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Synergistic upregulation of inducible nitric oxide synthase and cyclooxygenase-2 in gastric mucosa of Mongolian gerbils by a high-salt diet and *Helicobacter pylori* infection

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Summary. Aims: The intake of salt and salty food is known as a risk factor for gastric cancer. We have previously demonstrated that a high-salt diet dose-dependently enhances *Helicobacter pylori* (*H. pylori*)-associated gastritis and stomach carcinogenesis in Mongolian gerbils. In this study, we focused on the influence of excessive salt intake on the expression of inflammatory mediators involved in progression of *H. pylori*-induced chronic gastritis.

Methods and Results: A total of 45 stomach samples from Mongolian gerbils were evaluated by immunohistochemistry. The animals were infected with *H. pylori* and fed basal (0.32%) or a high-salt (10%) diet, and sacrificed after 40 weeks. Proliferative activity and expression of cyclooxygenase-2 (COX-2) in gastric mucosa were significantly increased in *H. pylori*-infected gerbils. The additional high-salt diet significantly up-regulated the expression of inducible nitric oxide synthase (iNOS) and COX-2 in *H. pylori*-infected groups ($P<0.01$ and $P<0.05$, respectively), while no significant effects were noted in non-infected animals. There was significant synergistic interaction between *H. pylori* infection and 10% NaCl diet on the expression of iNOS ($P<0.05$) and also a tendency for enhanced COX-2 expression ($P=0.0599$).

Conclusions: The present results suggest that a high-salt diet works synergistically with *H. pylori* infection to enhance iNOS and COX-2 expression in the gastric mucosa of Mongolian gerbils, and support the hypothesis that excessive salt intake may be associated with progression of *H. pylori*-induced gastritis.

Key words: Salt, Gastritis, *Helicobacter pylori*, iNOS, COX-2

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Introduction

Helicobacter pylori (H. pylori) is a major causative factor for gastric disorders and epidemiological evidence has accumulated indicating a significant relationship with gastric cancer development (Marshall and Warren, 1984; Uemura et al., 2001). In 1994, the World Health Organization/International Agency for Research on Cancer concluded that *H. pylori* is a "definite carcinogen" based on the epidemiological findings (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). Recently, the concept that inflammation is a critical component of tumor progression has received a great deal of attention (Coussens and Werb, 2002). It is now known that there is a strong association between *H. pylori*-induced chronic atrophic gastritis and development of gastric cancer (Correa, 1995). Mongolian gerbils can readily be infected with *H. pylori,* and the resultant chronic active gastritis, peptic ulcers and intestinal metaplasia resemble lesions also apparent in humans (Hirayama et al., 1996; Sugiyama et al., 1998). We have previously reported that the severity of gastritis plays an important role in *H. pylori*-associated gastric carcinogenesis in gerbils, with essential involvement of chronic inflammation and increased rates of cell proliferation (Cao et al., 2007). Thus, investigation of the progression mechanisms of gastritis and the search for crucial factors for chemoprevention of gastric cancer continues to be very important.

Environmental and host factors are also known to influence gastric carcinogenesis, and salt (sodium chloride, NaCl) and salty foods are probably of particular importance, based on evidence from a large number of case-control and other epidemiological studies (Joossens et al., 1996; Kono and Hirohata, 1996; Tsugane, 2005). In Japan, foods containing salt at concentrations up to 12% are commonly consumed, such as pickled vegetables (salt content: 1-10%) and salted fish roe or fish preserves (6-12%) (Tsugane et al., 2004), and it has been reported that restriction of salty food

intake may decrease the risk of gastric cancer (Tajima and Tominaga, 1985; Shikata et al., 2006). In addition, several studies in mice and gerbils indicate that chronic excessive salt in the diet exerts synergistic effects with *H. pylori* infection on progression of gastritis and mucosal hyperplasia, also enhancing *H. pylori* colonization (Fox et al., 1999; Gamboa-Dominguez et al., 2007). Thus, the association between *H. pylori* and NaCl appears to be important for the progression of gastritis and the associated carcinogenesis, although the detailed mechanisms remain to be resolved.

