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Effect of vitamin C-releasing acetylsalicylic acid on gastric mucosal damage before and after *Helicobacter pylori* eradication therapy

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Abstract

The interaction between *Helicobacter pylori* (*H. pylori*) and nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid is still controversial. This study was designed to compare the effect of acetylsalicylic acid and vitamin C-releasing acetylsalicylic acid on the gastric mucosal damage and microbleeding before and after eradication of *H. pylori* in 10 young healthy volunteers. Acetylsalicylic acid induced significantly more gastric lesions and higher microbleeding than acetylsalicylic acid–vitamin C. After successful *H. pylori* eradication therapy, acetylsalicylic acid induced significantly higher mucosal lesions and microbleeding than before eradication. In contrast, after acetylsalicylic acid–vitamin C, gastric lesion index was significantly lower and eradication therapy failed to aggravate it. All *H. pylori*-positive subjects showed significant up-regulation of antioxidant enzyme (superoxide dismutase, catalase, glutathione peroxidase). Plain acetylsalicylic acid stronger than acetylsalicylic acid–vitamin C reduced gastric gene expression of these antioxidant enzymes. *H. pylori* eradication significantly decreased expression of these enzymes and this was further enhanced by plain acetylsalicylic acid, but not acetylsalicylic acid–vitamin C. Under plain acetylsalicylic acid therapy, the expression of proinflammatory cytokines was increased before and after eradication of *H. pylori*. We conclude that vitamin C combined with acetylsalicylic acid, unlike plain acetylsalicylic acid without vitamin C, protects gastric mucosa in man probably due the attenuation of oxidative stress and proinflammatory cytokines.

Keywords: Acetylsalicylic acid; Vitamin C; Proinflammatory cytokine; Antioxidant enzyme

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) and *Helicobacter pylori* (*H. pylori*) are major causative factors in the pathogenesis of gastric mucosal injury in humans (Wolfe et al., 1999; Fiorucci et al., 2001; Huang et al., 2002). NSAIDs and *H. pylori* activate neutrophils that accumulate at the site of inflammation with a subsequent generation of reactive oxygen species (Yoshikawa and Naito, 2001). The increased production of free radicals is balanced by endogenous as well as exogenous antioxidants like superoxide dismutase, glutathione peroxidase, catalase or ascorbic acid. This delicate balance between generation of free radicals and antioxidants is of critical importance for functional integrity of the cells. The excessive production of free radicals or deficiency of antioxidants may lead to cellular destruction and gastric mucosal damage (Yoshikawa, 2002).

The gastric mucosal lesions induced by NSAID and *H. pylori* leads to the release of proinflammatory cytokines such as tumor necrosis factor alpha and interleukin-1beta (Yamaoka et al., 1996; Wallace, 2004). Moreover, *H. pylori* infection is accompanied by the overexpression of inducible

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nitric oxide (NO) synthase (iNOS), producing large amount of NO (Mannick et al., 1996) that may react with free radicals to form cytotoxic nitrogen oxides and peroxynitrites (Das, 2002). Moreover, the colonization of gastric mucosa by *H. pylori* leads to the activation of signal transduction pathways, especially early response transcription factor, nuclear factor kappa B (NF-κB), which contributes to the generation of proinflammatory cytokines and enhancement of apoptosis (Naumann and Crabtree, 2004). The importance of *H. pylori* in an initiation of neutrophil infiltration and subsequent induction of oxidative stress is further supported by the fact that the successful eradication of this infection results in the significant reduction of oxidative stress in the gastric mucosa (Drake et al., 1998; Pignatelli et al., 2001). NSAIDs, similarly to *H. pylori*, lead to increased oxidative stress in the gastric mucosa and this mechanism plays a crucial role in the damaging action of these drugs on the gastric mucosa (Wallace et al., 1990; Yoshikawa et al., 1993; Brzozowski et al., 2003). In the previous animal study, we demonstrated that the increase in the neutrophil-derived oxygen free radicals after exposure of the stomach to acetylsalicylic acid was significantly attenuated by the addition of vitamin C to plain acetylsalicylic acid (Brzozowski et al., 2001). This observation suggests that vitamin C may counteract the deleterious effects of acetylsalicylic acid on gastric mucosa. This was further supported by our previous study in humans showing that the addition of vitamin C to acetylsalicylic acid significantly attenuated the gastric damage due to its antioxidizing activity (Pohle et al., 2001).

Current evidence indicates that chronic *H. pylori* infection is accompanied by a significant decrease in vitamin C content in the stomach (Banerjee et al., 1994). The observed deficiency of vitamin C in the *H. pylori*-infected gastric mucosa may increase the damaging effect of acetylsalicylic acid on the gastric mucosa via accumulation of lipid peroxides. This could be reversed by the addition of vitamin C to native acetylsalicylic acid but no experimental evidence was provided in humans to support these notions.

The purpose of the present study was: (1) to compare the effects of acetylsalicylic acid with or without addition of vitamin C on gastric mucosal damage in *H. pylori*-infected subjects before and after eradication therapy; (2) to assess the effect of acetylsalicylic acid alone or combined with vitamin C on the gastric mucosal gene expression of antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase), proinflammatory cytokines (tumor necrosis factor-α, interleukin-1β) and inducible nitric oxide synthase, and (3) to compare the effects of acetylsalicylic acid alone or combined with vitamin C on the protein expression of catalase and inducible NO synthase in the gastric mucosa of *H. pylori*-positive subjects before and after eradication therapy and (4) to assess the effect of acetylsalicylic acid alone or combined with vitamin C on the activity of NF-κB in the presence or absence of *H. pylori* infection.

