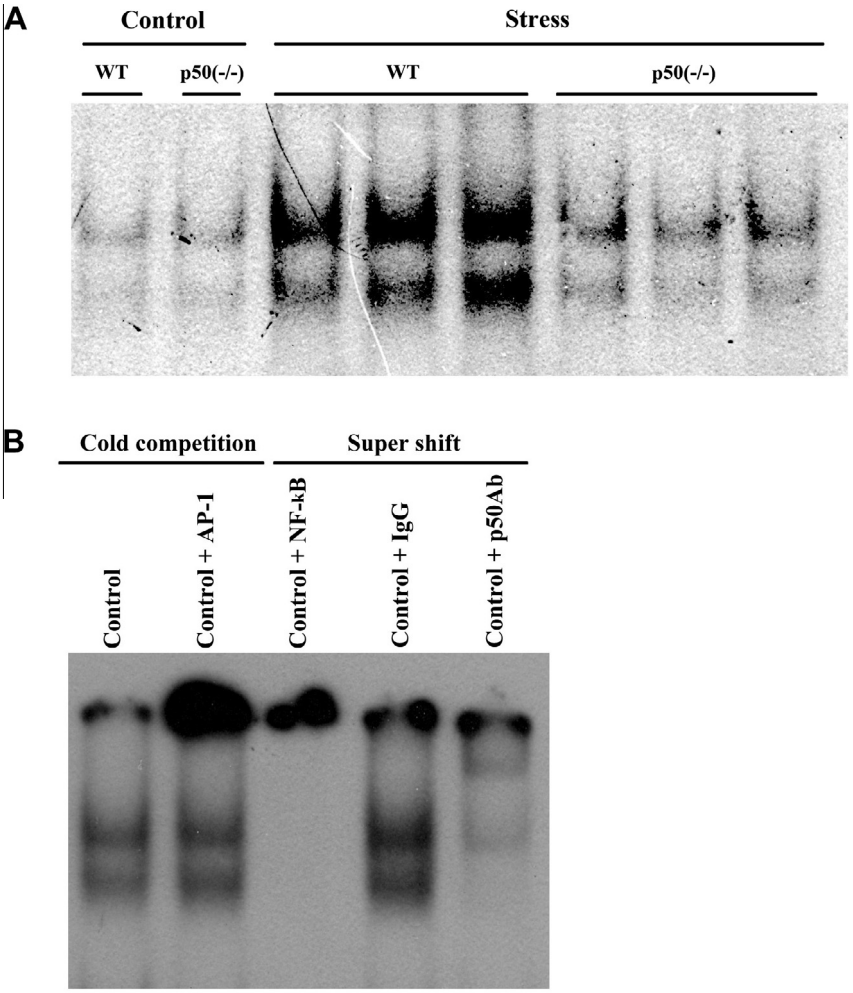

Fig. 1. Effects of p50 deficiency on stress-induced NF-κB activation. (A) Nuclear extracts from gastric mucosa of WT and p50$^{-/-}$ mice were analyzed by EMSA. The data shown are representative of three experiments. (B) Cold competition assays and antibody supershift assays. The extracts were incubated with labeled NF-κB probe in the presence of 100-fold molar excess of unlabeled specific NF-κB or nonspecific AP-1 probe or of anti-p50 antibody.

and MPO activity in supernatants was measured as described previously [8]. Results were expressed as units per g of protein per min.

2.5. Statistical analysis

All replicate results are here expressed as means ± SEM. Data were analyzed using Student–Newman–Keuls' *test*. Differences were considered statistically significant when two-tailed $P < 0.05$.

3. Results

3.1. Effects of p50 deficiency on stress-induced NF-κB activation

After cold immobilization stress, there was a significant increase in specific NF-κB binding activity in the gastric mucosa from WT mice (Fig. 1A). In contrast, NF-κB activity in $p50^{-/-}$ mice was completely abrogated. The specificity of NF-κB DNA-binding activity was confirmed by cold competition and supershift assays (Fig. 1B).