It has been reported that *H. pylori* infection induces the expression of pro-inflammatory cytokines and enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the gastric mucosa of rodents and humans (Jackson et al., 2000; Yamaoka et al., 2005; Bancel et al., 2006). In addition, a recent *in vitro* study demonstrated that NaCl could affect the production of interleukin (IL)-1ß, IL-6 and tumor necrosis factor-α induced by VacA, which is a virulence factor of *H. pylori*, in the AGS gastric cancer cell line (Sun et al., 2006). To our knowledge, however, there is limited information on the influence of long-term salt intake on *in vivo* expression of mediators of inflammation and proliferative activity. In the present study, we therefore examined whether a high-salt diet might increase epithelial proliferation and expression of iNOS and COX-2 assessed immunohistochemically in Mongolian gerbils at 40 weeks after *H. pylori* infection.

Materials and methods

Experimental design

The precise experimental design was as previously described (Kato et al., 2006). In the present study, 45 stomach samples from Mongolian gerbils (*Meriones unguiculatus*; MGS/Sea, Seac Yoshitomi, Fukuoka, Japan) were examined (Fig. 1). Briefly, the gerbils were divided into 4 groups (groups A-D). Groups A and B were inoculated with $1x10^8$ colony-forming unit of *H. pylori* (ATCC43504, American Type Culture Collection, Rockville, MD, USA) intra-gastrically, while groups C and D were inoculated with sterile Brucella broth (Becton Dickinson, Cockeysville, MD, USA). *H. pylori* was prepared by the same method as described previously (Shimizu et al., 1999). From weeks 1 to 40, the animals of groups A and C received a diet including 10% sodium chloride and those in groups B and D were maintained on basal diet (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) containing 0.32% NaCl. At week 40, all gerbils were intraperitoneally injected with 5'-bromo-2'-deoxyuridine (BrdU) at a dose of 100 mg/kg, 60 minutes before sacrifice. The animals were subjected to deep anesthesia and laparotomy with excision of the stomach. The excised stomachs were fixed in 10% neutral-buffered formalin and sliced along the longitudinal axis into 4 to 8 strips of equal width, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for histological examination. The experimental design was approved by the Animal Care Committee of Aichi Cancer Center Research Institute, and the animals were cared for in accordance with the institutional guidelines.

Immunohistochemistry to assess epithelial proliferation and inflammatory enzymes

Immunohistochemical analysis of BrdU, iNOS and COX-2 was carried out as previously described (Ikeno et al., 1999; Tanaka et al., 2006). Briefly, serial sections were deparaffinized and hydrated through a graded series of ethanols, and immersed in 0.3% hydrogen peroxide/methanol solution for inhibition of endogenous peroxidase activity. For antigen retrieval, sections for iNOS and COX-2 were microwaved in 10 mM citrate buffer (pH 6.0) for 10 minutes, and sections for BrdU were incubated in 5N hydrochloric acid for 30 minutes at room temperature. The sections were then incubated with primary antibodies: a mouse monoclonal anti-BrdU antibody (clone Bu20a, diluted 1:1,000, Dako, Glostrup, Denmark), a rabbit polyclonal anti-iNOS antibody (Saito et al., 2002) (diluted 1:500, Calbiochem, San Diego, CA, USA), or a mouse monoclonal anti-COX-2 antibody (Marrogi et al., 2000; Shiotani et al., 2001) (clone 33, diluted 1:100, BD Biosciences, San Jose, CA, USA). Staining for BrdU and COX-2 was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and the Fast Red Substrate System (Dako), respectively. Sections for iNOS were incubated with biotinylated secondary antibody (swine-anti rabbit IgG, Dako), and avidin-biotinylated horseradish peroxidase complexes were visualized using 0.05% 3,3'-diaminobenzidine. All sections were counterstained with hematoxylin.

