IL-18 mediates the formation of stress-induced, histamine-dependent gastric lesions

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STRESS ULCER was a highly prevalent occurrence, and acute gastric mucosal lesions represent a serious clinical problem. It is important to understand the mechanism of formation of stress ulcers, because it may suggest a means for the prevention and treatment of stress-related organ injury. Several factors including reactive oxygen species, cytokines, and bioactive amines have been implicated in the stress ulcer formation.

Water immersion and restraint stress (WRS) is commonly used to study stress-induced gastric lesions (26). Factors known to play a role in the induction of gastric lesions by WRS include gastric acid (13, 34) and oxygen-derived free radicals (18, 35).

It has been shown that histamine stimulates gastric acid secretion and prevent gastric lesion (13, 34). These results suggest that stress induces histamine to produce gastric acid excessively, leading to the formation of gastric lesions. However, how stress causes histamine synthesis is not clearly understood.

IL-18 was discovered as an IFN-γ-inducing proinflammatory factor (5, 20) but was later found to display multiple biological effects on immune and nonimmune systems (22). IL-18 is produced as a biologically inactive 24-kDa precursor and processed by caspase-1, proteinase-3, and Fas to a bioactive 18-kDa mature form (11, 19, 25, 29).

Recently, Sekiyama et al. (23) have reported that plasma IL-18 is upregulated in response to stress through a superoxide-mediated caspase-1 activation pathway, suggesting that IL-18 may influence pathological and physiological processes caused by stress. In the present study, we investigated the role of IL-18 in the induction of gastric lesions by stress, focusing on the involvement of histamine synthesis.

MATERIALS AND METHODS

Animals. Wild-type BALB/c mice were purchased from SHIMIZU Laboratory Supplies (Kyoto, Japan). BALB/c-background mice deficient of the IL-18 gene [IL-18 (knockout) KO mice, 8–10 wk old] were raised by backcrossing the original mutant mice for the IL-18 gene (27) with the BALB/c inbred background for more than eight generations at the National Institute for Agrobiological Sciences (Ibaraki, Japan). Homozygous mutant mice were used for breeding and experiments in our animal facilities. These mutant mice were healthy and devoid of apparent abnormalities in body weight and organ size for at least 16 wk after birth. They were kept at 22 ± 2°C. All experimental procedures were approved by the Animal Care Committee of Hyogo College of Medicine.

Reagents. The IL-18 ELISA kit, anti-mouse IL-18 monoclonal antibody (clone: 39-3F) for Western blot analysis, and horseradish peroxidase-conjugated goat anti-rat IgG were purchased from Medical Biological Laboratories (Tokyo, Japan). The histamine ELISA kit was purchased from Immunotech (Marseille, France). Protease inhibitor cocktail tablets, “Complete Mini,” were purchased from Roche Diagnostics (Mannheim, Germany). Monoclonal anti-β-actin antibody (A1978) for Western blot analysis and the histamine H2 receptor antagonist cimetidine (C4522) were obtained from Sigma (St. Louis, MO), and the glucocorticoid receptor antagonist mifepristone (RU-486) was from Roussel-UCLAF (Romainville, France). Recombinant mouse IL-18 (mIL-18) was kindly donated by GlaxoSmithKline Pharmaceuticals (PA, USA).

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Exposure of mice to WRS. Before each experiment, mice were deprived of solid food (MF, Oriental Yeast) for 24 h with access to tap water ad libitum. Animals were then restrained on an acrylic board by a plastic band and immersed in water at 25°C for 0, 1, and 2 h and killed with excess ether anesthesia.

Histological analysis of the stomach. The stomach of mice was removed, cut open with scissors, placed in PBS, and photographed by a Nikon digital camera (Tokyo, Japan). For histological evaluation, the stomach was fixed with 4% of neutralized phosphate-buffered paraformaldehyde (Sigma-Aldrich Japan, Tokyo, Japan), embedded in paraffin, cut into 5-µm sections, and stained with hematoxylin and eosin.