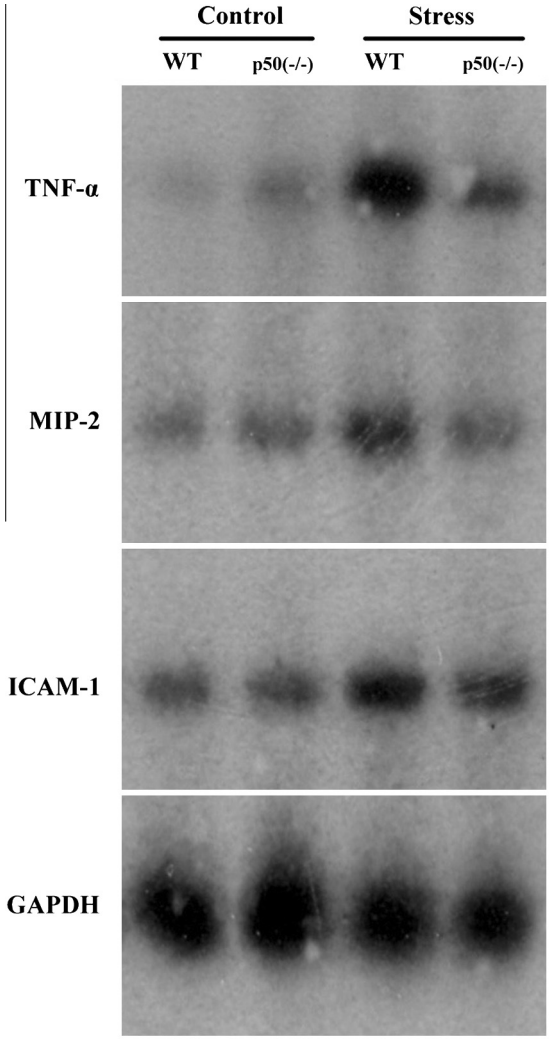

Fig. 2. Effects of p50 deficiency on stress-induced proinflammatory gene expression. TNF-α, MIP-2, and ICAM-1 mRNA in gastric mucosa of WT and $p50^{-/-}$ mice were measured by Northern blot analysis with GAPDH as an internal standard. The data shown are representative of three independent experiments.

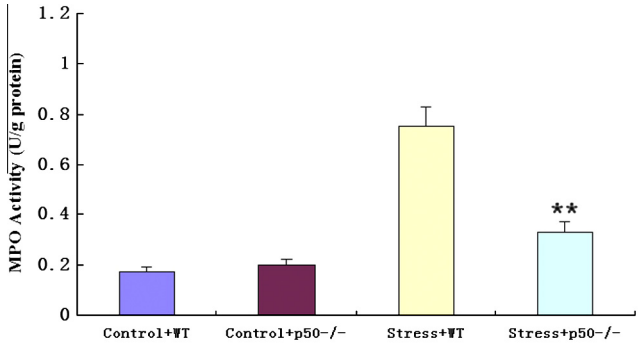

Fig. 3. Effects of p50 deficiency on stress-induced MPO activity. Tissue content of MPO in the gastric mucosa was measured as described in Section 2. Values are mean ± SEM for 6–8 animals. $^{**}P < 0.01$ versus stressed WT mice.

3.2. Effects of p50 deficiency on stress-induced proinflammatory gene expression

We next examined the role of p50 in stress-induced expression of the NF-κB target genes in the gastric mucosa. As shown in Fig. 2, TNF-α, MIP-2, and ICAM-1 mRNA were significantly up-regulated in the stressed WT mice, but their levels were markedly diminished in stressed $p50^{-/-}$ mice.

3.3. Effects of p50 deficiency on stress-induced MPO activity

Tissue MPO activity in the gastric mucosa from WT mice increased 3.5 h after initiation of stress. The stress-induced increase of MPO activity in the gastric mucosa was suppressed in $p50^{-/-}$ mice (Fig. 3).