The numbers of BrdU-labeled cells in gastric mucosa were counted under a microscope, and indices were determined as the mean percentages of positive epithelial cells among totals of 90 different arbitrarily selected glands (including 60 glands in each corpus and 30 in each antrum). The degree of iNOS immuno-

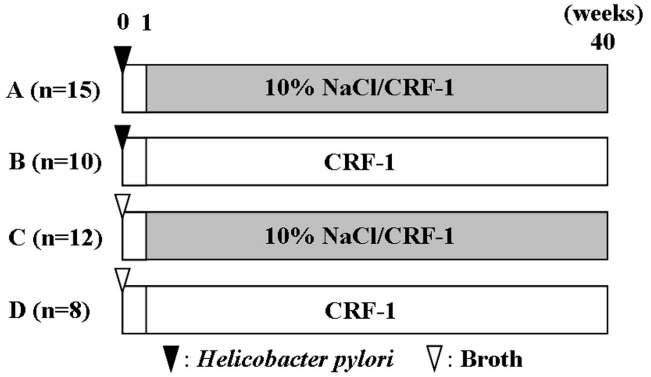

Fig. 1. Experimental design. Specific pathogen-free male, 4-month-old Mongolian gerbils were inoculated with *H. pylori* ATCC43504 strain (groups **A and B**) or Broth (groups **C and D**). Animals of groups A and C were given CRF-1 diet containing 10% NaCl from weeks 1 to 40.

positivity was expressed as the numbers of iNOS-positive cells in the total mucosal length. To quantitate the degree of COX-2 stainability, we measured the length of COX-2 positive areas per total mucosal length. The average mucosal lengths measured for evaluation of iNOS and COX-2 expression were 75.4±20.7 and 76.2±21.1 mm (means±SD), respectively.

Statistical analysis

Differences in data between groups were analyzed using the two-way factorial analysis of variance (ANOVA), followed by the Scheffe's multiple comparison procedure. P values <0.05 were considered to be statistically significant.

Results

Macroscopic and histological findings

The gastric mucosa of all gerbils in groups A and B (*H. pylori*-infected groups) was generally thickened and

