

• RAPID COMMUNICATION •

***Helicobacter pylori* upregulates prion protein expression in gastric mucosa: A possible link to prion disease**

Peter C Konturek, Karolina Bazela, Vitaliy Kukharskyy, Michael Bauer, Eckhart G Hahn, Detlef Schuppan

Peter C Konturek, Karolina Bazela, Vitaliy Kukharskyy, Michael Bauer, Eckhart G Hahn, Detlef Schuppan, Department of Medicine, University of Erlangen-Nuernberg, 91054 Erlangen, Germany

Supported by Bavarian Ministry of Health, Germany

Co-correspondents: Eckhart G Hahn

Correspondence to: Assistant Professor Dr Peter C Konturek, Department of Medicine I, University Erlangen-Nuremberg, Germany. peter.konturek@med1.imed.uni-erlangen.de

Telephone: +49-9131-8535210 Fax: +49-9131-8535212

Received: 2005-01-18 Accepted: 2005-07-08

Schuppan D. *Helicobacter pylori* upregulates prion protein expression in gastric mucosa: A possible link to prion disease. *World J Gastroenterol* 2005; 11(48): 7651-7656
<http://www.wjgnet.com/1007-9327/11/7651.asp>

Abstract

AIM: Pathological prion protein (PrP^{sc}) is responsible for the development of transmissible spongiform encephalopathies (TSE). While PrP^c enters the organism via the oral route, less data is available to know about its uptake and the role of gastrointestinal inflammation on the expression of prion precursor PrP^c, which is constitutively expressed in the gastric mucosa.

METHODS: We studied PrP^c expression in the gastric mucosa of 10 *Helicobacter pylori*-positive patients before and after successful *H. pylori* eradication compared to non-infected controls using RT-PCR and Western blotting. The effect of central mediators of gastric inflammation, i.e., gastrin, prostaglandin E₂ (PGE₂), tumor necrosis factor alpha (TNF- α) and interleukin 1 beta (IL-1 β) on PrP^c expression was analyzed in gastric cell lines.

RESULTS: PrP^c expression was increased in *H. pylori*-infection compared with non-infected controls and decreased to normal after successful eradication. Gastrin, PGE₂, and IL-1 β dose-dependently upregulated PrP^c in gastric cells, while TNF- α had no effect.

CONCLUSION: *H. pylori* infection leads to the upregulation of gastric PrP^c expression. This can be linked to *H. pylori* induced hypergastrinemia and increased mucosal PGE₂ and IL-1 β synthesis. *H. pylori* creates a milieu for enhanced propagation of prions in the gastrointestinal tract.

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Key words: Prions; *Helicobacter pylori*; Gastrin; Pro-inflammatory cytokines

Konturek PC, Bazela K, Kukharskyy V, Bauer M, Hahn EG,

INTRODUCTION

Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative diseases affecting both animals and human beings^[1]. They are characterized by typical cerebral histopathological findings such as amyloid deposition, neuronal loss, and spongiform changes. The prion protein (PrP) can exist in the normal cellular form (PrP^c) or in an "infectious" form (PrP^{sc}) that causes disease by converting apathogenic PrP^c into pathogenic PrP^{sc}^[2]. Previous studies have demonstrated that PrP^c is required for prion infection propagation and infectivity has been suggested to be a consequence of conformational modification of PrP^c by the infectious PrP^{sc}. Experiment on animals shows that animals lacking the PrP^c gene are not able to propagate prion infectivity and are not able to develop the disease^[3]. Both the prion isoforms differ dramatically in their physicochemical properties. Whereas PrP^c is soluble and easily digested by proteinase K, PrP^{sc} is rich in β -sheet structure, aggregates into fibrils, and is resistant to proteinase K.

The main entry for prions is the gastrointestinal tract. Recent animal studies have shown that after oral exposure to pathogenic prions, PrP^{sc} accumulates in gut lymphoid tissues or in the enteric nervous system prior to its appearance in the central nervous system^[4,5]. It has been postulated that prions then propagate from the enteric nervous system along the nerve pathways to ventral and dorsal root ganglia and further through the spinal cord into the brain cortex^[4,6].

