JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2001, 52, 1, 153–164

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HEAT SHOCK PROTEIN 70 (HSP70) IN GASTRIC ADAPTATION TO ASPIRIN IN *HELICOBACTER PYLORI* INFECTION

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We have recently shown that adaptation of gastric mucosa to aspirin (ASA) is disturbed in *Helicobacter pylori* (*H. pylori*)-infected human stomach, but can be restored by eradication of the *bacterium*. The aim of this study was 1) to evaluate the influence of *H. pylori* on expression of heat shock protein 70 (HSP70) during ASA ingestion in these subjects and in mice model and 2) to evaluate, whether altered HSP70 expression might be associated with different adaptation to ASA in *H. pylori*-positive and eradicated subjects. The gastric mucosal HSP 70 gene expression was determined by quantitative RT-PCR and Western blot and immunohistochemistry during 14 days of ASA ingestion (1 g bid) in the same 8 subjects before and 3 months after successful eradication of *H. pylori*. In addition, HSP70 mRNA and protein expression were examined in 30 mice without and with *H. pylori* infection and eradication. During 14 days of ASA treatment, human *H. pylori*-infected mucosa revealed a decrease of HSP70 expression, while after eradication a higher expression and further increase of HSP70 expression during ASA ingestion were observed. Mice inoculated with *H. pylori* also exhibited decreased gastric mucosal HSP70 mRNA expression that was restored after eradication therapy. Decreased basal and ASA-induced expression of HSP70 may partly be responsible for impaired gastric adaptation to ASA in *H. pylori*-positive subjects. We conclude that 1. The HSP70 gene and protein expression is reduced during infection with *H. pylori* in men and mice and that gastric adaptation to ASA in *H. pylori* eradicated subjects is accompanied by increased HSP70 expression; 2. It is reasonable to assume that decreased HSP70 expression might contribute to disturbed gastric adaptation in *H. pylori* infection in humans and 3. The expression of HSP70 plays an important role in the mechanism of gastric adaptation to ASA and that *H. pylori* infection interferes with this adaptation due to decrease of HSP70 expression in gastric mucosal cells.

Key words: *Heat shock protein 70, aspirin, gastric adaptation, H. pylori, gastric mucosa.*

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection and non-steroidal antiinflammatory drugs (NSAIDs) are the most common etiologic factors of gastritis and peptic

ulceration. The deleterious effects of NSAIDs on gastroduodenal mucosa are attributed to direct damage of mucosal cells, reduction in prostaglandin formation (1), reduced synthesis of endogenous growth factors (2) and impaired gastric microcirculation (3, 4). Gastric adaptation to repeated exposures to aspirin (ASA) is well documented (5), but the role of *Helicobacter pylori* in ASA-induced gastropathy and adaptation to ASA and the biochemical events underlying this adaptation remain unclear. Heat shock proteins (HSP) represent a nearly universal, phylogenetically highly conserved cellular response to a variety of environmental stresses or unfavorable conditions. They act as molecular chaperones in the folding of newly synthesized proteins in cells and assist in the refolding of damaged proteins (6).

Heat shock protein 70 (HSP70) can be induced by higher temperatures, toxic chemicals, anoxia, reactive oxygen intermediates and infections. It can antagonize the toxic effects of cytokines (7) and protects cells against the toxic effects of excessive amounts of nitric oxide (NO) (8). Thus, mild stress by inducing HSP70 may prepare cells to cope with subsequent stresses. Since this seems to be similar to the phenomenon of adaptation, it is tempting to speculate that HSP 70 might be involved in gastric adaptation. Hirakawa *et al.* have shown that geranylgeranyl-acetone induces the heat shock response in cultured guinea pig gastric mucosal cells and rat gastric mucosa while preventing ethanol-induced gastric damage (9).

In the present study, we, therefore, studied the role of HSP70 expression in *H. pylori* infection and gastric adaptation to repeated exposures to ASA. The endoscopic phenomenon of impaired gastric adaptation to ASA in *H. pylori*-positive otherwise healthy subjects and restoration of this adaptation after eradication has already been described by our group (10, 11). Using gastric mucosal biopsies originating from the previous studies (11) we now assessed HSP70 mRNA and protein expression during ASA treatment in non-infected healthy subjects and in *H. pylori* infected patients before and after eradication of *H. pylori*. Moreover, we corroborated our results by determination of HSP70 expression in mice with and without acute infection with *H. pylori* before and after eradication of this infection.