Assessment of gastric lesions. The bleeding index was determined by macroscopic assessments as follows: 0 points for no bleeding, 1 point for mild bleeding (a small amount of coagula in the stomach), 2 points for moderate bleeding, and 3 points for severe bleeding (contents of the stomach were filled with blood).

Measurement of IL-18. IL-18 in the serum was analyzed using the IL-18 ELISA kit. The sensitivity of the assay was 25.0 pg/mL. To assay gastric IL-18, the entire stomach was removed and cut open, and the gastric mucosal surface was exposed and rinsed with ice-cold PBS containing protease inhibitors. The stomach was homogenized and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was treated with a phosphorus cellulose powder (20 mg/ml) to remove histamine and centrifuged at 12,000 g for 40 min at 4°C. The supernatant (160 µl/H9262 was mixed with 40 µl of 0.1 M potassium phosphate buffer containing 0.2 mM DTT and 20 µM pyridoxal-5′-phosphate (Kinematica, Littau, Switzerland) and centrifuged at 12,000 g for 20 min at 4°C. The supernatant (0.2 ml) was poured into 3 ml of scintillation fluid and counted in a liquid scintillation counter.

Treatment of mice with IL-18 and receptor antagonists. Recombinant mouse IL-18 was dissolved in PBS containing heat-inactivated normal mouse serum (0.5%) and injected into mice subcutaneously at 2 µg/mouse 1 h before mice were exposed to WRS. Histamine H2 receptor antagonist cimetidine was dissolved in PBS and injected into mice subcutaneously at 100 mg/kg 1 h before mice were exposed to WRS. The glucocorticoid receptor antagonist mifepristone was dissolved in PBS containing 1% polyethylene glycol by chromatography in a Sephadex LH-20 column with added corticosterone antiserum (0.25 ml) and [3H]corticosterone (10,000 disintegrations/min) incubated at room temperature for 20 min. Ammonium sulfate (0.25 ml) was then added, and the samples were incubated for 10 min at room temperature and centrifuged for 10 min. The resulting supernatant (0.2 ml) was poured into 3 ml of scintillation fluid and counted in a liquid scintillation counter.

Measurement of serum corticosterone. Corticosterone levels in plasma were determined by an established radioimmunoassay. Plasma samples (0.1 ml) were diluted to 0.5 ml, extracted with ether (Sigma Chemical), and blown dry. Corticosterone in the extract was separated by chromatography in a Sephadex LH-20 column with added corticosterone antiserum (0.25 ml) and [3H]corticosterone (10,000 disintegrations/min) incubated at room temperature for 20 min. Ammonium sulfate (0.25 ml) was then added, and the samples were incubated for 10 min at room temperature and centrifuged for 10 min. The resulting supernatant (0.2 ml) was poured into 3 ml of scintillation fluid and counted in a liquid scintillation counter.

Assay of gastric histidine decarboxylase activity. The entire stomach was removed, frozen in liquid nitrogen immediately, and stored at −80°C until used. Histidine decarboxylase (HDC) activity was assayed according to Endo with a slight modification (15). The specimen was homogenized with Polytron homogenizer in ice-cold extraction buffer composed of 0.1 M potassium phosphate buffer (pH 6.8), 0.2 mM DTT, and 20 µM pyridoxal-5′-phosphate (Kinematica, Littau, Switzerland) and centrifuged at 12,000 g for 20 min at 4°C. The supernatant was treated with a phosphorus cellulose powder (20 mg/ml) to remove histamine and centrifuged at 12,000 g for 5 min at 4°C. The supernatant (160 µl) was mixed with 40 µl of 0.1 M potassium phosphate buffer containing 0.2 mM DTT and 20 µM pyridoxal-5′-phosphate (pH 6.8) and incubated with 1 mM L-histidine for 1 h at 37°C. The enzyme reaction was terminated by the addition of 100 µl of 6% HClO4, and histamine formed was quantified with the histamine ELISA kit.