However, the mechanisms of propagation of prions from the gut lumen, before they reach intestinal lymph follicles or the enteric nervous system remain unexplained. Recently, the 67-ku laminin, binding protein, which can act as a PrP^{sc} receptor was demonstrated on small intestinal epithelial cells^[7-9], suggesting that individuals with a high expression of this receptor could be at greater risk of developing TSE after oral challenge with PrP^{sc}. There is also evidence of transepithelial transport of pathogenic prions via intestinal M cells to adjacent lymph follicles (Peyer's patches)^[10]. However, it is unknown if and how far gastrointestinal inflammation may influence PrP^c expression

and thus potentially PrP^{sc} propagation in the GI tract.

MATERIALS AND METHODS

Patients

Ten *H. pylori* positive patients with non-ulcer dyspepsia (mean age 47 years, range 20-79 years) were included in this study. All patients underwent upper gastrointestinal endoscopy during which four biopsies from the antrum and corpus were obtained. Patients with *H. pylori* gastritis were graded according to the updated Sydney classification^[11]. All patients underwent a second endoscopy 4 wk after completing successful eradication therapy consisting of clarithromycin 500 mg twice daily, amoxicillin 1 000 mg twice daily and omeprazole 40 mg twice daily for 1 wk. *H. pylori* was considered to be successfully eradicated, if histology was normal and silver stainable organisms were not detected anymore in the follow-up endoscopy, during which again four biopsies from the antrum and corpus were obtained.

Cell culture

MKN45 and KATO III cell lines were obtained from the American Type Culture Collection. Cells were cultured in RPMI medium containing 10% of fetal calf serum (FCS), 2 mmol/L L-glutamine and antibiotics (1% penicillin-streptomycin, 0.5% gentamycin) at 37 °C in a water-saturated atmosphere of 95% air and 50 mL/L CO₂. Subconfluent MKN45 cells were incubated in RPMI without FCS and antibiotics for 24 h. Following serum starvation, cells were exposed to the increasing amounts of gastrin (Clinalfa, Switzerland, C-210), prostaglandin E₂ (PGE₂), interleukin 1 beta (IL-1β) or tumor necrosis factor alpha (TNF-α) (all from Calbiochem, Bad Soden, Germany).

Extraction of mRNA and RT-PCR analysis

Total RNA was extracted from biopsy specimens and cultured cells using TRIzol reagent (Gibco, Karlsruhe, Germany). Single stranded cDNA was generated from 5 µg RNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and oligo-(dT)-primers (both Stratagene, Heidelberg, Germany). Briefly, 5 µg of total RNA was uncoiled by heating (65 °C for 5 min) and then reverse transcribed (37 °C for 1 h) into complementary DNA (cDNA) in a 50 µL reaction mixture that contained 50 U MMLV-RT, 0.3 µg oligo-(dT)-primer, 40 U RNase Block Ribonuclease Inhibitor, 2 µL of a 100 mmol/L mixture of dNTPs, and 5 µL of buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl, 5 mmol/L MgCl₂, pH 8.3). The resultant cDNA (2 µL) was amplified in a 50 µL reaction volume containing 2 U Taq polymerase, dNTP (200 µmol/L each), 1.5 mmol/L MgCl₂, 5 µL 10× PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3) and specific primers at a final concentration of 1 mmol/L (all reagents from Takara, Shiga, Japan). Reactions were carried out at the following conditions: denaturation at 94 °C for 45 s, annealing at 60 °C (for GAPDH) and 67 °C (for PrPc) for 45 s and extension at 72 °C for 2 min.