2. Materials and methods

2.1. Subjects and study design

Ten *H. pylori*-positive healthy volunteers of both sexes (five males, five females) between 18 and 28 years of age, weighing 65–80 kg entered this study. All subjects were examined for the presence of *H. pylori* infection during the endoscopic examination using rapid urease test (HUT-test, ASTRA, Wedel, Germany). Pre-study screening ensured that all volunteers had a normal physical examination, full blood count, standard blood chemistry including coagulation tests, and that women had a negative pregnancy test. The study was approved by the appropriate Institutional Review Committee at Erlangen-Nuremberg University and all subjects gave written informed consent to participate prior to inclusion.

All subjects underwent routine endoscopy at day 0 (before acetylsalicylic acid without or with vitamin C or placebo treatment) during which multiple biopsies (Mannick et al., 1996) were taken from the oxyntic mucosa. On the day following endoscopy, patients were randomized to following treatment groups; acetylsalicylic acid or acetylsalicylic acid–vitamin C (Aspirin Plus C from Bayer, Leverkusen, Germany). The study medication was taken twice daily (0.8 g unbuffered acetylsalicylic acid or two tablets of acetylsalicylic acid (1.6 g) Plus C after breakfast at 08:00 h and before bed time summing up to a daily total dose of 1.6 g acetylsalicylic acid or 1.6 g acetylsalicylic acid plus 0.96 g vitamin C. The medication was given for 3 days. The controls were taking placebo tablets (containing only hydroxypropyl methylcellulose and starch without acetylsalicylic acid). For evaluation of gastric mucosal damage, in all subjects the gastric microbleeding was determined and the gastroscopy was performed on days 0 and 3 after start of treatment as described before (Konturek et al., 1981). Briefly, the rate of gastric microbleeding was determined as follows: each subject swallowed 16 French gauge orogastric tube. The stomach was rinsed of debris with 100 ml of distilled water, then 100 ml of test solution was instilled into the stomach for 10-min washing period. Mean gastric microbleeding for three 10-min washing periods was calculated and expressed as mean bleeding rate in ml/day. The standard, unsedated, upper gastrointestinal endoscopy was performed by one investigator using an Olympus GIF 100 endoscope and recorded on video tape that was evaluated for mucosal damage using the Lanza score system by the second investigator, being unaware of the treatment given and the *H. pylori* status. Grading score ranged from 0=normal to 4=large area of submucosal hemorrhage with active bleeding or widespread involvement of the stomach. During both gastroscopies, gastric mucosal samples were taken from corpus for the molecular analyzes (polymerase chain reaction, Western blot, nuclear factor kappa B activity). After the termination of both treatment regimens (acetylsalicylic acid or acetylsalicylic acid–vitamin C) or

placebo, all subjects underwent eradication using triple therapy including amoxicillin (2×1000 mg per day) plus clarithromycin (2×500 mg per day) plus pantoprazole (40 mg twice per day) given for 10 days. The *H. pylori* status was assessed using antigen stool-test, confirmed during endoscopy by rapid urease test as stated above. Four weeks after successful eradication of *H. pylori*, all subjects underwent in random order the same treatments and all above endoscopic procedures were repeated in the same manner as before eradication of *H. pylori*.

2.2. Reverse-transcriptase polymerase chain reaction (RT-PCR)

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, biopsy specimens were homogenized in 1 ml of TRIZOL (Total RNA Isolation Reagent, Gibco BRL, Karlsruhe, Germany) and RNA was recovered according to the manufacturer's instructions before resuspension in 10–20 μl of diethyl pyrocarbonate-treated water and quantification as described before (Yoshikawa et al., 1993).

Human complementary DNA (cDNA) was generated by reverse transcription of total RNA extracted from mucosal biopsy specimens using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene, Heidelberg, Germany) and oligi-(dT)-primers (Stratagene). The cDNA (2 μl) was amplified in a 50 μl reaction volume containing 2 U Taq polymerase, dNTP (200 μM each) (Pharmacia, Freiburg, Germany), 1.5 mM $MgCl_2$, 5 μl 10×polymerase chain reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH=8.3) and primers used at final concentration of 1 mM (all reagents from Takara, Shiga, Japan). The mixture was overlaid with 25 μl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT). The nucleotide sequence of the primers for superoxide dismutase, glutathione peroxidase, catalase, tumor necrosis factorα, interleukin-1β and inducible NO synthase were based on the sequences of the published cDNAs. All primers were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany). Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using 100-bp ladder (Takara) as a standard size marker. The intensity of bands was quantified using densitometry unit from Kodak Digital Science.

2.3. Western blot analysis

Using TRIZOL reagent, proteins were extracted from the same biopsy samples as mentioned above. Approximately 10 μg of total protein extracts was loaded on SDS-polyacrylamide gels and run 40 mA, followed by transfer on nitrocellulose membrane (Protran, Schleicher&Schuell, Dassel, Germany) by electroblotting. 3% bovine serum albumin (BCA) (Sigma Aldrich, Taufkirchen, Germany) in TBS/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris–HCl, pH 7.4, 0.1% Tween-20) was used to block filters for at least 1 h at room temperature. Specific primary antibody against iNOS (rabbit polyclonal, dilution 1:500; Santa Cruz, CA, USA), catalase (rabbit polyclonal, dilution 1:500; Santa Cruz) or β-actin (mouse monoclonal, dilution 1:5000; Sigma Aldrich) was added to the membrane, followed by an anti-rabbit-IgG or anti-mouse-IgG horseradish peroxidase conjugated secondary antibody (dilution 1:40 000 or 1:20 000) dissolved in 1% non-fat milk in TBS-Tween-20 buffer. Incubation of primary antibody was followed by three washes with TBS-Tween-20 buffer for 5-min incubation of the secondary antibody was followed by six washes for 5 min. Immunocomplexes were detected by the SuperSignal West Pico Chemiluminescent Kit (Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany).

