Synergism of *Helicobacter pylori* infection and stress on the augmentation of gastric mucosal damage and its prevention with α-tocopherol

Tae Young Oh\(^a\)\(^1\), Marie Yeoa\(^a\), Sang Uk Han\(^a\), Yong Kwan Cho\(^a\), Young Bae Kim\(^a\), Myung Hee Chung\(^b\), Yong Seok Kim\(^a\), Sung Won Cho\(^a\), Ki-Baik Hahm\(^a\)\(^*\)

\(^a\)Genomic Research Center for Gastroenterology, Ajou University School of Medicine, San 5 Wonchon-dong Yeongtong-gu, Suwon, 443-721, Korea

\(^b\)Department of Pharmacology, Seoul National University College of Medicine, Seoul, 110-799, Korea

Received 13 December 2004; revised 2 February 2005; accepted 2 February 2005

Available online 16 March 2005

Abstract

Despite evidence that *Helicobacter pylori* (*H. pylori*) infection is closely associated with stress in gastric ulcer patients, the underlying mechanism why ulcer recurrence after stress is augmented especially in patients with *H. pylori* remains unknown. In this study, we found that oxidative stress played a critical role in the augmented mucosal damage provoked by water immersion restraint stress (WIRS) in *H. pylori* infection and that an antioxidant, α-tocopherol, could ameliorate the aggravation of stress-associated gastric mucosal damage. Two hundred forty SD rats were divided into two groups according to *H. pylori* inoculation, and after 24 weeks of *H. pylori* infection, the water immersion restraint stress was imposed for 30, 120, or 480 min, respectively. To evaluate the therapeutic effects of an antioxidant, α-tocopherol was administrated 40 mg/kg daily prior to imposing WIRS. Remarkably increased hemorrhagic lesions and bleeding indexes were noted in the *H. pylori*-infected group with statistical significance (\(P < 0.05\)) compared to the noninfected group at the same duration of WIRS. Significantly higher oxidative stress documented by iNOS, lipid peroxides, and GSH level was detected in gastric homogenates of the *H. pylori*-infected group. Proteomic analysis using 2-dimensional electrophoresis showed a decrease of HSP27 and other chaperone proteins. α-Tocopherol pretreatment significantly prevented the gastric mucosal damage, caused by WIRS in the presence of *H. pylori*. α-Tocopherol induced HSP27 expression, which was well correlated with downregulation of iNOS mRNA. Conclusively, the presence of *H. pylori* caused significant deterioration of stress-induced gastric mucosal lesions through increased oxidative stress and thus antioxidant treatment such as α-tocopherol protected the gastric injuries.

© 2005 Elsevier Inc. All rights reserved.

Introduction

Since the clinical outcomes of *Helicobacter pylori* infection were influenced by an elaborate cross talk among the bacteria, infected host, and environmental situations, the existence of *H. pylori* itself does not explain fully the whole epto-pathogenesis of peptic ulcers [1–5]. This is further reflected by the fact that considerable populations of infected people did not develop ulcer disease and *H. pylori*-negative gastric ulcer patients were often found. Therefore, these clinical findings strongly suggest that other factors, such as stress, diet, smoking, sanitization, or host genetic background might contribute to the pathogenesis of peptic ulcers. Among these factors related to peptic ulcer disease, psychological stress has been revealed to trigger many ulcer diseases and impair the response to treatment [6–11].

Considerable prospective evidence has been gathered along with studies that have found a synergic relationship between *H. pylori* infection and psychological stress on ulcer formation. A study done immediately after the great
Hanshin Earthquake 1995 in Kobe, Japan, found that the recurrence rate of peptic ulcers in patients infected with *H. pylori* was much higher than that in patients in whom *H. pylori* had been eradicated [12]. Basically, and Yamamoto et al. [13] and Matsushima et al. [14] reported results suggesting the influence of *H. pylori* infection on the development of stress-induced gastric mucosal injury using animal experiments and human [13,14]. Despite these reports showing the close relationship between *H. pylori* infection and stress, the underlying mechanism was not revealed and therefore, no trial for prevention has been reported yet.