MATERIAL AND METHODS

Healthy volunteers — study design and biopsy specimen

The subjects (8 healthy *H. pylori*-negative controls and 8 *H. pylori*-positive subjects, 6 males, 2 females, all non-smokers, without history of gastrointestinal disease and without gastrointestinal symptoms) and the study design have been described previously (10, 11). Briefly, *H. pylori*-positive subjects ingested 2 g of unbuffered ASA (Bayer, Germany) per day for a total of 14 consecutive days ("group B, ASA, *H. pylori*-positive"). Three months after successful eradication of *H. pylori*,

subjects received the same ASA treatment again. They are referred to as "group C, ASA, *H. pylori*-negative after eradication". Healthy control subjects received placebo tablets ("group A, placebo, *H. pylori*-negative"). Gastric mucosal biopsies were taken before treatment and on days 3, 7 and 14 of ASA treatment during standard unsedated upper gastrointestinal endoscopy. Biopsy specimens were snap frozen in liquid nitrogen and stored at −80°C until further processing. Institutional Review Ethical Committee at Münster University Medical Faculty approved this study and all subjects gave informed consent to participate in the study.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blot analysis of HSP 70

Samples were homogenized in 10 mmol $NaHCO_3$/5% SDS using the microdismembrator (Braun, Melsungen, Germany). 160 µg proteins were loaded onto 7.5% SDS polyacrylamide gels. After electrophoresis the separated proteins were transferred to nitrocellulose membranes (Schleicher and Schull, Dassel, Germany) as described previously (13). A mouse monoclonal anti-HSP 70 antibodies was used at a dilution of 1:2000 (StressGen, Victoria, Canada). Thereafter, the membranes were probed with [^{125}I]-labeled anti mouse IgG (ICN, Meckenheim, Germany). Visualization and quantification of binding of the second antibody was performed with a Phosphor Imager System (Molecular Dynamics, Krefeld, Germany). A sample of known HSP70 content ("pool mix") was run on every gel as an internal standard, which allowed the correction for blotting efficiency.

Immunohistochemistry

Immunohistochemistry of HSP70 was performed in parallel paraffin sections with DAKO TechMate (DAKO, Copenhagen, Denmark). First antibody (DAKO polyclonal rabbit anti-HSP70) diluted 1:250 was applied for 1 hour at room temperature. Reaction was completed with appropriate DAKO Tech Mate peroxidase detection kit using AEC (3-amino--9-ethyl-carbazole) as a chromogen. Tissue was counterstained with hematoxylin.

H. pylori infection in mice — study design and assessments

Mice were infected with *H. pylori* as described previously (12). Briefly, 20 mice were inoculated intragastrically with a $CagA^+/VacA^+$ Hp strain (2×10^9 CFU per milliliter) at two following days. Ten of those mice inoculated with *H. pylori* were subjected to 5 day triple therapy starting 7 days upon *H. pylori* infection using omeprazole (20 mg s.c.), amoxycillin (25 mg/kg i.p.) and tinidazol (25 mg/kg i.g.), as described before (14). Control group of 10 mice received intragastrically (i.g.) vehicle (0.9 % saline) at a total volume of 0.2 ml. Animals were sacrificed on day 14 after first inoculation. The presence of *H. pylori* in the gastric mucosa was verified by culture, histological evaluation (Warthin-Starry silver staining), urease test and determination of cagA mRNA by RT-PCR.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) to detect messenger RNA (mRNA) for HSP70

The stomachs were removed from mice inoculated with *H. pylori* or treated with vehicle. Mucosal specimens were snap frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was extracted by a guanidium isothiocyanate/phenol chloroform method (15) using kit from Stratagene® (Heidelberg, Germany). Single stranded cDNA was generated from 5 µg of total

cellular RNA using Strata-Script reverse transcriptase and oligi-(dT)-primers (Stratagene, Heidelberg, Germany). The resultant cDNA (2 µl) was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) for 30 cycles (denaturation at 94°C for 1 min, annealing 60°C for 45 sec, and extension at 72°C for 2 min.). The nucleotide sequences of the primers for HSP 70 and GAPDH were based on data of the published cDNA encoding HSP70 and GAPDH (16, 17). The primers were synthesized by GIBCO/Life Technologies, Germany.