Polymerase chain reaction (PCR) products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Product size was confirmed by using a 100-bp ladder (Takara, Shiga, Japan) as standard. The gel was photographed under UV transillumination and the intensity of PCR products were measured using a video image analysis system (Kodak Digital Science). The signal for PrPc mRNAs was standardized against that of the GAPDH mRNA from each sample and the results were expressed as PrPc/GAPDH mRNA ratio. The following PCR primers were used based on published sequences: PrPc (sense) 5'-GGCAGTGACTATGAGGACCGTTAC-3'; PrPc (antisense) 5'-GGCTTGACCAGCATCTCAGGTCTA-3'; GAPDH (sense) 5'-GTCTTCACCACCATGGAGAAGGCT-3'; GAPDH (antisense) 5'-CATGCCAGTGAGCTTCCCGTTCA-3'. Expected product lengths were 528 bp for PrPc and 392 bp for GAPDH. All primer sequences were based on the sequences of the published cDNAs^[12,13] and synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany).

Real-time RT-PCR

PrPc transcript levels were quantified by real-time RT-PCR. Using the Primer Express software (Perkin Elmer, Tokyo, Japan) TaqMan probe and primer set were designed based on published sequences of human PrPc (GenBank accession no.: GI 11079225); PrPc sense (5'-CGCGAGCTTCTCCTCTCCTC-3'), PrPc antisense (5'-GCCCAGGTCACCTCCATGT-3') and beta-2-microglobulin (β2M, GenBank accession no.: XM_007650), β2M sense 5'-TGACTTTGT-CACAGCCCCAAGATA-3', β2M antisense primer 5'-AATCCA-AATGCGGCATCTTC-3'. Probes for PrPc (5'-TCGCCATAA-TGACTGCTCTGCCTCGGT-3') and β2M (5'-TGATGCTG-CTTACAT GTCTCGATCCCA-3') were synthesized and labeled with a reporter dye (FAM) at the 5' end and quencher dye (TAMRA) at the 3'-end at MWG Biotech AG (Ebersberg, Germany). For normalization of differences in RNA amounts and efficiencies in the reverse transcription reactions, the housekeeping gene β2M was amplified under the same conditions as PrPc. Real-time RT-PCR was performed on a LightCycler (Roche, Mannheim, Germany) in a reaction volume of 15 µL using the LightCycler FastStart DNA Master Hybridization Probes Kit (Roche Molecular Biochemicals, Mannheim, Germany). The reaction mix included FastStart Taq DNA-Polymerase, dNTP-mix, reaction buffer, MgCl₂ (3.0 mmol/L), primers (2 µmol/L of each) and probe (0.5 µmol/L). After pipetting 13.5 µL of this mixture into LC-capillaries and 1.5 µL template cDNA was added. The capillaries were sealed and placed into the thermal chamber of the LightCycler. Samples were amplified with a pre-cycling step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s and extension at 72 °C for 6 s.

Immunoblot

MKN45 cells were incubated with Gastrin 1-1 000 nmol/L, PGE₂ 1-100 nmol/L, TNF-α 1-10 ng/mL or IL-1β 1-10 ng/mL for 24 h. Cells were collected, washed