Recently, it is well known that heat-shock proteins (HSPs) might play a cytoprotective role in various gastrointestinal damage [15–20,27]. Nakamura et al. [18] demonstrated that cells inducing HSPs by heat-shock treatment showed considerable resistance against injury induced by 7.5% ethanol treatment in the guinea pig gastric mucosal cells and Hirakawa et al. [19] also reported that oral administration of geranylgeranylacetone (GGA), an antiulcer drug, rapidly induced HSP60, HSP70, HSC 70, and HSP90 in the rat gastric mucosal cells and these induced HSPs contributed to suppress ulcer formation by water immersion restraint stress. Therefore, drugs or treatments inducing HSPs could positively contribute to gastric mucosal defense and participate in definite cytoprotection [27].

α-Tocopherol (vitamin E) is a naturally occurring antioxidant in biological systems and is present in the cell membranes of various tissues, including the intestine and stomach [21–25]. Vitamin E prevents free radical-induced injury by blocking the free radical chain reaction. Previous studies have shown that deficiencies in vitamin E have resulted in peptic ulceration and that vitamin E supplementation to the diet has protective effects on the gastric mucosa [26].

Thus, we performed the current study to evaluate the fundamental mechanism of augmented mucosal injury by stress in the presence of *H. pylori* using experimental models of in vivo animals and its prevention with α-tocopherol. We found that the oxidative stress is principally involved in augmented mucosal damage after *H. pylori* infection and stress, and antioxidants, α-tocopherol in our study, can prevent the exaggeration of this gastric mucosal damage via induction of HSP27.

**Materials and methods**

**Animals**

Six-week-old specific-pathogen-free (SPF) Sprague-Dawley male rats (Charles River, Tokyo, Japan) were used for experiments. A total of 240 rats were fed a sterilized commercial pellet diet (Biogenicomics Co., Seoul, Korea), given sterile water ad libitum, and housed in an air-conditioned biohazard room designed for infectious animals, with a 12-h light and 12-h dark cycle. We divided the 120 rats into two groups; one group was a control group only with water immersion restraint stress (WIRS) (*n = 60*) and the other group was infected with *H. pylori* along with WIRS (*n = 60*). The remaining 120 rats were divided into another two groups, one group is *H. pylori*-infected rats with WIRS (*n = 60*) and the other (*n = 60*) was orally administered α-tocopherol 40 mg/kg in *H. pylori*-infected rats with WIRS. The 60 rats in each subgroup were further grouped into three time points of WIRS impose (30, 120, or 480 min). The rats were handled in an accredited animal facility in accordance with the AAALAC International Animal Care policies (Accredited Number of Ajou University School of Medicine: 2002-0611).

**H. pylori culture and infection**

*H. pylori* (SS1 strain) known to fulfill the Lausanne criteria were kindly provided in a frozen state by Prof. Adrian Lee. The bacteria were characterized with the typical S shape, gram-negative rods with modified Gram stain, and positive CagA, VacA, oxidase, urease, and catalase. For the liquid culture, *H. pylori* were inoculated into Brain Heart Infusion (BHI) broth supplemented with 10% sheep blood, 5% horse serum, and Skirrow’s supplement in an anaerobic jar with the micro-aerophilic gas-generating kit Campy-Pack-Plus (Becton Dickinson, USA) at 37°C and 5% O₂/10% CO₂/85% N₂ for 4 days. The rats were dosed three times for 3 weeks with 1 ml of bacterial suspension (approximately 1 × 10⁸ CFU/ml) by an oro-gastric tube designed for rats. Control rats were the given same amount of saline and were housed in isolators in order to prevent the risk of infection.

**Water immersion restraint stress**

Twenty-four weeks after inoculation of *H. pylori*, the rat was exposed to the water immersion restraint stress. The animals were deprived of food, but allowed free access to water 24 h before exposure of WIRS. Twenty rats in each group were placed in strain cages and immersed in water for 30, 120, or 480 min, respectively. Animals were sacrificed immediately after the end of the water immersion. Stomachs were removed and opened along the greater curvature, followed by rinsing with phosphate-buffered saline. In order to confirm the presence of *H. pylori* infection, a rapid urease test called CLO test (Pronto Dry, Solothurn, Switzerland) was applied to each rat. Isolated tissues were used for the following experiments including histological examination, ELISA, and Western blotting.