Statistics

Results were expressed as mean ± SEM. The significance of differences between means was evaluated using Mann-Whitney test with a confidence value at $p < 0.05$.

RESULTS

Expression of HSP70 in human gastric mucosa assessed by Western blot

In human gastric mucosal biopsies HSP70 protein was detectable as a single band of 70 kDa (*Fig. 1*, top right). Preincubation of the first antibody with a "blocking peptide", (i.e. the peptide against which the antibody had been raised) attenuated the signal (*Fig. 1*, top left), thus indicating the specificity of the first antibody. The signal was proportional to the amount of loaded protein in a range between 100 and 220 µg protein (*Fig. 1*, top right and bottom). Hence, 160 µg of protein was chosen for subsequent experiments.

Before ASA treatment (day 0) the HSP70 expression in human antral mucosa was significantly lower before than 3 months after eradication of *H. pylori* (*Fig. 2*). During ASA treatment, *H. pylori*-positive subjects did not exhibit any significant change of HSP70 protein expression. After eradication, however, ASA treatment resulted in an increase in HSP70 expression on day 14 in the same subjects. Placebo-treated controls did not exhibit any significant change of HSP70 expression during the whole treatment period indicating that the procedure of gastroscopy and multiple biopsies did not affect HSP70 expression. In human gastric corpus before eradication of *H. pylori* ASA treatment resulted in a decrease of HSP70 expression (*Fig. 3*). The initial signal was slightly diminished on day 3 and further decreased on days 7 and 14. After eradication, ASA treatment was accompanied by a sustained expression of HSP70 expression.

Quantitative Western blot revealed that initial values of HSP70 (before ASA intake) were significantly higher after *H. pylori* eradication (8.7 ± 1.2) than before this eradication ($5.4 \pm 0.8 \times 10^4$ Phosphor Imager units) (*Fig. 3*). During ASA administration, in *H. pylori*-infected subjects the HSP70 content significantly decreased from 5.4 ± 0.8 on day 0 to $3.5 \pm 0.5 \times 10^4$ Phosphor Imager units on day 14, while after eradication HSP70 increased from 8.7 ± 1.2 on day 0 to $12.8 \pm 1.5 \times 10^4$ Phosphor Imager units on day 14.