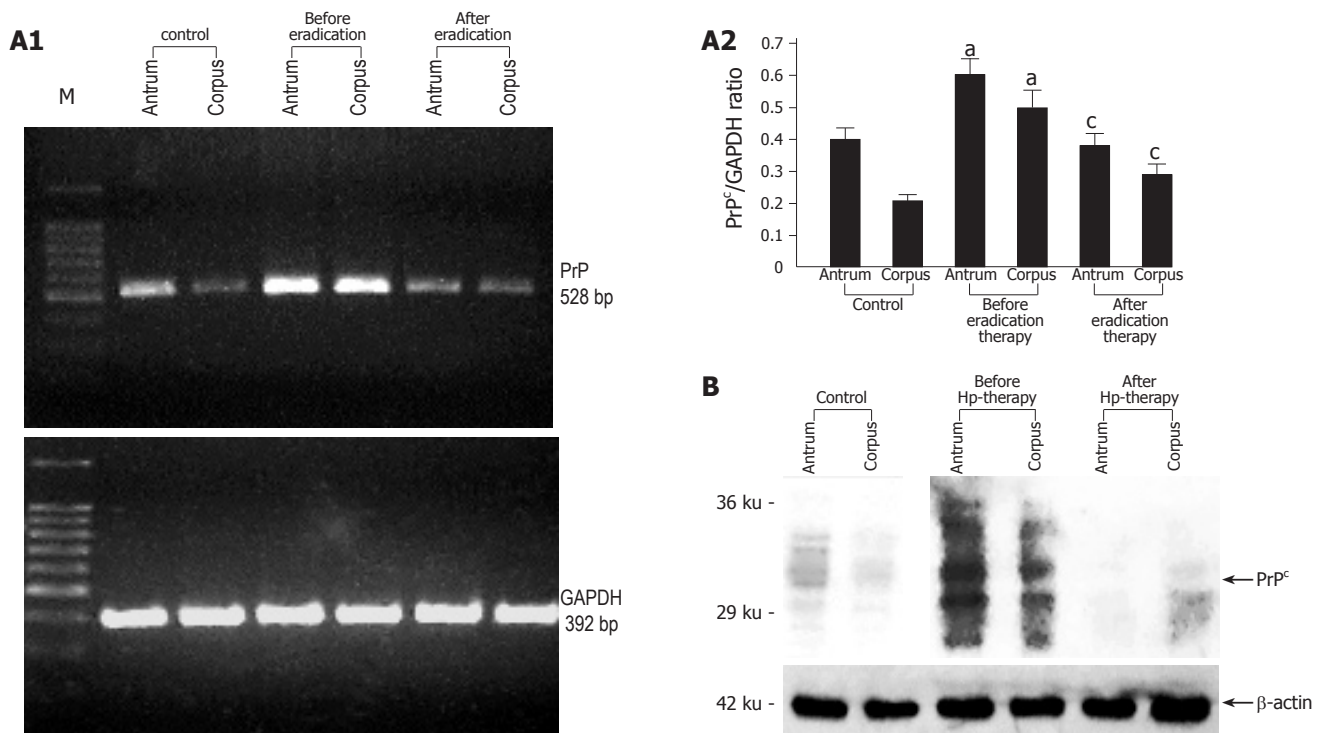


Figure 1 A: Representative RT-PCR and densitometric analysis showing PrPc mRNA expression in the gastric mucosa colonized with *H. pylori* before and after successful eradication therapy ($n = 10$). Data are expressed as means \pm SE. $^aP < 0.05$ vs the control group, $^cP < 0.05$ vs the expression before eradication therapy; **B:** Representative immunoblot analysis showing PrPc protein expression in the gastric mucosa colonized with *H. pylori* before and after successful eradication therapy.

twice with PBS, and lysed in 0.4 mL of lysis buffer (0.06 mol/L Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% beta-mercaptoethanol, 0.0025% bromophenol blue). DNA was sheared by a needle, the solution heated at 95 °C for 5 min and centrifuged at 15 000 g for 2 min at 4 °C. Twenty-five micrograms of the total protein was loaded on SDS-polyacrylamide gel, run at 40 mA and transferred to nitrocellulose (Protran, Schleicher&Schuell, Germany) by electroblotting. Filters were blocked with 3% bovine serum albumin (BSA, Sigma Aldrich, Germany) in TBS/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris-HCl, pH 7.4, 0.1% Tween-20) before incubation with antibodies against PrPc (mouse monoclonal anti-PrP antibody 6H4, 1:2 000 dilution; Prionics, Switzerland), or β -actin (mouse monoclonal, dilution 1:5 000; Sigma Aldrich, Germany), followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit-IgG secondary antibody (dilution 1:20 000; Promega, WI, USA) dissolved in 1% non-fat milk in TBS/Tween-20. Immune complexes were detected by the SuperSignal West Pico Chemiluminescent Kit (Pierce, USA) and exposed to an X-ray film (Kodak, Wiesbaden, Germany).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney Wilcoxon's test. The level of significance was set at $P < 0.05$.