**Gross and histological observation**

The stomach isolated from the rat was placed in 10% buffered formalin and embedded in paraffin, and 4-μm
sections were cut. In order to determine the presence of *H. pylori* and for pathological findings, hematoxylin and eosin (H&E) staining and Warthin-Silver stainings were employed on formalin-fixed sections. According to the criteria of Yamamoto et al. [13], the bleeding index was evaluated on formalin-fixed sections and the bleeding rate (%) was calculated. Simply stated, index 0 means no bleeding at all; index 1 is mild bleeding, the presence of small amounts of coagula in the stomach; index 2 is moderate bleeding, intermediate between 1 and 3 points; index 3 is severe bleeding, contents of the stomach were filled with bleedings including coagula.

**TBA-reactive substance and cytokine measurement**

The concentration of thiobarbituric acid-reactive substances (TBARS), an index of lipid peroxidation, was measured in the gastric mucosal homogenates using the method of Ohkawa et al. [28]. The level of TBARS in the mucosal homogenate was expressed as picograms of malondialdehyde per milligram of protein using 1, 1, 3, 3-tetramethoxypropane as a standard.

The concentration of IFN-γ and TNF-α in the gastric tissue of the rat was measured by enzyme-linked immunosorbent assay (ELISA). The specimen was then homogenized for 90 s in phosphate-buffered saline (PBS) containing 0.1% sodium dodecyl sulfate (SDS) and 0.1% Tween 20. After lysis at 4°C overnight, it was centrifuged at 400 *g* for 15 min at 4°C. The supernatant was analyzed with a Biotrak Rat Cytokine ELISA System (Amersham International, Buckinghamshire, England).

**Two-dimensional gel electrophoresis (2-DE)**

The pooled samples of proteins from 7 rats of each group were used for 2-DE. Protein separation by isoelectric point was performed with an isoelectric focusing system (IPG-phor, Amersham Biosciences). Immobiline dry strips (18 cm, nonlinear pH 3–10, Amersham) were rehydrated for 16 h at room temperature in a rehydration buffer (8 M urea, 0.5% Chaps, 10 mM DTT, 0.002% BPB). Each hydrated strip containing 800 µg of protein sample was kept at 20°C in an electric field programed at 100 V for 2 h; 200 V for 1 h; 500 V for 1 h; 1000 V for 1 h; and 8000 V for 6.5 h. The IPG gel strip was incubated for 15 min with 1% DTT in water and then incubated overnight at 4°C with primary antibodies for HSP27, HSP70, or α-tubulin (Santa Cruz Biotech, Santa Cruz, CA), followed by probing with 1:2000 diluted HRP-conjugated secondary antibody. The immunocomplex were detected using the ECL detection kit (Amersham Pharmacia Biotech).

**Western blotting**

Gastric mucosa was homogenized in ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, 300 mM sucrose, 2 mM phenylmethylsulfonyl fluoride (PMSF) with a tissue homogenator at 4°C. Thirty micrograms of the protein was subjected to electrophoresis on an 8% SDS-PAGE gel and transferred onto PVDF membrane. The blots were blocked with 5% nonfat dry milk and then incubated overnight at 4°C with primary antibodies for HSP27, HSP70, or α-tubulin (Santa Cruz Biotech, Santa Cruz, CA), followed by probing with 1:2000 diluted HRP-conjugated secondary antibody. The immunocomplex were detected using the ECL detection kit (Amersham Pharmacia Biotech).

**In-gel digestion and peptide mass fingerprinting by MALDI-TOF MS**

Stained protein spots were excised from the gel and digested with trypsin (Promega, Madison, WI). After washing with 10 mM ammonium bicarbonate and 50% acetonitrile, gel pieces were swollen in digestion buffer (50 mM ammonium bicarbonate, 5 mM CaCl₂, 12.5 ng/µl trypsin) and incubated at 37°C for 16 h. The peptides were recovered by stage extraction using 50 mM ammonium bicarbonate and 100% acetonitrile. The peptide extracts were pooled, lyophilized in a vacuum centrifuge, and stored at −20°C. Dried samples were dissolved in 0.5% TFA solution. To improve the ionization efficiency of MALDI-TOF MS, sample peptides were purified with Zip-tip C18 (Millipore, Bedford, MA) before MS analysis. Equal volumes of peptide and matrix solution were mixed and crystallized on the sample plate. The matrix solution consisted of 10 mg α-cyano-4-hydroxy-cinnamic acid dissolved in 1 ml of a solution containing 50% acetonitrile and 0.5% TFA. The peptide solution was analyzed using a modified Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA). Internal mass calibration was performed using trypsin autodigestion products. The MSFit (http://www.prospector.ucsf.edu/ucsfhtml4.0/msfit.html) search program was used to search interpreted tandem mass spectra of tryptic digest peptides against the National Center for Biotechnology Information (NCBI) and SWISS-PROT data bases which are accessible at http://www.ncbi.nlm.nih.gov/ and http://www.expasy.com.