2.4. Measurement of nuclear factor kappa B (NF-κB) activity

Nuclear extracts were prepared by a mini-extraction procedure (Konturek et al., 1981). Biopsy specimens were homogenized in 0.8 ml of buffer A (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris pH 7.5 and 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.1% Tween) and after 15 min of incubation centrifuged for 10 min at 400×*g*. Pellets were resuspended in 0.6 ml of buffer A without Tween and samples were centrifuged at 1400×*g* for 10 min. After centrifugation, lysis buffer was added, probes were shaken for 30 min and then centrifuged at 14 000×*g* for 10 min. After centrifugation at 4 °C, the supernatant proteins were measured by a BCA method using QuantitatiPro™ BCA Assay kit. The rest of the supernatant was frozen in a 10% glycerol.

The activity of NF-κB p65 was measured using TransAM method—the first enzyme-linked immunosorbent assay (ELISA)-based kit to detect and quantify transcription factor activation (Active Motif; Rixensart, Belgium). TransAM NF-κB kit contains a 96-well plate on which has been immobilized oligonucleotide containing the NF-κB consensus site (5′ -GGGACTTTCC-3′). The active form of NF-κB contained in cell extract specifically binds to this oligonucleotide. After binding, a specific epitope on p65 is accessible and is recognized by primary antibody. Addition of a secondary antibody conjugated to horseradish peroxidase provides a sensitive colorimetric reaction. The reaction product is quantified by measuring the absorbance at the respective wavelength using a spectrophotometer. Whole procedure was performed as per the manufacturer's protocol.

Nuclear extracts samples (~2 μg diluted in 20 μl of lysis buffer) and 30 μl of binding buffer were loaded into each well and plate was incubated for 1 h at room temperature on

a rocking platform (100 rpm). Then, wells were washed 3× with washing buffer. Subsequently, 100 μl diluted NF-κB antibody was added and incubated additional 1 h at room temperature without agitation. Incubation of primary antibody was followed by three washes with washing buffer. Incubation with diluted horseradish peroxidase-conjugated/secondary antibody (100 μl) was performed for 1 h and after 4× washing the immune complexes were detected by the subsequent substrate reaction. Probes were measured in an ELISA reader at 450 nm.

2.5. Statistical analysis

Results are expressed as means±standard error of the mean (S.E.M.). The significance of the difference between means was evaluated using analysis of variance followed by Duncan's test or, when appropriate, by Wilcoxon's rank sum test with a level of confidence at $P<0.05$.

3. Results

3.1. Gastric mucosal injury

All subjects completed the study and no major side effects were recorded. In the control group treated with placebo at days 0 and 3, the mucosal damage was negligible and these results are not included for the sake of clarity. Plain acetylsalicylic acid caused significant ($P<0.05$) increase in gastric mucosal damage according to Lanza score both in *H. pylori*-positive and negative subjects as compared to that in these subjects treated with acetylsalicylic acid–vitamin C (Fig. 1). After successful eradication (the eradication rate was 100%), acetylsalicylic acid induced significantly more lesions (about twofold) than before eradication therapy, but after therapy with acetylsalicylic acid–vitamin C of the *H. pylori*-eradicated patients, the gastric lesion index was significantly lower than that in those treated with plain acetylsalicylic acid.

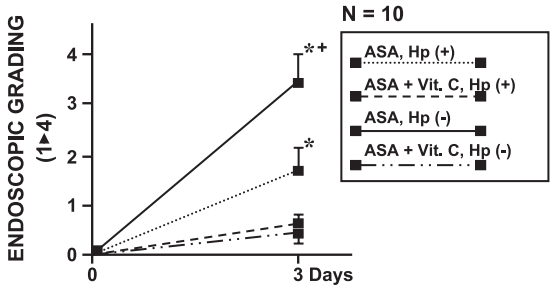

Fig. 1. Endoscopic grading of gastric injury according to the LANZA score in patients treated with acetylsalicylic acid (ASA) or acetylsalicylic acid–vitamin C (ASA–C) before (Hp+) and after successful eradication (Hp−). Asterisk indicates significant ($P<0.05$) increase above the value obtained with acetylsalicylic acid–vitamin C treatment. Cross indicates significant increase as compared to the values obtained in (Hp−) subjects treated with plain acetylsalicylic acid (ASA).

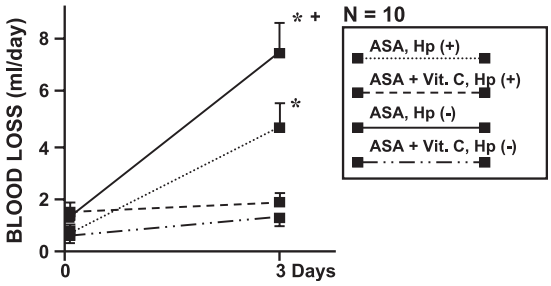

Fig. 2. Gastric microbleeding in patients treated with acetylsalicylic acid or acetylsalicylic acid–vitamin C before (Hp+) and after successful eradication (Hp−). Asterisk indicates significant (P<0.05) increase above the value obtained with acetylsalicylic acid–vitamin C (ASA–C) treatment. Cross indicates significant increase as compared to the values obtained in (Hp−) subjects treated with plain acetylsalicylic acid (ASA).