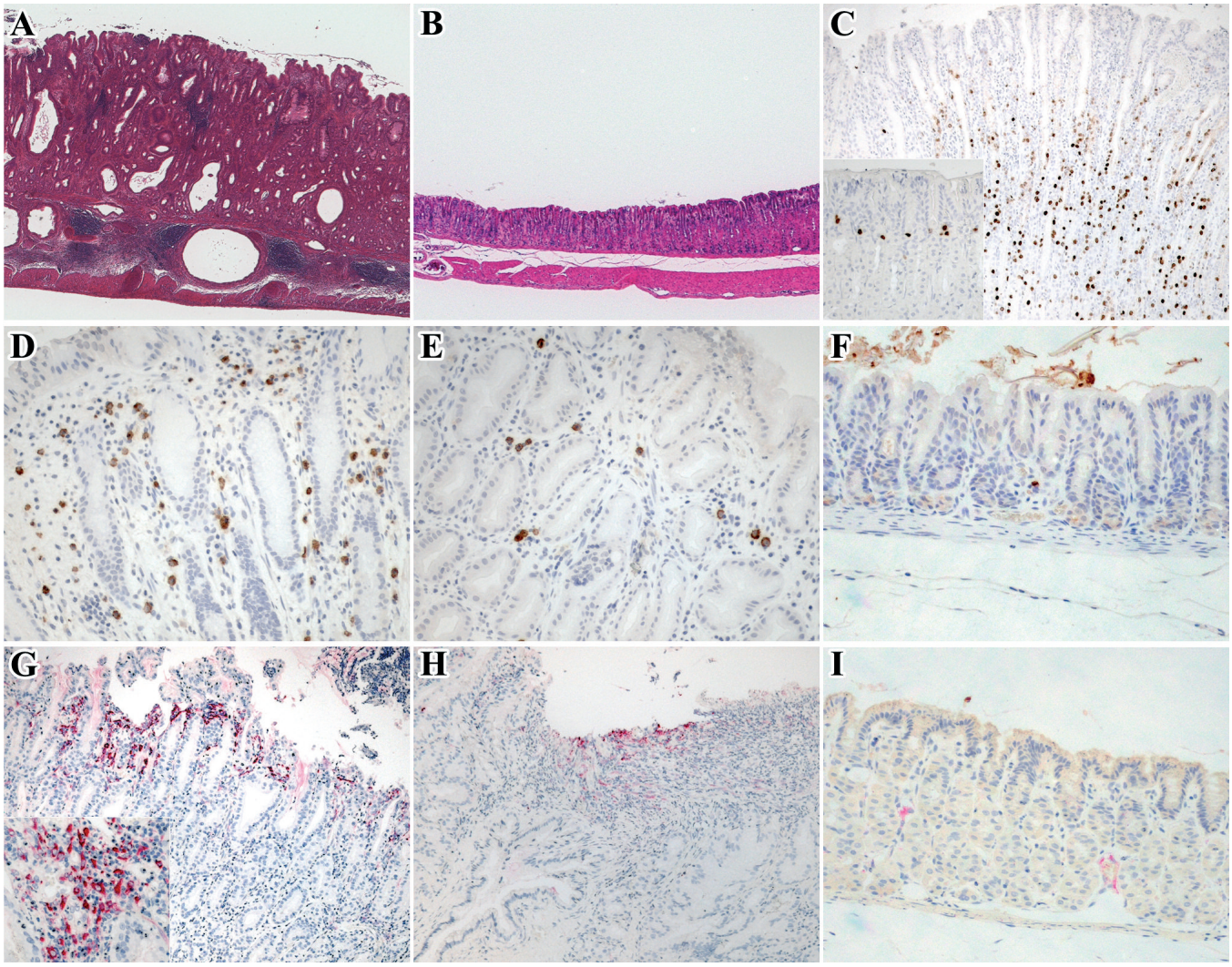

Fig. 2. Histopathology and immunohistochemistry of gastric mucosa of Mongolian gerbils. *H. pylori* + 10% NaCl group (**A, C, D and G**), *H. pylori* + basal diet group (**E and H**) and Broth + 10% NaCl group (**B, inset in C, F and I**). A and B. H&E staining. **A.** Note severe gastritis with infiltration of inflammatory cells, heterotopic proliferative glands, mucosal hyperplasia, and intestinal metaplasia at 40 weeks post-infection. x 50. **B.** No lesions were observed in gastric mucosa of non-infected and 10% NaCl diet-treated gerbils. x 50. **C.** Immunohistochemistry for BrdU. Large numbers of BrdU-positive cells are apparent in hyperplastic mucosal epithelium, while much fewer are present in the proliferative zone of a non-infected animal (inset). x 100. **D-F.** Immunohistochemistry for iNOS. **D and E.** Expression of iNOS mainly in mononuclear cells infiltrating in the lamina propria. x 200. **F.** In non-infected group, iNOS-positive cells were rarely observed in the lamina propria. x 200. **G-I.** Immunohistochemistry for COX-2. **G and H.** COX-2 is predominantly localized at the rims of areas of erosion or ulceration. Note expression localized in the cytoplasm of infiltrating mononuclear cells, fibroblasts and endothelium (**inset of G**). x 125. **I.** In the non-infected group, COX-2 staining was occasionally found in macrophages and endothelium. x 200.

edematous, occasionally with erosion and ulcers. In groups A and B, marked infiltration of neutrophils and mononuclear cells and formation of heterotopic proliferative glands were observed in the lamina propria and submucosa, occasionally with formation of lymphoid follicles. The histological examination also revealed various degrees of hyperplasia of the mucosa and intestinal metaplasia (Fig. 2A). Such macroscopic and histological lesions were not recognized in the stomachs of groups C and D (non-infected groups) (Fig. 2B). Detailed data for gastritis, including inflammation scores, have previously reported by our colleagues (Kato et al., 2006).