Measurement of gastric histamine. The entire stomach was removed and homogenized in ice-cold Dulbecco’s PBS containing protease inhibitors. The homogenate was centrifuged at 12,000 g for 30 min at 4°C, and the supernatant was stored at −80°C until used. Histamine in the supernatant was measured by the histamine ELISA kit. The sensitivity of this assay was 0.1 pmol/ml.

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400 and injected into mice subcutaneously at 10 mg/kg 1 h before mice were exposed to WRS.

**Statistical analysis.** Data are expressed as means ± SD. The statistical significance of the difference between two means was evaluated using Student’s unpaired t-test. For analyzing multiple mean values, Dunnett’s multiple-comparison test was used after ANOVA. In these tests, P values of <0.05 were considered to be significant.

### RESULTS

**Effect of WRS on IL-18 concentration in the serum and stomach of wild-type mice.** Exposure of wild-type mice to WRS for 1 h caused significant increases in IL-18 levels in both the serum (Fig. 1A) and stomach (Fig. 1B). Western blot analysis showed that IL-18 in the gastric tissue before WRS was primarily in the 24-kDa precursor form (Fig. 1C), but after WRS, precursor protein levels decreased with concomitant increases in 18-kDa mature IL-18 levels (Fig. 1C).

**Induction of gastric lesions by WRS in wild-type and IL-18 KO mice.** In wild-type mice, 2 h of WRS caused extensive hemorrhage in the gastric mucosa (Fig. 2, B and C). In IL-18 KO mice, hemorrhage was also observed, but to a much lesser extent (Fig. 2, E and F). When IL-18 KO mice were pretreated with rmIL-18 and exposed to WRS, extensive hemorrhage similar to that in wild-type mice was observed (Fig. 2, H and I, and Table 1). Treatment of wild-type mice with rmIL-18 for 2 h caused no macroscopic gastric mucosal lesions, ruling out that IL-18 mediates mucosal hemorrhage (data not shown).

**Effect of WRS on gastric acid secretion in wild-type and IL-18 KO mice.** The effect of WRS on gastric acid secretion was compared between wild-type and IL-18 KO mice. Before WRS, gastric acid levels were not significantly different in wild-type and IL-18 KO mice, but after WRS, gastric acid secretion in IL-18 KO mice was much less than that in wild-type mice. Administration of rmIL-18 to IL-18 KO mice before WRS resulted in increases in gastric acid secretion to the same level as in wild-type mice after WRS (Fig. 3). On the other hand, rmIL-18 had no effect on the basal gastric acid secretion before WRS (Fig. 3).

**Effect of IL-18 on the induction of gastric histamine by WRS.** We analyzed the effect of WRS on gastric histamine concentrations in wild-type and IL-18 KO mice. After 2 h of WRS, there were significant increases in gastric histamine concentrations in wild-type mice but not in IL-18 KO mice (Fig. 4).

### Table 1. Comparison of bleeding index in wild-type and IL-18 KO mice after WRS

<table>
<thead>
<tr>
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<th>1 h</th>
<th>2 h</th>
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<tr>
<td>Wild-type</td>
<td>1.70±0.47</td>
<td>2.57±0.51</td>
</tr>
<tr>
<td>IL-18 KO</td>
<td>0.91±0.60</td>
<td>1.09±0.51</td>
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<tr>
<td>IL-18 KO treated with rmIL-18</td>
<td>1.52±0.51</td>
<td>2.39±0.58</td>
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Values are expressed as means ± SD; n = 22 mice/group. KO, knockout; WRS, water immersion and restraint stress; rmIL-18, recombinant mouse IL-18. *P < 0.05, IL-18 KO mice compared with wild-type mice or compared with rmIL-18-pretreated IL-18 KO mice.