RESULTS

Analysis of gastric biopsy samples obtained endoscopically from patients infected with *H. pylori*, which is found in ap-

proximately 50% of the world's population^[14] and which causes gastric inflammation and ulceration^[15] demonstrated highly increased PrPc expression compared to uninfected controls. After treatment with antibiotics which usually lead to *H. pylori* eradication, PrPc mRNA and protein expression decreased to control levels (Figures 1A and B).

Using RT-PCR and immunoblotting we demonstrated the expression of PrPc mRNA and protein in two different gastric cell lines (MKN45 and KATO III) (Figure 2). In order to assess possible mechanisms responsible for the upregulation of PrPc during chronic *H. pylori* gastritis, we analyzed the effect of increasing doses of key physiological modulators of the gastric mucosa on PrPc expression in these gastric cell lines. These modulators included gastrin (hypergastrinemia is a hallmark of chronic *H. pylori* infection^[16,17]), PGE₂ chronic *H. pylori* infection is accompanied by an increased mucosal production of PGE₂^[18], and the pro-inflammatory cytokines TNF- α and IL-1 β , which are implicated as key promoters of *H. pylori*-induced gastritis and ulceration. When exposed to gastrin, cellular PrPc expression increased in a dose-dependent manner, reaching a peak value of 100 nmol/L (Figure 3A). Similarly, PGE₂ induced maximal PrPc mRNA and protein expression at 10 μ mol/L (Figure 3B). While IL-1 β increased PrPc expression in a dose-dependent manner at the mRNA and protein level (Figures 3C and D), TNF- α showed no effect (Figure 3E).

DISCUSSION

Considering the importance of the human gastrointestinal

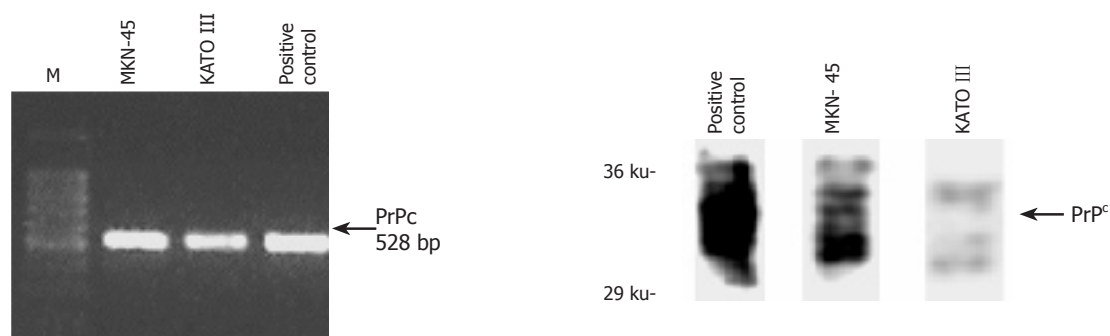


Figure 2 Protein expression of PrPc in two gastric cell lines (MKN45 and KATO III); the positive control is from bovine brain.