**Cell culture and H. pylori infection**

The human gastric epithelial (AGS) cells were cultured in RPMI 1640 (Gibco BRL, Gaithesberg, MD), supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FBS in a humidified 5% CO₂ atmosphere. For inoculation, *H. pylori* were resuspended in PBS to an *A*₅₀ of 1.2 units, corresponding to 5 × 10⁸ CFU/ml, and cocultured with AGS cells at a final concentration of 5 ×
$10^7$ CFU/ml. After 6 h of infection, the cells were washed with serum-free RPMI 1640 media three times and then treated 5 μM α-tocopherol for 8 h.

**Statistical assay**

Results are expressed as the mean ± SE. The data were analyzed by one-way analysis of variance (ANOVA), and the statistical significance between groups was determined by Duncan’s multiple range test. Statistical significance was accepted with a $P < 0.05$.

**Results**

*The synergism of both *H. pylori* and stress on augmented gastric mucosal damage*

While psychological stress by water immersion restraint for 30 min did not provoke significant gastric mucosal damage, WIRS over 120 min induced apparent hemorrhagic erosions in the rat stomach. After 480 min of WIRS, the gastric injury was aggravated by more severe hemorrhagic lesion with multifocal, deep, and large erosions, suggesting that the severity of the hemorrhagic erosion was dependent on the duration of exposure to WIRS (Fig. 1A, upper panel). Infection of *H. pylori* alone provoked gastric inflammation, but neither ulcerative lesion nor tumorous lesion were observed in the stomach up to 24 weeks after *H. pylori* infection in a rat model. After imposing WIRS to rats, we tested the presence of *H. pylori* using a biopsied rat stomach with the rapid urease test (CLO test), which is used frequently in the clinic to check the presence of *H. pylori* infection due to its convenience despite its slightly lower sensitivity compared to the urea breath test or pathology examination. Briefly, a small piece of stomach of the sacrificed rat was put onto the CLO test kit and defined as positive showing red color within 30 min. The colonization percentage of *H. pylori* in our rat model was 84.4% (152/180) in SD rats. Non-*H. pylori*-infected rats (15.6%, 28/180 rats) were excluded to

Fig. 1. Gross and microscopic findings showing the augmented gastric mucosal lesions in rats infected with *H. pylori* even after the same exposure of restraint water immersion stress (WIRS). Gross pathology of the stomach (A), bleeding index (B), and representative microscopic photography (C). At 30, 120, and 480 min of restraint water immersion stress without *H. pylori* infection (a–c); at 30; 120, and 480 min of restraint water immersion stress with *H. pylori* infection (d–f), respectively (magnification ×100).
subject to further patho-biological studies as well as statistical analysis. Infection of \textit{H. pylori} significantly aggravated this WIRS-induced gastric lesion in the same time points of WIRS than rats without \textit{H. pylori} infection (Fig. 1A, lower panel). The bleeding index was measured in each SD rat and the mean index is presented in Fig. 1B, which clearly demonstrates that \textit{H. pylori} infection provoked more severe degree of WIRS-induced gastric damage with statistical significance ($P < 0.05$). Especially, a bleeding index of 480 min WIRS alone was scored by 0.8, but WIRS with \textit{H. pylori} infection significantly increased up to 1.35 of bleeding score at the same WIRS time (Fig. 1B). We found that bleeding rate (%) also increased in the WIRS group with \textit{H. pylori} infection compared to the group with WIRS alone.

Microscopic pathological evaluation showed similar findings. As observed in gross observations the WIRS caused serious pathological changes in the stomach, severity of which was significantly increased in accordance with the duration of WIRS (Fig. 1C, upper panel). After 120 min of WIRS, focal erosions and inflammatory cell infiltrations were observed, the lesions of which were worsened to hemorrhagic necrosis of gastric mucosa at 480 min of WIRS (Fig. 1C, upper panel). However, in rats affected by both stress and \textit{H. pylori} infection, the gastric lesions were more aggravated and worsened than rats without \textit{H. pylori} infection under the same times of stress (Fig. 1C, lower panel). Many cells sloughed off into the gastric lumen and focal area of deep hemorrhagic necrosis was noted at 480 min of stress and \textit{H. pylori} infection. Conclusively, macro- and microscopic findings clearly demonstrated that \textit{H. pylori} infection synergistically aggravated the WIRS-induced gastric mucosal damage.