3.4. Effects of p50 deficiency on stress-induced gastric mucosal injury

No lesions were observed in non-stressed animals, but large numbers of hemorrhagic necrotic lesions were observed in the corpus mucosa in WT mice subjected to 3.5 h of stress. Deficiency in p50 prevented the formation of stress-induced lesions as assessed by gross and histological examination (Fig. 4A), causing a remarkable decrease in ulcer index as macroscopically evaluated (Fig. 4B).

4. Discussion

Inflammation plays an important role in human stress ulceration and in its animal model of cold immobilization stress-induced gastric ulcers [4,5], but the molecular mechanisms contributing to stress-induced gastric inflammation are unclear. The current study showed that deficiency of the NF-κB subunit p50 abrogates the κB DNA-binding activity in gastric mucosa and decreases cytokine expression, neutrophil recruitment, and mucosal damage in mice subjected to cold immobilization stress. These results demonstrated a crucial role for p50 in the gastric inflammatory in response to stress.

NF-κB, most commonly a heterodimer of p50 and p65, is an important and highly inducible transcriptional factor with a pivotal role in inducing genes involved in stress and inflammatory responses [6]. In this study, we used p50-knockout mice to investigate whether NF-κB activation is causally related to stress-induced gastric inflammation. We showed that the absence of p50 abolishes stress-induced activation of NF-κB and expression of NF-κB target genes, including TNF-α, MIP-2, and ICAM-1, suggesting that the induction of these inflammatory mediators in vivo by stress is an NF-κB-dependent process. We further found that corresponding with the decrement in expression of