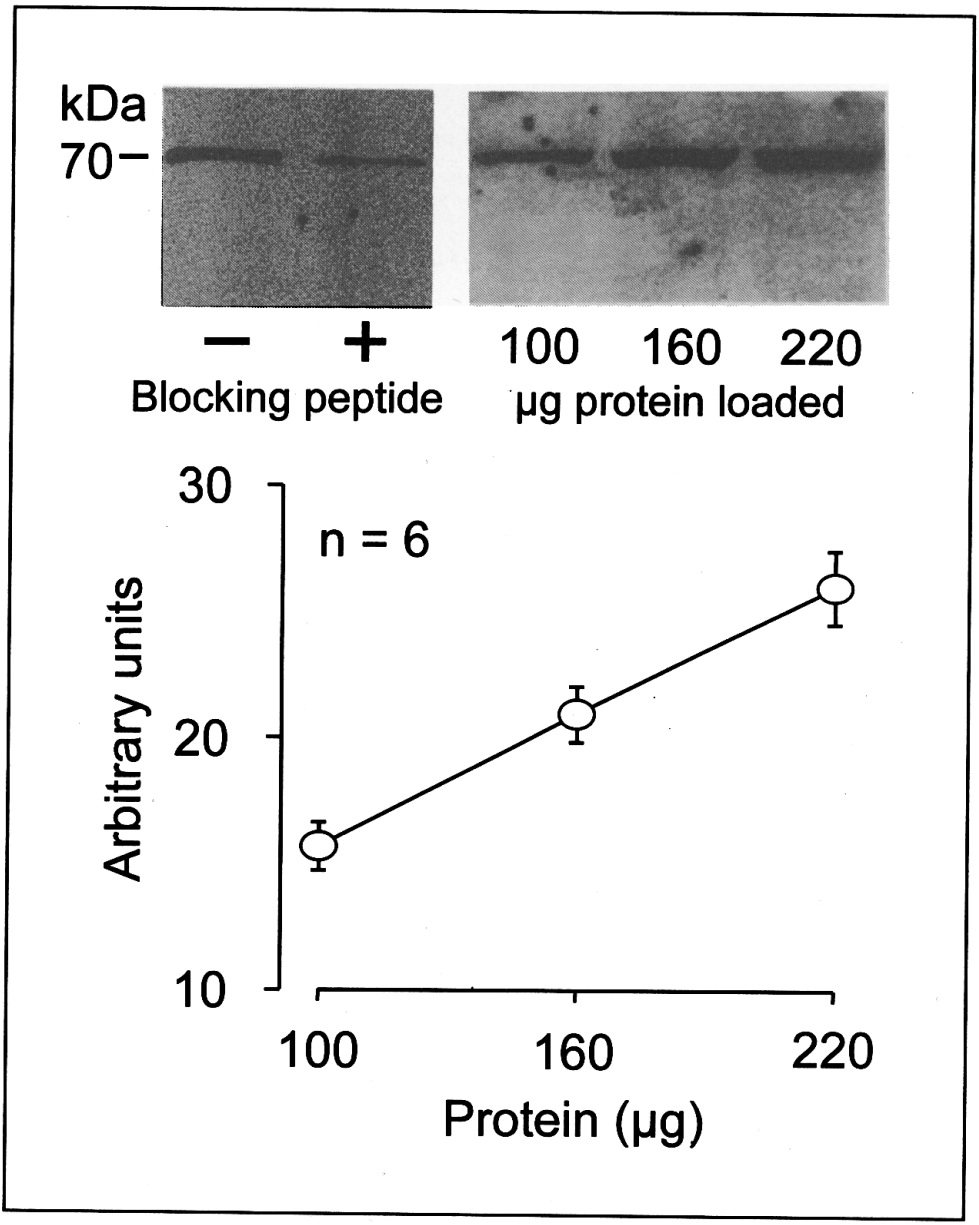

Fig. 1. Expression and quantification of HSP 70 in human gastric mucosa. Top panel left): Blocking experiment for 40 µg of protein with and without preincubation of the first antibody with blocking peptide (250 µg/ml). Top panel (right): Autoradiogram of Western blot of HSP 70 in human gastric mucosa with different amounts of protein per lane. Lower panel: quantification of the signal in a range between 100 and 220 µg of loaded protein per lane (n = 6). Abscissa: amount of protein loaded (µg). Ordinate: pixels (arbitrary Phosphor Imager units) of HSP 70 quantification.