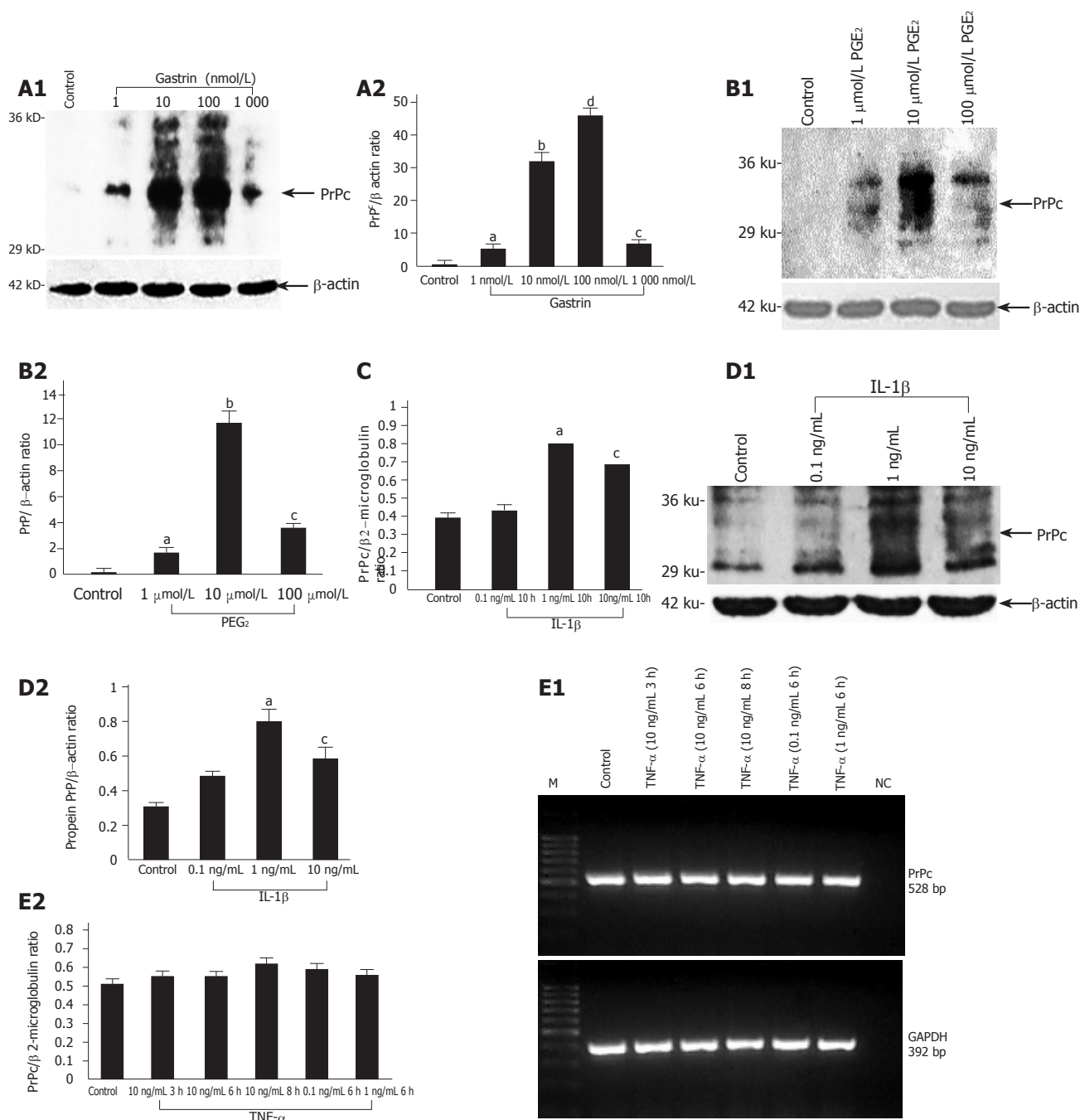


Figure 3 PrPc protein and mRNA expression in MKN45 cells incubated with increasing doses of gastrin (1-1000 nmol/L) (A), PGE₂ (1-100 μmol/L) ^a*P*<0.05 vs control, ^b*P*<0.001 vs control, ^c*P*<0.05 vs control, ^d*P*<0.001 vs control (B), interleukin 1β (0.1-10 ng/mL) (C and D) or TNF-α (0.1-10 ng/mL) ^a*P*<0.05 vs control, ^b*P*<0.001 vs control, ^c*P*<0.05 vs control (E). Data represent means ± SE of three independent experiments. At the mRNA level, the expression of PrPc was normalized to β2-microglobulin and at the protein level to β-actin.