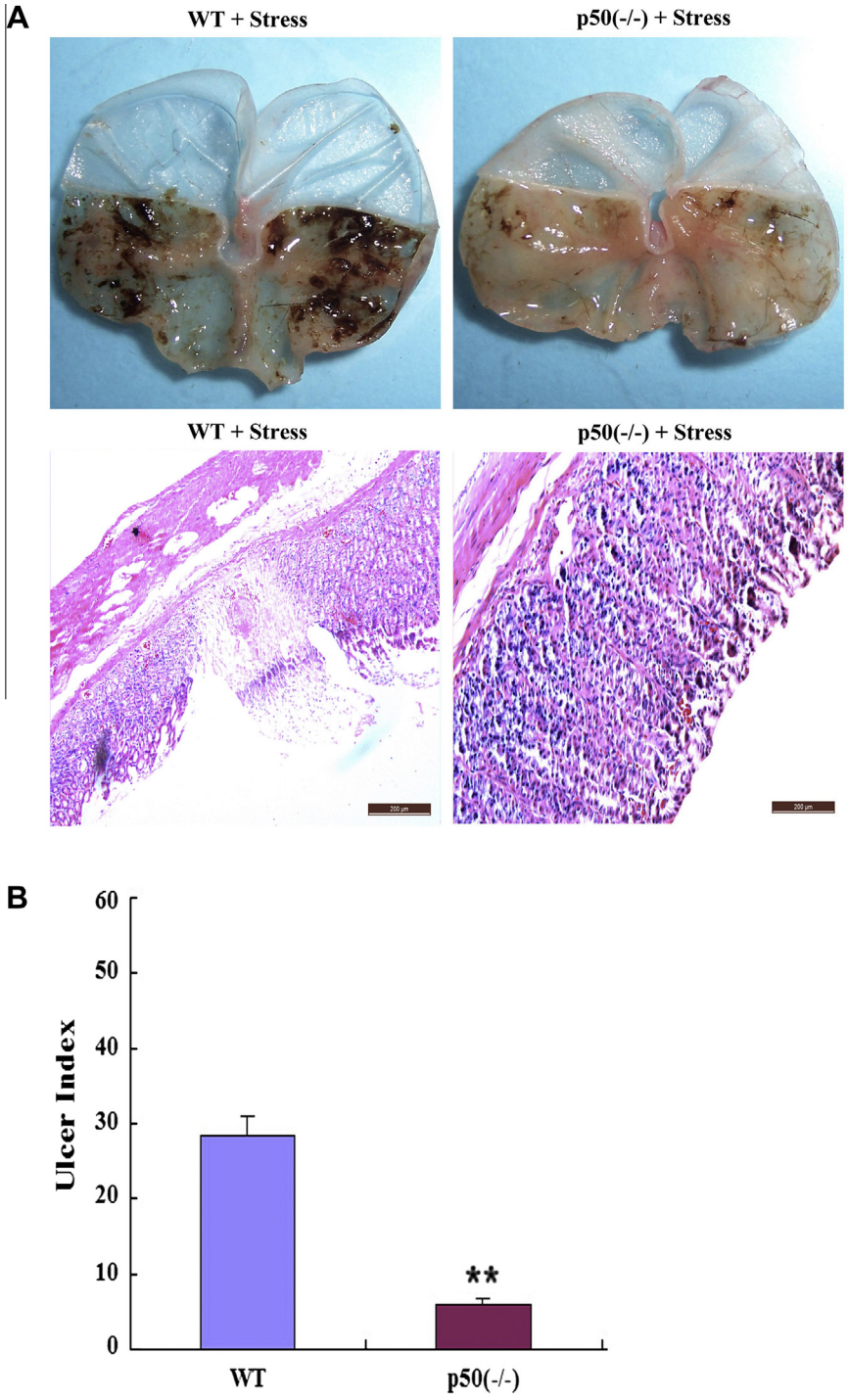

Fig. 4. Effects of p50 deficiency on stress-induced gastric injury. (A) Gross and microscopic appearance of gastric mucosa of stressed WT and $p50^{-/-}$ mice ($n = 6$). Note the numerous hemorrhagic and necrotic lesions in the corpus mucosa in stressed WT mice and the absence of lesions in stressed $p50^{-/-}$ mice. (B) Stress-induced gastric mucosal lesions were assessed macroscopically. Results were expressed as ulcer index. Values are mean ± SEM for 6–8 animals. $^{**}P < 0.01$ versus stressed WT animals.

inflammatory genes, neutrophil sequestration as assessed by tissue MPO activity and gastric damage are markedly reduced in $p50^{-/-}$ mice compared with WT mice undergoing stress. Together with our previous report using pharmacological inhibitors for NF-κB [7], the results of the present study with the use of specific genetic approach definitively defined a crucial role of NF-κB in the pathogenesis of stress-induced gastric mucosal inflammation and injury.

In our previous study [7], cold immobilization stress induced the nuclear translocation of NF-κB in the gastric mucosa, including p50/p65 homodimers as well as p50/p50 heterodimers. This study showed that the majority of κB DNA-binding activity in gastric mucosa induced by stress is abrogated from $p50^{-/-}$ mice. These data indicated that the protective effect of p50 deficiency in this model is mediated through a combination of deficient p50/p65 and p50/p50 activity. Among the NF-κB subunit family, p65 has transcriptional activity whereas p50 lacks transcription activation domains but is involved in transcriptional regulation [10,11]. In contrast to the essential role of p65 in inducing gene expression

[12], p50 can either increase or decrease gene expression [13,14]. In different inflammation-associated disease models, p50 deficiency has been reported to decrease [15], increase [16,17], or not affect [18] inflammation in vivo. For example, in the case of LPS-induced pulmonary inflammation, the absence of p50 was associated with an increased expression of proinflammatory genes [16]. In experimental models of the inflammation induced by ischemia/reperfusion, p50 deletion has an anti-inflammatory effect against myocardial ischemia/reperfusion injury [15] but it does not alter the hepatic inflammatory response to ischemia/reperfusion [18]. Reasons for these discrepancies may be the different experimental models used. Our results showed that the cold immobilization stress-induced activation of NF-κB and the resultant expression of NF-κB-dependent genes, including TNF-α, MIP-2, and ICAM-1 mRNA, were abolished in the $p50^{-/-}$ mice, suggesting that p50 is an essential component of the activated NF-κB complex and that p50 plays a pro-inflammatory role in our experimental condition and inflammatory context.

In summary, we found that deletion of the NF-κB subunit p50 decreases the activation of NF-κB and the inflammatory response induced by cold immobilization stress. Our findings demonstrated a central role for p50 in the pathogenesis of stress-induced gastric mucosal inflammation and gastric injury.

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