Enhancement of oxidative stress by \textit{H. pylori} infection in WIRS rats

The contents of gastric mucosal thiobarbituric acid-reactive substances, a marker of lipid peroxidation [28], were significantly increased in the WIRS group with \textit{H. pylori} infection compared to the WIRS alone group (Fig. 2A). At 480 min of WIRS, TBARS of \textit{H. pylori}-infected rats were increased by 4-fold than the \textit{H. pylori}-uninfected group. The contents of the reduced form of glutathione, GSH, were measured in rat gastric mucosal homogenates and showed significant decrease in the \textit{H. pylori}–infected group than noninfected rats under the same burden of WIRS (Fig. 2B). These changes of significantly increased lipid peroxidation and reduced GSH indicated that considerably higher burdening of oxidative stress occurred after WIRS in \textit{H. pylori}-infected rats. The data suggested that oxidative stress might contribute to the \textit{H. pylori}/WIRS-augmented gastric damage.

In the same time, we also measured the changes of INF-$\gamma$ and TNF-$\alpha$, both of which are effective cytokines for gastric inflammation. Production of INF-$\gamma$ was significantly increased in WIRS with \textit{H. pylori} infection than without \textit{H. pylori} infection in a time-dependent manner (Fig. 2C). At

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Biochemical changes between the \textit{H. pylori}-uninfected group and the \textit{H. pylori}-infected group according to times after restraint water immersion stress. The contents of TBARS (A), glutathione, reduced form (GSH) (B), INF-$\gamma$ (C), and TNF-$\alpha$ (D) were measured in gastric mucosa of rats. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively.}
\end{figure}
480 min of WIRS, IFN-γ was at a maximum level (79 pg/mg protein) and the levels were slightly decreased after 720 min. The group without *H. pylori* infection did not change levels of IFN-γ. The contents of TNF-α were increased with time dependency in both WIRS with *H. pylori* and without *H. pylori* infection (Fig. 2D), but the cytokine did not show a significant difference between two groups. The results indicated that IFN-γ might be a factor for the stress response in *H. pylori*-infected rats.

**Shift of protein profiling in augmented gastric mucosal damage of both *H. pylori* and stress**

We performed comparative proteomic analysis to evaluate the changes of protein expression profiling involved in the augmented gastric mucosal damage after both *H. pylori* infection and WIRS compared to WIRS alone. Proteins extracted from the rat stomach homogenate were subjected to proteomic analysis using 2-dimensional electrophoresis (Fig. 3A). We found a significantly decreased 23 spots in density in the *H. pylori*-infected rat compared to the noninfected control rat. Each was digested with trypsin and analyzed by MALDI-TOF MS and 18 proteins were identified (Table 1). Most of them were cellular structure-related proteins and some metabolic enzymes were included. Of all, we were interested in the decrease of several heat-shock proteins including HSP90, HSP70, and HSP60 because heat-shock proteins are well known to have a cytoprotective role in various gastrointestinal damage. A HSP90 spot disappeared after 120 min of WIRS in the *H. pylori*-infected rat (Fig. 3B, upper panel) and the basic form of HSP70 was decreased by WIRS treatment (Fig. 3B,

![Fig. 3](image_url)

*Fig. 3.* Proteomic analysis of gastric mucosa after restraint water immersion stress in *H. pylori*-infected rat stomach. (A) Gel images of 2-dimensional electrophoresis, (B) change of heat-shock protein expression (C), which was identified with MS spectrum analysis of the tryptic digestion of spots shown in (B).
In the animal experiment, at 120 min of WIRS, tocopherol (40 mg/kg) significantly attenuated the severity of WIRS-induced gastric mucosal damage. As shown in Fig. 4, treatment of infected rats, we evaluated whether treatment with antioxidants such as α-tocopherol can ameliorate the augmented gastric mucosal damage. As shown in Fig. 4, treatment of α-tocopherol (40 mg/kg) significantly attenuated the severity of WIRS-induced gastric mucosal damage in H. pylori-infected rats. In the animal experiment, at 120 min of WIRS, the H. pylori-infected rats had hemorrhagic lesions of the stomach and gastric damage was more aggravated according to exposure time of WIRS (Figs. 4A and C, upper panel). However, administration of α-tocopherol significantly prevented the gastric mucosal damage induced by 120 min WIRS in H. pylori-infected rats (Figs. 4A and C, lower panel). The bleeding index also indicated that pretreatment of α-tocopherol clearly attenuated the severity of the WIRS-induced gastric damage in H. pylori-infected rats (Fig. 4B). The levels of gastric mucosal TBARS were significantly decreased in rat groups pretreated with α-tocopherol than WIRS in the presence of H. pylori (Fig. 4D).