3.2. Gastric microbleeding

In *H. pylori*-infected patients, the pre-treatment value of gastric microbleeding was 1.3±0.6 ml/day and it was not significantly different from the value recorded in *H. pylori*-negative controls (1.2±0.4 ml/day) (Fig. 2). After 3 days of treatment with plain acetylsalicylic acid, the gastric microbleeding increased significantly ($P<0.001$) in all subjects tested, reaching significantly higher value in *H. pylori*-eradicated subjects (7.6±1.2 ml/day) than in *H. pylori* positive (4.3±0.5 ml/day) subjects. No significant changes in gastric blood loss was observed in subjects treated with acetylsalicylic acid combined with vitamin C.

3.3. Gastric mucosal gene expression of antioxidant enzymes; superoxide dismutase, glutathione peroxidase and catalase

In all *H. pylori*-positive subjects, a significant ($P<0.05$) up-regulation of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase was observed as compared to *H. pylori*-negative subjects (Fig. 3).

In *H. pylori*-positive subjects (before eradication therapy), the treatment with plain acetylsalicylic acid resulted in a significant ($P<0.05$) down-regulation of the mRNA expression for superoxide dismutase, glutathione peroxidase and catalase, while treatment with acetylsalicylic acid–vitamin C of these *H. pylori*-positive subjects enhanced significantly ($P<0.05$) the gene expression of all three enzymes. After eradication therapy, a significant ($P<0.05$) decrease in the mRNA for antioxidant enzymes was observed as compared to initial value in *H. pylori*-positive patients before this therapy. In subjects successfully eradicated, therapy with plain acetylsalicylic acid led to a significant ($P<0.05$) down-regulation of the mRNA for antioxidant enzymes (except for glutathione peroxidase). In contrast, in these *H. pylori*-eradicated subjects receiving acetylsalicylic acid–vitamin C, this down-regulation of the expression of mRNA for antioxidant enzymes was not

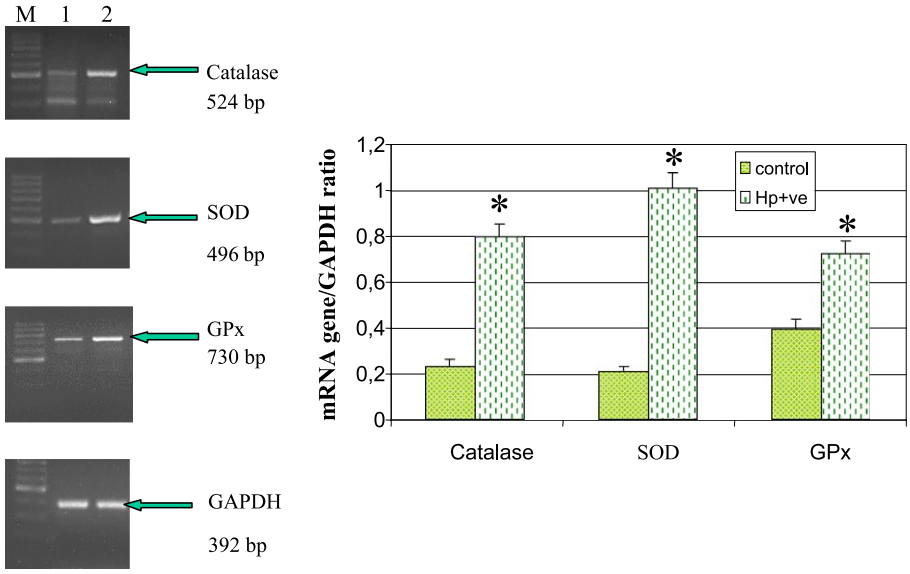

Fig. 3. Representative RT-PCR and densitometric analysis of the mRNA expression of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, catalase) in the gastric mucosa of human Hp+ve volunteers compared to Hp−ve controls (2). Asterisk indicates significant ($P<0.05$) increase above the value obtained in Hp−ve control patients.