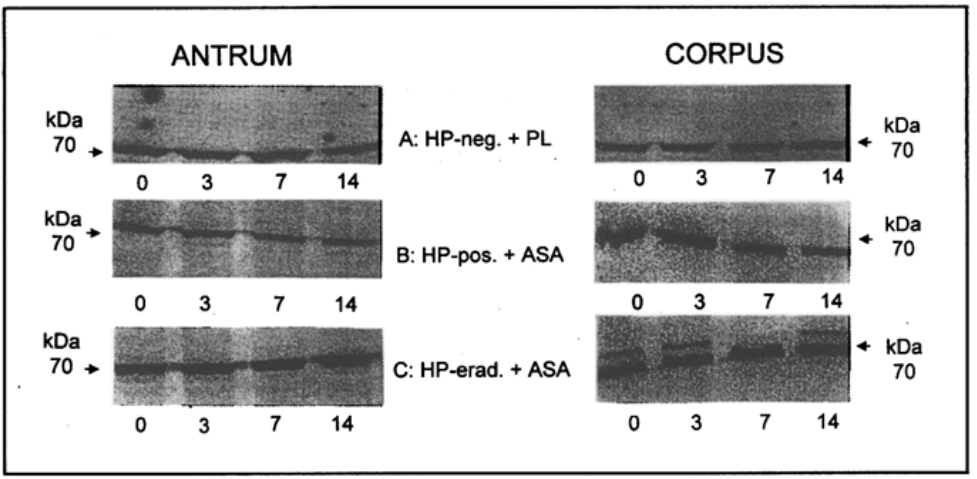

Fig. 2. Expression of HSP70 in human gastric mucosa (antrum: left panel and corpus: right panel) in *H. pylori*-negative, placebo-treated controls (A) and in subjects with *H. pylori* infection before (B) and after (C) eradication treated with ASA for 14 days. Autoradiogram of representative Western blots.

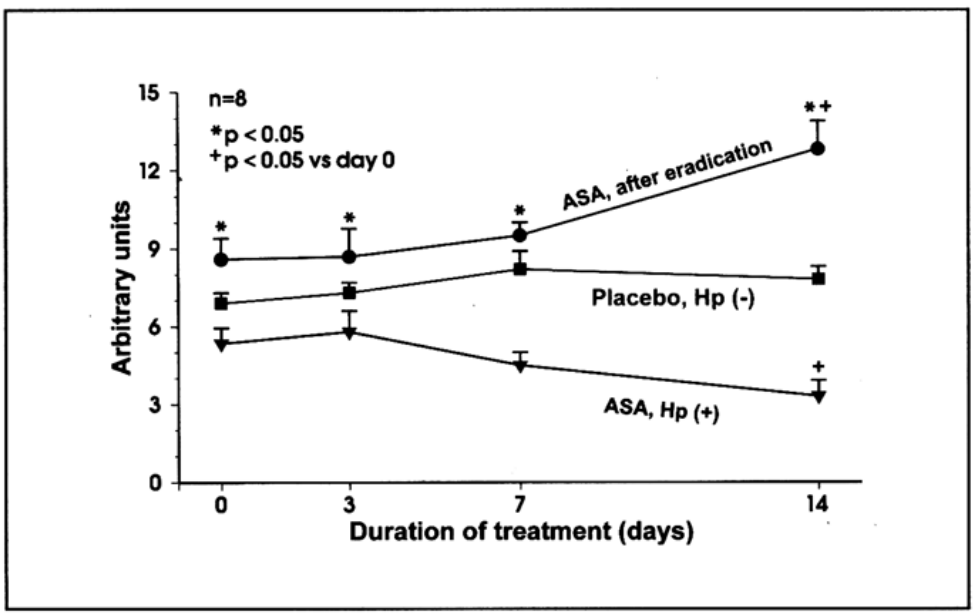

Fig. 3. Expression of HSP70 protein (in arbitrary units) as assessed by Western Blot in biopsies of human gastric mucosa in *H. pylori*-negative, placebo-treated controls (A), and in subjects with *H. pylori* infection before (B) and after eradication (C) treated with ASA for 14 days. Loaded protein: 160 μg of protein per lane. *for $P < 0.05$ vs. B and + for $P < 0.05$ vs. day 0. Abscissa: Duration of treatment (days). Ordinate: pixels (arbitrary Phosphor Imager units) of HSP70 quantification.

In control biopsies from healthy subjects without treatment or prior to medication, the HSP70 immunostaining in antral mucosa was weak to moderate in most of surface and foveolar epithelial cells and was present only in some mononuclear lamina propria cells (data not shown). In parietal cells unspecific binding of link antibody was noticed (reaction remained positive in control without the first antibody (without anti-HSP70). In *H. pylori*-positive subjects chronic active gastritis was associated with more irregular expression of HSP70, but generally increased expression was not observed. In those subjects after prolonged ASA administration mild foveolar hyperplasia was not accompanied by markedly increased expression of HSP70 (*Fig. 4A* vs. *Fig. 4B*). However, an increase in HSP70 expression in foveolar epithelium was noticed in subjects on ASA after eradication of *H. pylori*, in which regression of gastritis was present (*Fig. 4C* and *D*; arrows). In oxyntic mucosa HSP 70 was more irregular and generally did not change significantly during or after eradication and ASA treatment (data not shown).