tract for the propagation of prions from the gut to the central nervous system, we analyzed the expression of PrPc in human beings with *H. pylori* infection (determined by histology) before and after eradication therapy. This study provides the first *in vitro* and *in vivo* evidence that *H. pylori* infection is accompanied by a dramatic upregulation of PrPc expression in the gastric mucosa. The physiological importance of the observed PrPc overexpression in *H. pylori* infected gastric mucosa remains elusive. However, multiple physiological functions of PrPc were identified only recently. Thus, PrPc is involved in signal transduction, from the extracellular space to cells, participates in intracellular signaling and regulation of cell survival, protects against oxidative stress, and interacts preferentially with some of the heat-shock-proteins^[19-20]. Therefore, the main role of increased PrPc expression in the stomach appears to be the protection of the gastric mucosa against oxidative stress induced, for example by chronic *H. pylori* infection^[21]. This assumption is further supported by a previous study demonstrating that PrPc upregulates antioxidant enzyme activities^[22]. Another possible role of PrPc in the gastric mucosa could be the modulation of apoptosis induced by *H. pylori*^[23], since PrPc was shown to protect cells against Bax-mediated cell death^[24,25]. But this issue remains controversial, as other investigators demonstrated that PrPc may sensitize cells to apoptosis^[26,27]. An explanation could be the preferential use of various neuronal cell lines^[28] *in vitro* which necessitates further *in vitro* and *in vivo* studies on modulation of apoptosis gastric epithelial cells by PrPc.

In the present study, using quantitative RT-PCR and Western blot analysis we demonstrated an increased PrPc expression in the *H. pylori* infected mucosa which significantly decreased after a successful eradication therapy. The mechanisms behind this phenomenon appears to be linked to the hypergastrinemia observed during *H. pylori* infection which is supported by our *in vitro* data showing a dose-dependent increase in PrPc expression in gastric epithelial MKN45 cells after incubation with gastrin. According to previous studies, this finding shows that PrPc expression may be modulated by different growth factors^[29,30].

Since *H. pylori* infection is associated with an increased generation of prostaglandins in the gastric mucosa^[18], we analyzed the effect of prostaglandin PGE₂ on PrPc expression in MKN45 cells. At the protein level, we observed a dose-dependent increase in PrPc expression which reached its maximum at the physiological concentration of 10 µmol/L. This could represent another mechanism by which the *H. pylori*-induced inflammatory response triggers protective mechanisms in the gastric mucosa.

According to previous studies, chronic infection with *H. pylori* is accompanied by a significantly increased generation of pro-inflammatory cytokines, especially IL-1β and TNF-α, in the gastric mucosa. Thus, both cytokines could be responsible for the upregulation of PrPc in the gastric mucosa colonized by *H. pylori*. Here we have demonstrated a significant dose-dependent increase of PrPc expression in MKN45 cells incubated with IL-1β, while in contrast,

TNF-α showed no effect. We did not further analyze the difference in the action of these two cytokines, but it has been shown by others that these two cytokines evoke different signaling cascades in gastric epithelial cells^[31,32].

Gastrin, IL-1β and prostaglandins are not the sole candidates for stimulation of PrPc expression in *H. pylori* infected gastric mucosa. Although not investigated in this study, heat shock proteins could represent additional important factors responsible for the upregulation of PrPc. Previous studies demonstrated that exposure of the gastric mucosa to *H. pylori* lipopolysaccharide leads to a strong upregulation of heat shock proteins which could in turn stimulate the PrPc expression in gastric epithelial cells^[33]. In a previous study, it was found that cellular stress upregulates PrPc expression through its interaction with the heat shock elements (HSE) on the PrPc gene promoter^[34]. Together these findings suggest a regulation of PrPc expression by heat shock proteins. All these findings demonstrate the complexity of the regulation of PrPc expression and underscore the need to further analyze the precise link between *H. pylori* infection and PrPc expression.

In conclusion, our results indicate that (1) *H. pylori* infection is accompanied by a dramatic upregulation of PrPc expression in the gastric mucosa; (2) this is linked to *H. pylori*-induced hypergastrinemia, increased mucosal prostaglandin synthesis and enhanced mucosal generation of IL-1β; (3) Thus, *H. pylori* infection may promote uptake and propagation of alimentary prions from the gastrointestinal tract by upregulation of PrPc expression.

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