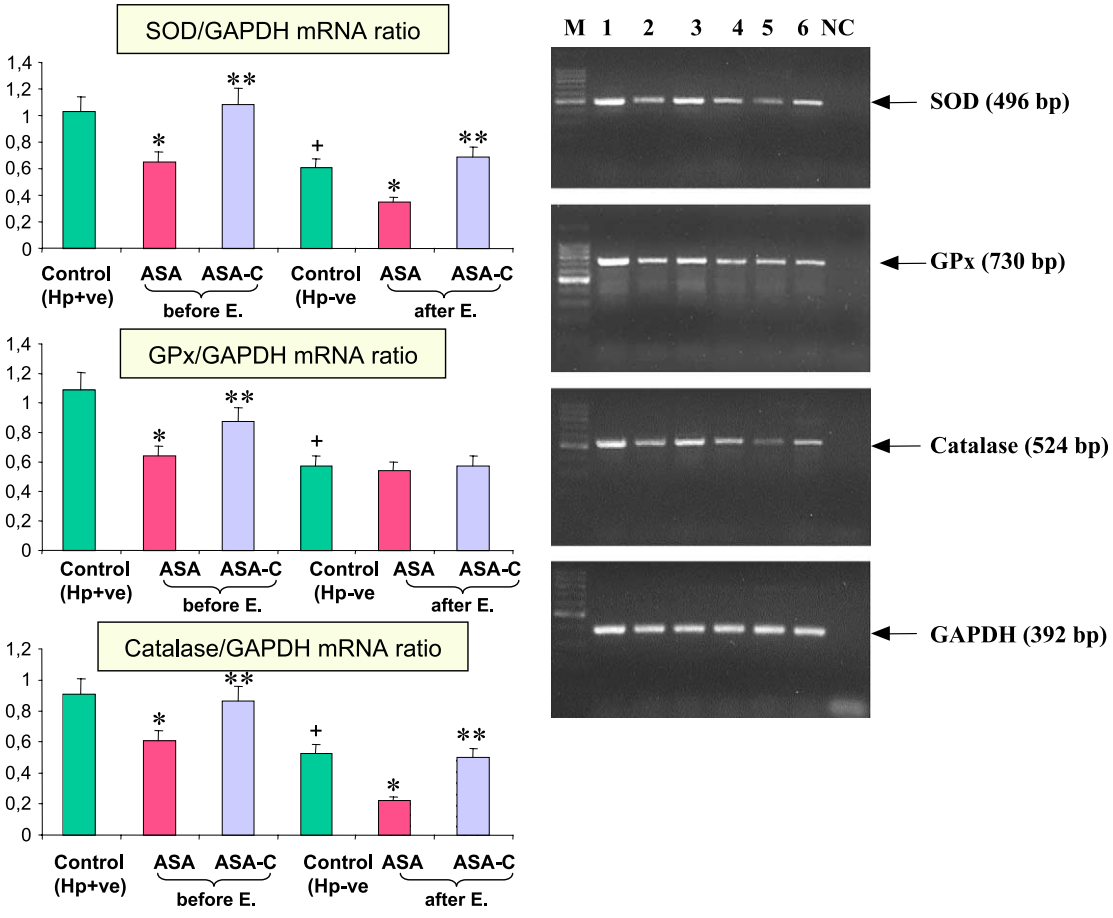

Fig. 4. Representative RT-PCR and densitometric analysis of the mRNA expression of antioxidant enzymes (catalase, superoxide dismutase—SOD, glutathione peroxidase—GPx) in the gastric mucosa of human Hp+ve volunteers treated with acetylsalicylic acid (ASA) or acetylsalicylic acid–vitamin C (ASA–C) before and after successful eradication (E). Asterisk indicates significant ($P<0.05$) decrease below the value obtained in Hp±ve or Hp−ve controls. Cross indicates significant decrease as compared to the values obtained in Hp±ve controls. Double asterisks indicates significant increase above the value obtained after treatment with plain acetylsalicylic acid (ASA). Cross indicates significant decrease as compared to Hp+ve control.

observed and this expression was significantly higher than in plain acetylsalicylic acid treated *H. pylori*-negative subjects (Fig. 4).

3.4. Inducible NO synthase (iNOS), tumor necrosis factor alpha (TNFα) and interleukin-1beta transcripts in the gastric mucosa

In all *H. pylori*-positive subjects, the expression of iNOS, TNFα and interleukin-1beta was detectable. In *H. pylori*-positive subjects receiving plain acetylsalicylic acid, a strong and significant ($P<0.001$) increase in the expression of iNOS, TNFα and interleukin-1beta was observed. In contrast, after acetylsalicylic acid–vitamin C therapy a significant ($P<0.05$) decrease of the expression of iNOS, TNFα and interleukin-1beta was found. After successful eradication therapy, in control subjects treated with placebo, the expression of both iNOS and two proinflammatory cytokines (TNFα and interleukin-1beta) was negligible and significantly ($P<0.05$) lower than that in these subjects before eradication therapy. Treatment with plain acetylsalicylic acid of eradicated subjects resulted in a strong and significant ($P<0.001$) expression of TNFα and iNOS but the expression of interleukin-1beta was not detectable. In eradicated subjects receiving acetylsalicylic acid–vitamin C treatment, the expression of TNFα and iNOS was significantly attenuated as compared to that in plain acetylsalicylic acid treated subjects, while the expression of both proinflammatory cytokines, TNFα and interleukin-1beta, was not detectable (Fig. 5).

3.5. Nuclear factor kappa B (NF-κB) activity in the gastric mucosa

In *H. pylori*-positive subjects treated with placebo, the activity of NF-κB was 0.29 ± 0.05. In *H. pylori*-positive subjects receiving acetylsalicylic acid, a tendency for increase in NF-κB activity was observed, but this change was not significant ($P<0.07$). In contrast, after acetylsalicylic acid–vitamin C treatment, a significant ($P<0.05$) decrease in NF-κB activity as compared to that obtained with plain acetylsalicylic acid was found. After successful eradication, no significant change in NF-κB activity was found in the vehicle-treated gastric mucosal controls. In subjects eradicated, plain acetylsalicylic acid caused a significant ($P<0.05$) increase in NF-κB activity as compared to the control value while after treatment with acetylsalicylic acid–vitamin C a significant ($P<0.05$) decrease in activity of NF-κB was observed and found to be similar to that in the control group (Fig. 6).