BrdU labeling indices for epithelial cells

BrdU-labeled epithelial cells in gastric mucosa were distributed mostly in the neck region of the hyperplastic polyps or in the proliferative zone of the non-hyperplastic mucosa (Fig. 2C). At week 40, BrdU labeling indices in *H. pylori*-infected groups were significantly greater than in non-infected groups (P<0.0001) (Fig. 3). The high-salt diet showed no significant effects of enhancement of epithelial proliferation both in *H. pylori*-infected groups and non-infected groups. There was no significant correlation between *H. pylori* infection and 10% NaCl diet in BrdU labeling indices (P=0.4785).

Immunohistochemistry of iNOS

In *H. pylor*i-infected gerbils, immunostaining for iNOS was located mainly in the cytoplasm of infiltrating mononuclear cells both in the lamina propria and submucosa (Fig. 2D,E). Expression was also detected in endothelium, segmented leukocytes, and gastric epithelial cells at lower frequency. At 40 weeks, iNOS expression in group A (*H. pylori*-infected and 10% NaCl diet-treated group) was significantly higher than in

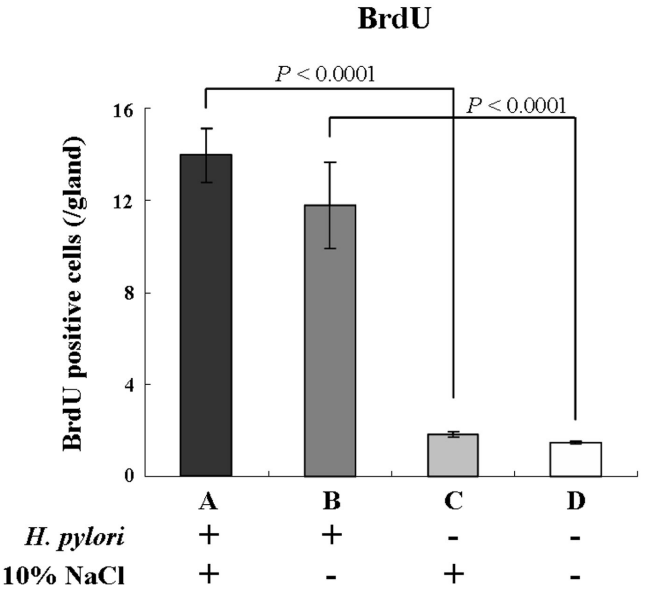

Fig. 3. Immunohistochemical analysis of epithelial cell proliferation in gastric mucosa of Mongolian gerbils by BrdU staining. Data are mean ± SE values.

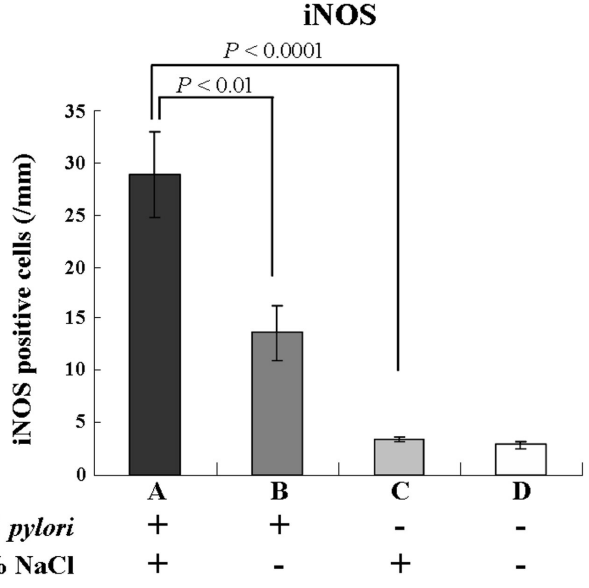

Fig. 4. Immunohistochemical analysis of iNOS expression in gastric mucosa of Mongolian gerbils. Data are mean ± SE values.