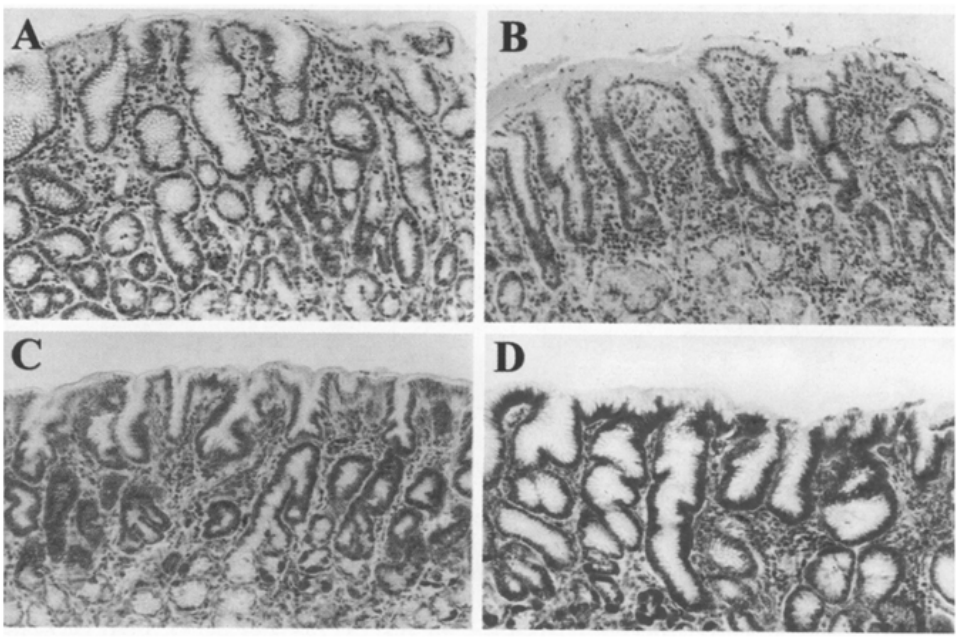

Fig. 4. A: Antral mucosa of *H. pylori*-positive patient (group B) before ASA treatment. Mild expression of HSP70 is noticed in foveolar epithelial cells. B: Another antral biopsy of the *H. pylori*-positive patient during prolonged ASA treatment. Mild to moderate HSP70 immunoreactivity is present. C: Antral mucosa in *H. pylori*-negative patient (*H. pylori*-negative after eradication, group C) before ASA treatment. Moderate HSP 70 immunoreactivity is present especially in surface and foveolar epithelium. Few parietal cells show nonspecific reaction. D: Antral mucosa after eradication and prolonged ASA treatment. Mild foveolar hyperplasia is already present. Strong HSP 70 immunoreactivity is present especially in surface and foveolar epithelium (arrows). Indirect immunohistochemistry. Magn. 240×.

HSP70 expression in mice stomach infected with H. pylori

Since humans with freshly acquired *H. pylori* infection are difficult to identify and intentional infection was not possible for ethical reasons, we assessed the HSP70 expression after *H. pylori* infection and *H. pylori* eradication in a mouse model, which was established recently (11). RT-PCR revealed that expression of HSP70 mRNA was lower in gastric tissue of *H. pylori*-infected mice as compared to vehicle-treated, non-infected mice (*Fig. 5*, left). Quantification of the signal revealed a decrease of HSP70/GAPDH ratio from 1.6 ± 0.1 to 0.9 ± 0.1 ($n = 8$) (*Fig. 5*, right). Eradication of *H. pylori* restored in part the HSP70/GAPDH ratio to the value not different from that observed in the intact mucosa without *H. pylori* infection (*Fig. 5*, right).