HSPs restoration by pretreatment of α-tocopherol

We evaluated change of HSPs expression after α-tocopherol treatment by Western blot analysis. HSP27 and HSP70 were significantly upregulated in the α-tocopherol-treated group compared to the group without α-tocopherol treatment in 30 min WIRS/H. pylori infection (Fig. 5A). Especially, HSP27 was not detected in the WIRS group with H. pylori infection, but its expression was increased by 3.63-fold in the group administrated with α-tocopherol. We previously reported that introduction of HSPs by HSPs inducers such as noncytotoxic heat shock or GGA treatment prevented iNOS expression provoked by the H. pylori infection response, which clearly suggested that the restored HSPs confer a cytoprotective role in H. pylori-infected gastric epithelial cells. We examined the biological significance of HSP27 induced by α-tocopherol in response to H. pylori infection [29]. Hence, To examine the possible cytoprotective effect of HSPs induction by α-tocopherol treatment on H. pylori-induced iNOS upregulation, we did immunohistochemical staining of iNOS and the result of HSPs restoration by pretreatment of α-tocopherol on the response of iNOS in a cell culture model. H. pylori infection definitely increased the expression of iNOS mRNA at 6 h of infection and decreased HSP27 expression conversely (Fig. 6A and B). The H. pylori-infected iNOS expression was completely suppressed by α-tocopherol treatment and then expression of iNOS was well correlated reversely with expression of HSP27 (Fig. 6). The induction of HSP27 showed the ability to attenuate iNOS expression. These results strongly suggested that induction of HSP27 confers cytoprotection through suppressing H. pylori-induced iNOS expression.

Discussion

The present study showed that H. pylori infection influenced the outcome of stress-induced gastropathy, for
which oxidative stress and dysregulated stress protein responses were principally responsible. Our findings show that the incidence or recurrence of gastric ulcers is highly prevalent in the population with *H. pylori* infection under a stressful environment or conditions, suggesting a possible susceptibility for psychosocial stress in *H. pylori*-infected cohorts and these events were closely associated with oxidative stress and deranged cytoprotective proteins of affected gastric mucosal cells in its pathogenesis. Thus, treatment of antioxidants such as $\alpha$-tocopherol can effectively prevent the aggravation of stress-induced gastric damage in *H. pylori* infection. The agent rescues cells from stress/*H. pylori*-induced damage via induction of HSPs, which are able to attenuate the oxidative stress response of iNOS.

The pathogenic relationship between *H. pylori* infection and various stresses has been revealed previously in several studies. Matsushima et al. reported that immediately after the Great Hanshin Earthquake in Kobe, the recurrence rate of peptic ulcers was higher in patients infected with *H. pylori* than in patients in whom *H. pylori* had been eradicated [12,14]. In previous data, the stress impose of WIRS for 30 min resulted in a significantly higher bleeding rate and bleeding index among *H. pylori*-infected mice compared with those in uninfected animals [13,15]. However, the mechanism and causal factor contributing to aggravation of gastric injury induced by emotional stress in a presence of *H. pylori* infection remain unknown. Hence here, we suggested for the first time that oxidative stress induced by *H. pylori* infection was responsible for stress-associated gastric injury, and that treatment of antioxidant potently prevented *H. pylori*/stress-induced gastric damage.