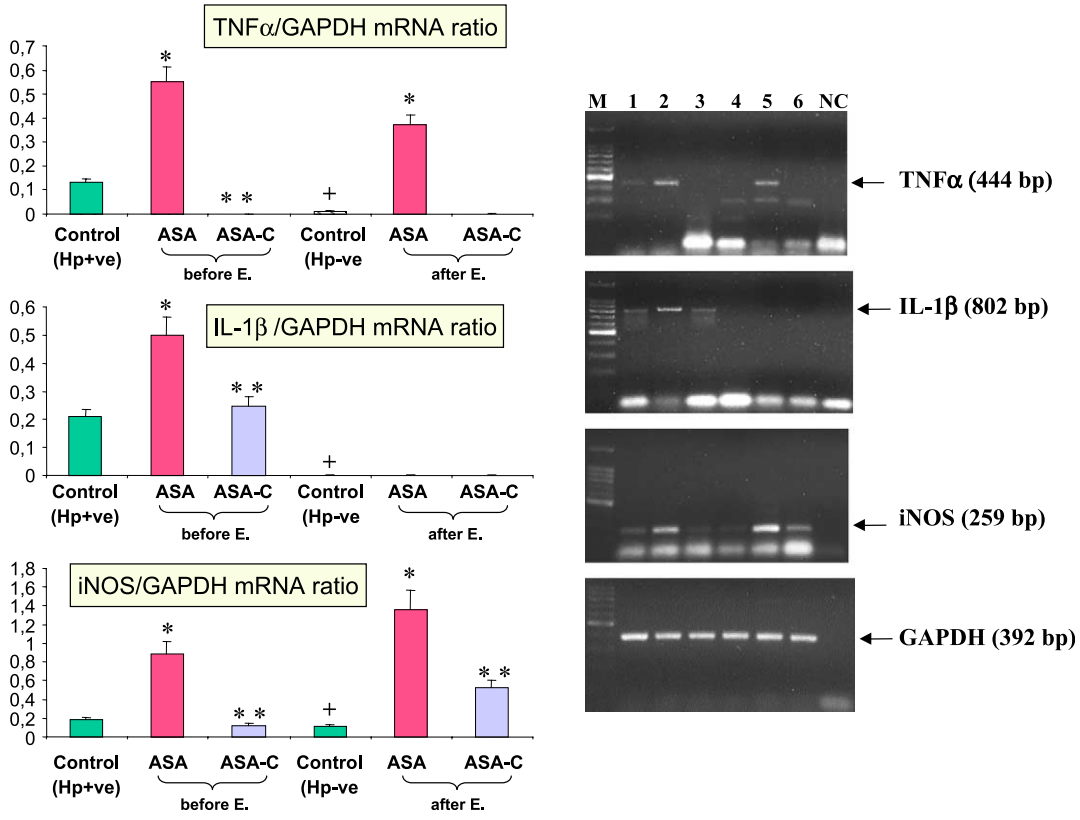

Fig. 5. Representative RT-PCR and densitometric analysis of the mRNA expression of proinflammatory cytokines (TNF-α, interleukin-1beta) and inducible nitric oxide synthase (iNOS) in the gastric mucosa of human Hp+ve volunteers treated with acetylsalicylic acid (ASA) or acetylsalicylic acid–vitamin C (ASA–C) before and after successful eradication (E). Asterisk indicates significant ($P<0.05$) increase above the value obtained in Hp+ve or Hp−ve controls. Double asterisks indicates significant decrease as compared to the values obtained in Hp+ve or Hp−ve subjects treated with plain acetylsalicylic acid (ASA). Cross indicates significant decrease as compared to Hp+ve control.

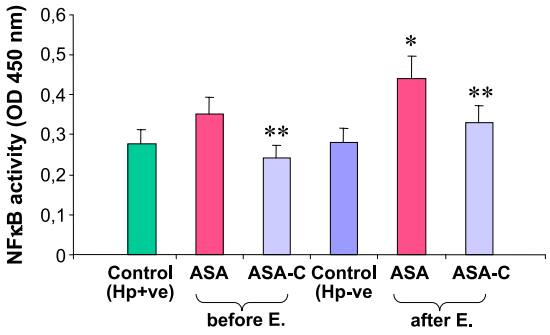

Fig. 6. NF-κB activity measured in the gastric mucosal biopsy obtained from human Hp+ve volunteers treated with acetylsalicylic acid (ASA) or acetylsalicylic acid–vitamin C (ASA–C) before and after successful *H. pylori* eradication (E). Asterisk indicates significant increase above the value obtained in controls. Double asterisks indicates significant decrease below the value obtained with plain acetylsalicylic acid (ASA) in subjects before or after *H. pylori* eradication (E).

3.6. Protein expression of iNOS and catalase

Internal control with β-actin showed intense signals in all samples tested, indicating a good quality of protein isolated from gastric mucosal biopsies. The iNOS expression (Fig. 7) was detected in the gastric mucosa of *H. pylori*-positive controls. Following administration of plain acetylsalicylic acid, the ratio of iNOS over β-actin increased significantly ($P<0.05$) indicating that the protein expression of iNOS was significantly up-regulated by plain acetylsalicylic acid. In subjects taking acetylsalicylic acid–vitamin C, the iNOS expression was significantly ($P<0.05$) reduced as compared to that observed in acetylsalicylic acid-treated group. The eradication therapy led to a small but significant ($P<0.05$) down-regulation of iNOS expression in the gastric mucosa of