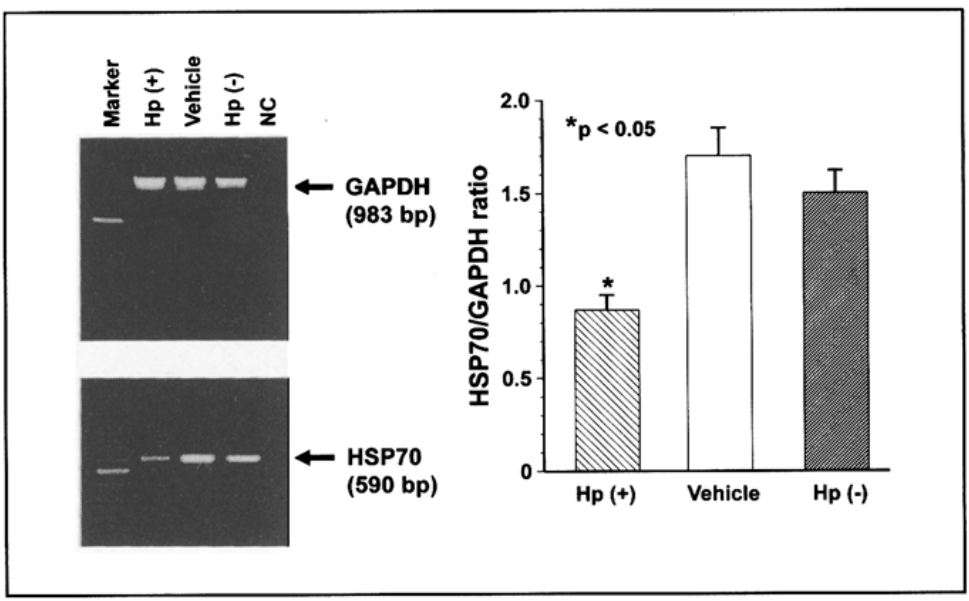

Fig. 5. HSP70 mRNA expression after inoculation of mice stomach with *H. pylori* and eradication of the infection. Left panel: RT-PCR analysis of GAPDH mRNA (top) and HSP 70 mRNA (bottom) in gastric tissue of mice successfully inoculated with *H. pylori*, inoculated with vehicle (saline) or *H. pylori* eradicated in mice. Each column represents mean of ten examinations on 10 mice. Arrow indicates expected PCR product size for HSP70 (590 bop) and GAPDH (983 bp). M = DNA size marker (100 bp DNA ladder from Gibson BRL, Germany). NC = negative control (water). Right panel: The ratio of HSP70 mRNA over GAPDH in gastric tissue of mice inoculated with *H. pylori* or vehicle or in mice with eradicated *H. pylori*. Asterisk indicates significant decrease as compared to vehicle (saline) value.

DISCUSSION

Gastric adaptation is characterized by the resolution of mucosal damage despite further exposure to the drug. Adaptation to ASA and other NSAIDs is

a well-recognized phenomenon, but little is known about the influence of *H. pylori* infection on this adaptation, although it has been shown, that eradication of *H. pylori* may be of benefit in patients undergoing NSAID therapy (18). Analyzing healthy *H. pylori*-positive volunteers before and after eradication we were able to demonstrate recently, that *H. pylori* infection impairs gastric adaptation to ASA and that eradication of the *bacterium* restores this process (10, 11). However, it has not been fully elucidated how *H. pylori* exerts its negative effect on gastric adaptation in these patients. Therefore, we tested the hypothesis, whether alteration in HSP70 expression might accompany the process of adaptation in these healthy volunteers and in *H. pylori* infected subjects before and after eradication. For this purpose, mucosal biopsies that had been taken during the previous study (11) were now assessed for expression of HSP70.

One of the most surprising findings was, that *H. pylori* infection is accompanied by decreased expression of HSP70. HSP70 was detectable in mucosal biopsies of *H. pylori*-positive and eradicated subjects as well as in *H. pylori*-negative controls. To the best of our knowledge, expression of HSP70 in human stomach has not yet been reported. It might be argued that endoscopy per se might alter HSP70 expression. However, at least no difference was observed in placebo-treated controls upon repeated endoscopies during the whole treatment period. Caloric restriction in rats results in an increased HSP70 expression (19). However, all subjects underwent endoscopy at the same time at 8 a.m. Therefore, caloric restriction as well as endoscopy itself is unlikely to have influenced the comparative results of the study. Finally, since other inducible proteins like cyclooxygenase 2 (COX-2) are expressed in macroscopically uninvolved gastric mucosa (20), basal expression of HSP70 before drug therapy might reflect the fact that gastric mucosa is constantly challenged by a variety of environmental stresses.