The defense mechanism of the gastric mucosa against several aggressive factors, such as hydrochloric acid, bile...
acid, and drugs like NSAIDs, mainly consisted of functional, humoral, and neuronal factors [30–33]. In detail, mucus-alkaline secretion, mucosal microcirculation, and gastric motility act as functional factors, while prostaglandins and nitric oxide act as humoral factors, and capsaicin-sensitive sensory neurons and substance P act as neuronal factors. In recent years, HSPs like HSP70 and HSP27 have been implicated to be an emerging important factor engaging in immediate and critical defense mechanisms of gastric mucosa at the intracellular levels. Mechanistically HSPs might contribute to mucosal defense mechanisms and ulcer healing, most probably through protecting key enzymes related to anti-inflammation, cytoprotection, and regeneration. Therefore, to keep appropriate levels of intracellular HSPs might be very important in the recovery of cells from stress and other cellular damage. In general, heat-shock proteins are crucial for the maintenance of cell integrity both during normal cell growth and under pathophysiological conditions. By controlling binding and release, HSPs function mainly as molecular chaperones, which participate in the folding and assembly of nascent and unfolding proteins and facilitate protein transport to subcellular compartments [34,35]. These HSPs are classi-
fied into four major families according to their biological activities and apparent molecular weights: HSP90, HSP70, HSP60, and small HSPs including HSP27. While HSP60, HSC70, and HSP90 are constitutively expressed, HSP70 and HSP27 are induced by various conditions, including heat, oxidative stress, or drug exposure [36]. Among the HSPs, HSP27 acts as an ATP-independent chaperone that interact with misfold or oxidized polypeptides creating reservoirs of folding intermediates. In response to oxidative stress, Hsp27 forms transient structures with native molecular masses ranging from 100 to 800 kDa that subsequently undergo phosphorylation-dependent deaggregation. The transient formation of a large oligomeric structure is responsible for the chaperone and protective activity of Hsp27 against oxidative stress [37].

In the current experiment, significant decreases in HSP70 and HSP27 expressions were noted after H. pylori infection, suggesting that a pathogenic mechanism of H. pylori infection in the stomach might be deranged cytoprotective action and these attenuated HSP expressions were further worsened in cases with both H. pylori infection and stress. Another finding in the current experiment related to deranged HSP27 after stress in the presence of H. pylori was that iNOS, one of the key mediators released to H. pylori infection, was significantly increased, suggesting that H. pylori can exert either its inflammatory stimuli or carcinogenic actions through iNOS [38,39]. Heat-shock response in many organs or many diseases status showed a correlation between iNOS downexpression, suggesting that HSPs inhibits iNOS DNA binding activity, sequentially resulting in the attenuation of transcription of iNOS, one of the major target genes of NF-κB transcriptional factors [40–42]. We also previously published that HSPs, especially Hsp70 and Hsp27, could export a cytoprotective role in a host response against H. pylori infection via suppression of iNOS expression [29].

Therefore, drug or chemicals possessing antioxidative and HSP-inducing action can prevent augmented gastric mucosal damage by both H. pylori infection and restraint stress. We selected α-tocopherol since α-tocopherol has been known to be a potent antioxidant and cytoprotective, especially in the stomach with H. pylori infection. Significantly lower levels of α-tocopherol and ascorbic acid were reported in the stomach of H. pylori infection [43], which is regarded as one of the critical mechanisms leading to H. pylori-associated atrophic gastritis and even gastric cancer. As anticipated, α-tocopherol pretreatment (40 mg/kg) significantly prevented the augmented injury of both H. pylori and WIRS-associated gastric mucosa. In accordance with effective protection of gross and microscopic findings, significantly attenuated levels of TBARS and considerable restoration of GSH, HSP70, and HSP27 were observed with α-tocopherol pretreatment, signifying that oxidative stress and dysregulated cytoprotective responses in the stomach wall were responsible for augmented gastric mucosa damage caused by H. pylori/WIRS and these could be corrected with the administration of α-tocopherol.

Conclusively, our study suggested that stress, one of the environmental factors, critically modified the outcomes of H. pylori infection and an antioxidative strategy can prevent augmented gastric mucosal damage related to stress and H. pylori infection. Therefore, the countries confronted with serious H. pylori-associated gastric lesions like gastric cancer or complicating ulcers must pay attention to relieving environmental or social stress and encouraging a high-risk population the intake of antioxidants.

Acknowledgments

This study was supported by a grant of The Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (01-PJ10-PG6-01GN14-0007, 03-PJ10-PG6-PG01-002) and R05-2004-12749-0 from the Ministry of Science and Technology. The authors thank Mr. W.J. Kim for assistance in preparing the manuscript and several researchers (D.K. Kim, Y.M. Won, H.K. Park, K.H. Park, J.S. Cho, J.H. Jin) working in the Genomic Research Center for Gastroenterology for experimental assistance.

References