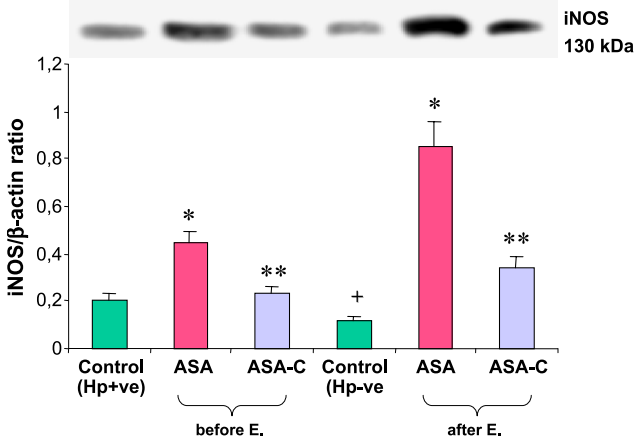

Fig. 7. Representative Western blot and densitometric analysis of the protein expression of iNOS in the gastric mucosa of human Hp+ve volunteers treated with acetylsalicylic acid (ASA) or acetylsalicylic acid–vitamin C (ASA–C) before and after successful eradication. Asterisk indicates significant ($P<0.05$) increase above the value obtained in Hp±ve or Hp−ve controls. Double asterisks indicates significant decrease as compared to the values obtained in Hp±ve or Hp−ve subjects treated with plain acetylsalicylic acid (ASA). Cross indicates significant decrease as compared to Hp+ve control.

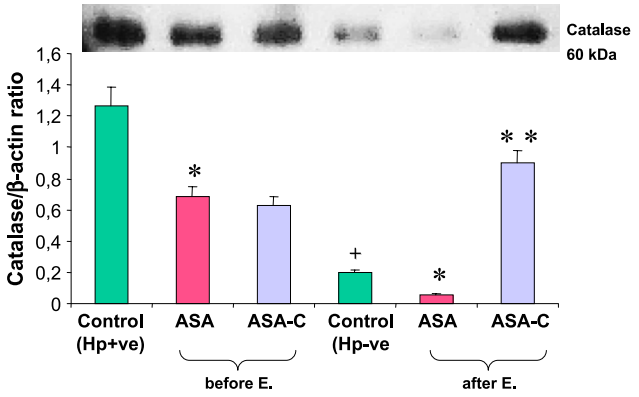

Fig. 8. Representative Western blot and densitometric analysis of the protein expression of antioxidant enzyme catalase in the gastric mucosa of human Hp+ve volunteers treated with acetylsalicylic acid (ASA) or acetylsalicylic acid–vitamin C (ASA–C) before and after successful eradication. Asterisk indicates significant ($P<0.05$) decrease below the value obtained in Hp±ve or Hp−ve controls. Double asterisks indicates significant increase as compared to the values obtained in Hp−ve subjects treated with plain acetylsalicylic acid (ASA). Cross indicates significant decrease as compared to Hp+ve control.

control subjects. In contrast, in subjects successfully eradicated and taking acetylsalicylic acid, a strong ($P<0.05$) up-regulation of iNOS expression was detected that reached several times higher level than before eradication therapy. In subjects treated with acetylsalicylic acid–vitamin C, this increase in iNOS protein expression was significantly ($P<0.05$) attenuated, but was still higher than before eradication therapy.

The expression of catalase (Fig. 8) was detected as a strong signal in all gastric biopsy samples obtained from the gastric mucosa of *H. pylori*-positive subjects. In these subjects taking plain acetylsalicylic acid, similarly to those taking acetylsalicylic acid–vitamin C, a significant ($P<0.05$) down-regulation of catalase expression was found. The expression of catalase in gastric mucosa was remarkably and significantly ($P<0.03$) decreased as compared to that found in the mucosa before eradication therapy. After administration of plain acetylsalicylic acid, the expression of catalase was significantly ($P<0.05$) smaller than in *H. pylori*-negative control but following treatment with acetylsalicylic acid–vitamin C, this expression showed strong and significant ($P<0.03$) increase (Fig. 8).

4. Discussion

Our present study confirms numerous previous reports showing that plain acetylsalicylic acid damages gastric mucosa and that this effect is accompanied by the increased expression of proinflammatory cytokines such as TNFα and interleukin-1beta as well as iNOS. Unexpectedly, the damaging effect of acetylsalicylic acid on gastric mucosa was higher after eradication therapy, indicating the possible protective influence of *H. pylori* infection against damage caused by acetylsalicylic acid, at least in this human model

of acute gastric injury. However, the interaction between *H. pylori* and acetylsalicylic acid is still a controversial issue. Pathophysiological studies suggest that the effect of acetylsalicylic acid and *H. pylori* on gastric mucosal damage is not a simple one and may be either synergistic or antagonistic. This interaction is affected by various factors such as history of gastroduodenal lesions (peptic ulceration), the use of acetylsalicylic acid or other types of NSAIDs, duration of treatment, whether the patient is at high or normal risk and whether the patient is a new or established user of NSAID (Sung et al., 2000).

The major finding of the present study was that the treatment with acetylsalicylic acid–vitamin C induces significantly less gastric mucosal lesions when compared to that attained with plain acetylsalicylic acid. This protective effect of acetylsalicylic acid–vitamin C is probably due to its antioxidant effects as we postulated before (Brzozowski et al., 2001; Pohle et al., 2001). Moreover, we compared the effects of plain acetylsalicylic acid and acetylsalicylic acid–vitamin C on the expression of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase and found that plain acetylsalicylic acid stronger than acetylsalicylic acid–vitamin C reduced the gastric mucosal mRNA expression for these enzymes. This finding indicates that the damaging effect of acetylsalicylic acid on gastric mucosa is, at least partly, due to suppression of the expression of antioxidant enzymes and results mainly from the deficiency of antioxidant mucosal defence. This may result in the disturbance in the balance between the formation of free radicals and endogenous antioxidants leading to the increased lipid peroxidation and the cell destruction (Kohen and Nyska, 2004).