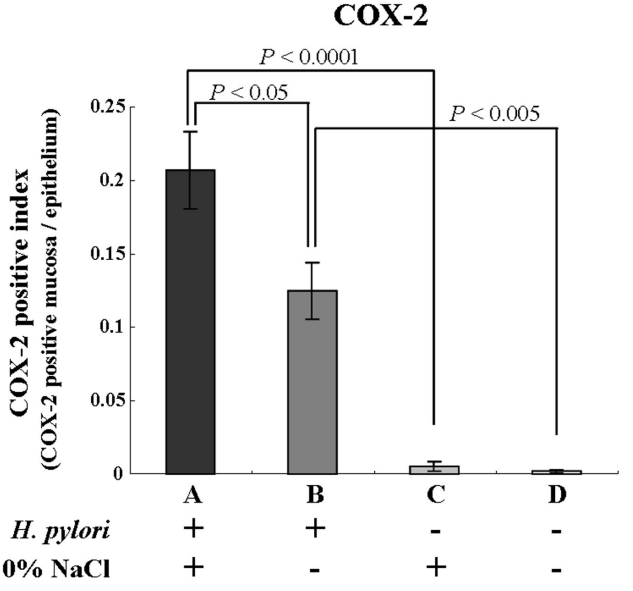

Fig. 5. Immunohistochemical analysis of COX-2 expression in gastric mucosa of Mongolian gerbils. Data are mean ± SE values.

group C (non-infected and 10% NaCl diet-treated group) (P<0.0001) (Fig. 4). Two-way factorial ANOVA revealed a significant interaction between *H. pylori* infection and excessive salt intake on iNOS expression (P<0.05). In *H. pylori*-infected groups, the numbers of iNOS-positive cells in group A (10% NaCl diet-treated) (28.8±4.12 cells/mm; means ± SE) were significantly higher than in group B (basal diet-treated) (13.7±2.63) (P<0.01). In non-infected groups, a high-salt diet showed no significant influence on frequency of iNOS expression (Fig. 2F).

Immunohistochemistry of COX-2

In *H. pylori*-infected groups, COX-2 protein was mainly detected in infiltrating mononuclear cells, fibroblasts, and endothelium in the lamina propria, particularly at the rims of erosion and ulcers (Fig. 2G,H), while a little COX-2 staining was observed in mononuclear cells and endothelium of non-infected gerbils (Fig. 2I). At 40 weeks, COX-2 expression in groups A and B (*H. pylori*-infected) was significantly greater than in groups C and D (non-infected) (P<0.0001 and *P*<0.005, respectively) (Fig. 5). Two-way factorial ANOVA showed a tendency for interaction between *H. pylori* infection and 10% NaCl diet on COX-2 expression, although this was not statistically significant (P=0.0599). In *H. pylori*-infected groups, the COX-2 positive index in group A (10% NaCl diet-treated) (0.21±0.03; means ± SE) was significantly higher than that in group B (basal diet-treated) (0.12±0.02) (*P*<0.05). There were no significant effects of salt on COX-2 immunoreactivity between the non-infected groups.

Discussion

It has been recognized that iNOS and COX-2 are involved in the processes of inflammation, carcinogenesis and its progression (Prescott and Fitzpatrick, 2000; Jaiswal et al., 2001). iNOS is expressed both by inflammatory cells and epithelial cells and the generated nitric oxide contributes to carcinogenesis during chronic inflammation. COX-2 is an inducible form of cyclooxygenase, which catalyzes the conversion of arachidonic acid to pro-carcinogenic eicosanoids such as prostaglandin, and is increased by various cytokines, growth factors and reactive oxygen species. A number of previous findings suggest that both iNOS and COX-2 are associated with *H. pylori*-induced gastritis in humans (Mannick et al., 1996; Jackson et al., 2000; Bhandari et al., 2005). In the present study, we showed COX-2 expression in gastric mucosa to be significantly enhanced by *H. pylori* infection in Mongolian gerbils, consistent with previous immunohistochemical studies in humans (Fu et al., 1999; Chen et al., 2006). In addition, our results demonstrated that a high-salt diet can further upregulate the expression of these two enzymes in *H. pylori*-infected gerbils. To our knowledge, this is the first report of synergistic effects of salt and *H. pylori* infection on the expression of iNOS and COX-2 in the glandular stomach of Mongolian gerbils. Rajnakova et al. (2001) reported using immunohistochemistry that iNOS and COX-2 expression may promote gastric cancer progression associated with an accumulation of p53. Furthermore, prognosis in patients expressing both iNOS and COX-2 appears to be significantly poorer than in those with single or no expression of these two genes (Chen et al., 2006). The results thus indicate a possibility that the co-expression of iNOS and COX-2 may not only promote gastric inflammation but also be a determinant factor for *H. pylori*-associated gastric carcinogenesis and prognostic outcome.