Induction of HSP70 has been reported to occur in primary cultures of guinea pig gastric surface mucous cells in response to numerous irritants such as ethanol or stresses (9, 21). Water immersion and restraint stress was shown to rapidly activate heat shock factor 1 (ASF1) and to induce HSP70 mRNA expression and protein accumulation (21). In our study, however, HSP70 mRNA and protein expression in subjects infected with *H. pylori* was not enhanced, but reduced. To the best of our knowledge, this is the first report on reduced expression of HSP 70 in the stomach during bacterial infection. The mechanism mediating this reduced HSP70 expression in *H. pylori* infection remains to be elucidated yet. Snyder *et al.* (7) have reported an inverse relationship between the levels of TNF-α and HSP70. In fact, in the gastric fluid of our study patients, TNF-α was increased in *H. pylori* infection in humans and was diminished after eradication (22). Similarly, an extensive expression of TNF-α and IL-1β was found previously in animals infected with

H. pylori (14). These results seem to support the hypothesis of an inverse relationship between HSP70 and TNF-α expression and possible contribution of cytokines in HSP70 expression in *H. pylori* infected human or animal gastric mucosa. This is further reinforced by our present finding that HSP70 expression is restored upon elimination of *H. pylori* eradication therapy that also abolished the excessive production of cytokines in these animals (14).

Upon challenge with ASA, HSP70 expression showed further decrease in *H. pylori*-positive subjects, but increased when after eradication the same subjects were challenged with ASA again and showed typical adaptation to ASA (23). Several groups have reported induction of HSP70 by ASA or related compounds. Ritossa (24) noted that sodium salicylate was able to mimic the heat shock response in Drosophila. Furthermore, Bugiel and Betts (25) reported that ASA, salicylate, indomethacin and dexamethasone induced HSP70 synthesis directly or facilitated the heat shock response at 39°C in human peripheral blood mononuclear cells. Salicylate can induce heat shock protein gene expression by enhancing binding of heat shock transcription factor to DNA (26). On the other hand, Schett *et al.* (27) reported an increased HSP70 expression in synovial tissue of patients with rheumatoid arthritis as compared to osteoarthritis and aseptic bone necrosis. Using synovial fibroblast-like cells they found an increase of HSP70 expression brought about by proinflammatory cytokines like TNF-α. NSAIDS (indomethacin, aspirin, dexibubrufen and meloxicam) by themselves do not induce HSP70 protein expression. Fawcett and Holbrook (28) found that ASA alone failed to affect the mRNA expression for HSP70 in isolated lung, liver and kidney but the rise of body temperature by heat application greatly potentiated the HSP70 response to ASA. Since nonsteroidal anti-inflammatory drugs (NSAID) are potent inhibitors of prostaglandin (PG) generation it was worthwhile to test whether the PG could affect the HSP expression in the gastric mucosa adapting to ASA. Jin *et al.* (29) found in rat gastric mucosa adapting to ASA that PG level was almost completely suppressed without interfering with HSP60 and HSP90 expression. Towndrow *et al.* (30) using renal cannaliculi failed to observed any influence of endogenous PG on HSP expression. Thus, the role of PG in ASA adaptation of gastric mucosa and in HSP expression in rats seems to be questionable. Another possible candidate for induction of HSP might be nitric oxide (NO) that was reported to induce HSP72 in rat gastric mucosa after application of NO donor such as S-nitro-N-acetyl-penicillamine (31). The question remains, however, whether the amounts of NO released by the gastric mucosa treated with ASA or infected with *H. pylori* are sufficient to alter the expression of HSP70.

In our *in vivo* study in humans and mice the effect of ASA on HSP70 expression seems to be dependent on the presence or absence of *H. pylori*, thus confirming the impact of environmental conditions such as infection and

related increase in cytokine level (22) on induction of HSP70 expression. The difference in HSP70 expression before and after eradication was more pronounced in antral mucosa, possibly due to the fact, that in our study *H. pylori* infection was localized predominantly in the antrum, while oxyntic mucosa was not infected in these subjects.

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Received:
Accepted:

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