**Fig. 2.** Effect of WRS on gastric tissue of wild-type, IL-18 knockout (KO), and recombinant mouse (rm)IL-18-pretreated IL-18 KO mice. A and B: stomach of wild-type mice before (A) and after (B) WRS. C: microscopic analysis of the gastric mucosa of a wild-type mouse after WRS. D and E: stomach of an IL-18 KO mouse before (D) and after (E) WRS. F: microscopic analysis of the gastric mucosa of an IL-18 KO mouse after WRS. G and H: stomach of rmIL-18-pretreated IL-18 KO mice before (G) and after (H) WRS. I: microscopic analysis of the gastric mucosa of a rmIL-18-pretreated IL-18 KO mouse after WRS. Bar = 5 mm. Images in C, F, and I were stained with hematoxylin and eosin. Magnification: ×10.

**Fig. 3.** Secretion of gastric acid in wild-type, IL-18 KO, and rmIL-18-pretreated IL-18 KO mice before and after WRS. Data are presented as means ± SD; n = 4–6. *P < 0.05 and **P < 0.01 compared with before WRS; #P < 0.05 and ##P < 0.01, IL-18 KO mice compared with wild-type mice or compared with rmIL-18-pretreated IL-18 KO mice.
Pretreatment of IL-18 KO mice with rmIL-18 resulted in increases in WRS-induced gastric histamine concentrations to the level observed in wild-type mice (Fig. 4). In the absence of stress, IL-18 had no effect on gastric histamine concentrations in both wild-type and IL-18 KO mice (data not shown).

**Effect of IL-18 on the induction of gastric HDC activity by WRS.** To explore the mechanism by which IL-18 mediates WRS-induced gastric histamine secretion, gastric HDC activity was measured in wild-type and IL-18 KO mice. In both wild-type and IL-18 KO mice, WRS caused marked increases in HDC activity, although the absolute values of HDC activity were lower in IL-18 KO mice than in wild-type mice (Fig. 5). Pretreatment of IL-18 KO mice with rmIL-18 resulted in an elevation of gastric HDC activity both before and after WRS to similar levels as observed in wild-type mice (Fig. 5).

**Effect of cimetidine on WRS-induced secretion of gastric acid and IL-18 and formation of gastric lesions in wild-type mice.** To examine the participation of histamine in WRS-induced gastric acid secretion, effect of cimetidine, a histamine H2 receptor antagonist, on the secretion of gastric acid was analyzed in wild-type mice. Cimetidine significantly decreased gastric acid secretion after WRS (Fig. 6A). Omeprazole, a proton pump inhibitor that inhibits acid secretion independently of H2 receptors, also decreased gastric acid secretion (Fig. 6A). Cimetidine decreased WRS-induced gastric lesions (Table 2) but did not affect the WRS-induced elevation of gastric IL-18 levels (Fig. 6B).

**Effect of IL-18 on the elevation of serum corticosterone levels induced by WRS.** It is well known that serum corticosterone levels increase under stressed conditions and that glucocorticoids mediate stress-induced ulcer formation. To examine whether corticosterone was involved in IL-18-mediating stress-induced gastric hemorrhage, we measured corticosterone in the serum of wild-type and IL-18 KO mice. Serum corticosterone levels in IL-18 KO mice were almost half of those in wild-type mice before WRS (Fig. 7A). After 2 h of WRS, serum corticosterone levels increased 1.6-fold in wild-type mice and 1.7-fold in IL-18 KO mice (Fig. 7A). The absolute value of serum corticosterone in IL-18 KO mice after WRS was 10.220.33.6 by 10.220.33.6 on October 21, 2017 http://ajpgi.physiology.org/ Downloaded from...
wild-type mice treated with mifepristone. Data are presented as means ± SD; n = 4–6. **P < 0.01 compared with before WRS; #P < 0.01, wild-type mice compared with IL-18 KO mice (by Student’s t-test). B: gastric IL-18 concentrations in wild-type mice treated with mifepristone. Data are presented as means ± SD; n = 4–6. *P < 0.05 compared with before WRS (by Student’s t-test). C: gastric histamine concentrations in wild-type mice treated with mifepristone. Data are presented as means ± SD; n = 4–6. *P < 0.05 and **P < 0.01 compared with before WRS (by Student’s t-test).