We have previously reported that excessive salt intake enhances *H. pylori*-associated gastritis and gastric cancer development in gerbils through alteration of the gastric mucus microenvironment (Kato et al., 2006). The present study showed that chronic salt administration enhances iNOS and COX-2 expression in gastric mucosa of *H. pylori*-infected gerbils. In non-infected gerbils, on the other hand, salt alone induced almost no lesions in stomach mucosa and had no promoting effects on iNOS and COX-2 expression. Furthermore, we found a significant synergistic effect between excessive salt intake and *H. pylori* infection on iNOS expression and a tendency to enhance COX-2 expression. The results suggest that increased expression of iNOS and COX-2 was induced not by a high-salt diet alone but by promoting effects of salt on *H. pylori*-activated inflammatory responses, and that excessive salt intake may be associated with the progression of *H. pylori*-induced gastritis. Further analysis is needed to clarify the interaction between co-expression of iNOS and COX-2 and progression of gastritis, because over-expression is not directly linked to functional activation.

In the present study, COX-2 immunostaining was observed in infiltrated mononuclear cells, fibroblasts and endothelium in the lamina propria, particularly at the edges of erosions and ulcers, consistent with previous reports on ulcerated gastric mucosa in humans and rodents (Mizuno et al., 1997; Takahashi et al., 1998; Jackson et al., 2000). These studies suggested that the localization of COX-2 may be associated with repair of mucosal injury. Our present results showed that excessive salt intake could significantly increase COX-2 expression in *H. pylori*-infected gerbils, without any influence on the localization. Since salt alone had no significant effects on COX-2 expression in non-infected gerbils, further up-regulation of COX-2 by salt intake in *H. pylori*-infected gerbils may be related to enhancement of *H. pylori*-induced gastritis rather than direct mucosal damage.

Several studies in rats have demonstrated that acute exposure to a highly concentrated NaCl solution may cause direct injury of the gastric surface epithelium, followed by rapid recovery with increased regenerative cellular proliferation (Charnley and Tannenbaum, 1985; Furihata et al., 1996). In the present study, on the other

hand, a 10% NaCl-containing diet had no significant effects on epithelial proliferation in gastric mucosa, independent of *H. pylori* infection. In addition, our previous study demonstrated that intermittent (once a week) administration of saturated NaCl solution for 40 weeks had no promoting effects on *H. pylori*-associated gastritis and carcinogenesis in gerbils (Kato et al., 2006). Therefore, we consider that continuous exposure to salt, rather than short-term and highly-concentrated salt intake, may be important to enhance *H. pylori*-induced gastritis, associated with increased expression of iNOS and COX-2 in the gastric mucosa. A recent study reported that osmoprotective genes promote cell survival against NaCl-induced hypertonic stress (Neuhofer et al., 2007). The osmoprotective activity might be one of the determinant factors for outcome with gastric epithelial cells exposed to various concentrations of NaCl, although detailed functions in the stomach are little understood.

In conclusion, the present study showed synergistic effects of salt with *H. pylori* infection on iNOS and COX-2 expression in the gastric mucosa of Mongolian gerbils. The results provide further support for the hypothesis that salt promotes progression of *H. pylori*-induced gastritis, and also raise the possibility that reduction of salt intake may decrease the risk of *H. pylori*-associated gastric cancer, compatible with previous epidemiological and experimental findings.

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