The combination of vitamin C with acetylsalicylic acid appears to attenuate the deleterious effect of plain acetylsalicylic acid on the gastric mucosa. The protective effect of vitamin C is mainly due to its antioxidant activity (Young and Woodside, 2001). Previous experimental studies demonstrated that pretreatment of gastric mucosa with free radical scavengers exhibited protective activity against NSAID-induced gastric mucosal injury (Yoshikawa et al., 1993; Tanaka and Yuda, 2004). However, this does not seem to be the only mechanism responsible for the observed protection afforded by acetylsalicylic acid–vitamin C. It is of interest that acetylsalicylic acid combined with vitamin C significantly attenuated the mucosal mRNA expression of two key proinflammatory cytokines (interleukin-1beta and TNF-α). This indicates that acetylsalicylic acid–vitamin C differs from plain acetylsalicylic acid in limitation of inflammatory response and attenuation of cytokine release in the gastric mucosa. This observation agrees with our previous animal studies showing that acetylsalicylic acid combined with vitamin C failed to produce a rise in plasma interleukin-1beta levels in rats with experimentally induced gastric ulcers (Brzozowski et al., 2001).

In the present study, we demonstrated that the eradication of *H. pylori* increases the susceptibility of gastric mucosa against damaging effect of plain acetylsalicylic acid. This could be explained by still pronounced expression of TNF-α in the *H. pylori*-eradicated gastric mucosa of subjects treated with acetylsalicylic acid as suggested by previous studies (Wallace et al., 1990). Parallel to the increased expression of TNF-α mRNA in gastric mucosa under acetylsalicylic acid therapy, we found a strong increase of NF-κB activity in *H. pylori*-eradicated mucosa treated with plain acetylsalicylic acid. The demonstration of increased activity of NF-κB is of particular importance in view of its implication in the inflammatory process in the gastric mucosa. NF-κB is an ubiquitous transcription factor that is activated in response to a variety of pathogens, including endotoxins, oxidative stress and proinflammatory cytokines. Under normal conditions, NF-κB is retained in the cytoplasm bound to the inhibitory proteins of the I-κB family. Following the exposure of cell to the inflammatory mediators, a numerous signal transduction pathways lead to degradation of I-κB proteins, resulting in nuclear translocation of NF-κB with subsequent transcriptional activation of proinflammatory genes and induction of the expression of some of the proinflammatory factors that initially induce its activation. By this mechanism NF-κB acts as an amplifier to maintain inflammation by positive feedback (Baldwin, 2001). In contrast to plain acetylsalicylic acid, vitamin C-releasing acetylsalicylic acid had an inhibitory effect on the activation of NF-κB. This was more pronounced after eradication of *H. pylori*, which by itself up-regulates NF-κB in the gastric mucosa (Munzenmeier et al., 1997). One possible explanation of this phenomenon is that vitamin C inhibits TNF-α induced activation of NF-κB as evidenced by the in vitro studies (Carcamo et al., 2002). These data indicate that vitamin C may influence the inflammatory process in the gastric mucosa via inhibition of NF-κB activation.

Finally, the present study demonstrated a significant up-regulation of iNOS expression at mRNA and protein level in the gastric mucosa of *H. pylori*-positive subjects taking acetylsalicylic acid. This indicates that the damage of gastric mucosa by acetylsalicylic acid in the presence of *H. pylori* leads to a significant increase of NO production. The excessive NO release has been reported to exert detrimental effects attributed to the production of nitrogen oxides and peroxynitrate (Lamarque and Whittle, 1996). It is of interest that in subjects taking acetylsalicylic acid combined with vitamin C, the gastric mucosa expression of iNOS was significantly attenuated as compared with that obtained with plain acetylsalicylic acid. This finding suggests that the protective effect of vitamin C-releasing acetylsalicylic acid on the gastric mucosa could be due to the inhibitory effect of this compound on the iNOS expression and subsequent reduction in NO formation. The exact mechanism of inhibition of iNOS expression by vitamin C-releasing acetylsalicylic acid has not been clarified in this study, but it may be due to the action of

vitamin C rather than acetylsalicylic acid on this expression. Previous studies postulated that the down-regulation of NF-κB may result in the decrease in iNOS expression (Lamarque and Whittle, 1996) and this effect could result from the action of vitamin C released from acetylsalicylic acid–vitamin C that was found in the present study to be more effective in reduction of NF-κB and iNOS expression than plain acetylsalicylic acid.

In conclusion: (1) Vitamin C-releasing acetylsalicylic acid in comparison with plain acetylsalicylic acid induces less gastric mucosal damage and this protective effect is probably due to the attenuation of oxidative stress in gastric mucosa; (2) the protective effect probably results from the presence of vitamin C in acetylsalicylic acid–vitamin C and may be due to its stronger inhibitory effect on NF-κB activity and the mucosal gene expression of proinflammatory cytokines, and (3) eradication of *H. pylori* increases the susceptibility of gastric mucosa against damaging effect of acetylsalicylic acid and this is accompanied by the increased activity of NF-κB and enhanced expression of inducible NOS.

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