WRS was still below that in wild-type mice before WRS (Fig. 7A). Pretreatment of wild-type mice with a glucocorticoid receptor antagonist, mifepristone, caused the formation of much more severe gastric lesions after 1 h of WRS (Table 2). Mifepristone had no effect on levels of gastric IL-18 (Fig. 7C) and histamine (Fig. 7C) with or without WRS.

**DISCUSSION**

Stress-related mucosal bleeding is a significant cause of morbidity and mortality in critically ill patients (16). It is likely that multiple factors play a role in the induction of gastric lesions, but little is known about mechanisms involved.

WRS used in this study has been widely used to induce gastric lesions in animals, providing a model system for studying stress-induced ulcers in humans (4, 21, 26, 30). Considering the severe nature of the WRS procedure, we first conducted a time-course study for gastric hemorrhage induction and applied the minimum time required, 1–2 h, in all the experiments in this study.

It has been demonstrated that stress induces superoxide anions, activates caspase-1, which mediates the conversion of IL-18 precursors to mature proteins to be secreted into serum (23). In this study, our results suggested that IL-18 may play a role as a mediator in the formation of stress-induced gastric lesions, acting in collaboration with other factors induced by stress. It has been shown that stress causes increases in plasma norepinephrine and epinephrine levels, and inhibitors of adrenaline receptors or sympathetic nerve excitation reduce stress-induced syndromes (1). Thus, the collaboration of IL-18 and excessive peripheral sympathetic nerve excitation may exacerbate gastric hemorrhage after WRS.

It has been reported that overproduction of gastric acid is the most important factor in the formation of gastric lesions caused by stress (14). Histamine, a multifunctional biogenic amine involved in intercellular communications and inflammatory processes, is a strong stimulator of gastric acid secretion via H2 receptor activation (6, 12, 28, 31). In the present study, we observed that the production of WRS-induced gastric lesions was related to excessive gastric acid secretion stimulated by histamine via the H2 receptor. In IL-18 KO mice, WRS caused little elevation of gastric histamine levels and gastric injury, suggesting that IL-18 mediates the stress-induced secretion of histamine. Cimetidine, which inhibits the interaction between histamine and the H2 receptor, reduced gastric acid secretion with no effect on IL-18 production, indicating that IL-18 functions upstream of the histamine-H2 receptor interaction.

Histamine biosynthesis includes a decarboxylation step catalyzed by HDC (17). It has been shown that gastric HDC activity is elevated by various types of stress including WRS and prolonged walking (3). IL-18 has also been reported to induce HDC activity in the lung, liver, spleen, and bone in mice (33), suggesting that WRS elevates gastric HDC activity via IL-18. This is supported by the observation that in IL-18 KO mice, the HDC activity was low and, although it was elevated by WRS, the level was equivalent to that in wild-type mice without WRS. Moreover, pretreatment of IL-18 KO mice with rmIL-18 resulted in increases in gastric HDC activity to similar levels observed in wild-type mice both before and after WRS.

It is known that stress causes release of glucocorticoids at high levels to induce gastric ulcers, possibly by changing the microcirculation (2, 24, 32). In this study, however, we found that the glucocorticoid receptor antagonist mifepristone aggragated stress-induced gastric hemorrhage, as reported by others (8–10), without affecting the induction of gastric IL-18 and histamine by WRS. These results indicate that corticosterone released after WRS might be protective rather than injurious to the stomach. In addition, we found that the basal serum corticosterone level was lower in IL-18 KO mice than in wild-type mice even after WRS. Although WRS caused an elevation of serum corticosterone levels in IL-18 KO mice, it failed to induce gastric lesions in these mice. Thus, we conclude that glucocorticoids are not involved in the IL-18-mediated formation of stress-induced gastric lesions.

In summary, we demonstrated that stress-induced IL-18 enhanced HDC enzyme activity to stimulate histamine synthesis, which in turn leads to the activation of H2 receptors and the formation of gastric lesions. Our findings suggest that IL-18 mediation of stress-induced gastric injuries.
may be a novel therapeutic target to prevent stress-induced gastric ulcers.

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