SYSTEMATIC REVIEWS

Oxidative and nitrosative stress enzymes in relation to nitrotyrosine in *Helicobacter pylori*-infected humans

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Abstract

AIM: To compare a possible relation between *Helicobacter pylori* (*H. pylori*) and the oxygen- and nitrogen radical system in humans.

METHODS: Mechanisms for *H. pylori* to interfere with the oxygen and nitrogen radical system is of great importance for understanding of the *H. pylori* persistence and pathogenesis. Biopsies were obtained from the gastric wall of 21 individuals. Ongoing infection with *H. pylori* was detected using direct analyze from the biopsies using campylobacter-like organism test (CLO-test) and/or by using ^{14}C-urea breath test. The individuals were divided in a negative *H. pylori* and a positive *H. pylori* group. Expression in the gastric mucosa of inducible nitric oxide syntase (iNOS), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) myeloperoxidase (MPO), and nitrotyrosine were assessed by Western blotting.

RESULTS: The individuals who undervent gastroscopy were divided in a *H. pylori* neg. [*n* = 13, m/f = 7/6, age (mean) = 39] and a *H. pylori* pos. group [*n*= 8, m/f = 5/3, age (mean) = 53]. Using western blot analysis iNOS was detected as a 130 kDa band. The iNOS expression was upregulated in the antrum of H. pylori infected individuals in comparison to the controls, mean ± SD being 12.6 ± 2.4 *vs* 8.3 ± 3.1, *P* < 0.01. There was a markedly upregulated expression of MPO in the antrum of *H. pylori* infected individuals in comparison to the control group without infection. In several of non-infected controls it was not possible to detect any MPO expression at all, whereas the expression was high in all the infected subjects, mean ± SD being 5.1 ± 3.4 *vs* 2.1 ± 1.9, *P* < 0.05. The NADPH-oxidase expression was analysed by detecting the NADPH-oxidase subunit p47-phox expression. P47-phox was detected as a 47 kDa band using Western blot, and showed a significantly higher expression of p47-phox in the antrum of the *H. pylori* infected individuals compared to the controls, mean ± SD being 3.1 ± 2.2 *vs* 0.3 ± 0.2, *P* < 0.01. Regarding nitrotyrosine formation, Western blot did not show any significant increase or decrease compared to controls, 7.0 ± 0.9 *vs* 6.9 ± 1.1, not significant.

CONCLUSION: iNOS, MPO and NADPH-oxidase was up-regulated among *H. pylori* infected. Regarding nitrotyrosine no difference was found. This support an *H. pylori* related inhibition of radical formation.

Key words: *Helicobacter pylori*; Radical; Myeloperoxidase; Nicotinamide adenine dinucleotide phosphate; Nitrotyrosine; Gastric

Core tip: The present project was performed to compare a possible relation between *Helicobacter pylori* (*H. pylori*) and the oxygen- and nitrogen radical system in humans. Expression of inducible nitric oxide syntase, myeloperoxidase and nicotinamide adenine dinucleotide phosphate-oxidase was upregulted in the antrum of the group with *H. pylori* infection. Regarding nitrotyrosine formation, Western blot did not show any significant increase or decrease compared to controls. The present study illustrates the complex picture of the oxidative stress in relation to *H. pylori* infection. The present study supports the theory of an *H. pylori* related inhibition of the enzymes involved in the oxy- and/or nitro-radical formation pathway.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a pathogen colonizing the human gastric mucosa playing a significant role in the development of gastric ulcer, gastritis, and gastric cancer[1] Until recently there was insufficient knowledge about how *H. pylori* could avoid being eliminated by the acute host defence and establish a chronic infection in the gastric mucosa of humans. Recent studies have shown that *H. pylori* interferes with reactive oxygen species (ROS) such as superoxide anion (O_2^-) that is of importance in the elimination of invading microorganisms[2,3]. At the same time reactive nitrogen intermediates such as nitric oxide (NO) represent another class of oxidants. NO can be formed as a nitrogenous product of nitric oxide synthase (NOS). Peroxynitrite, formed by NO and O_2^-, is a very powerful oxidant. It is unstable with dimensions related to the hydroxyl radical[4]. In neutrophils and macrophages large amounts of reactive oxygen and nitrogen species are presented to the invading microorganism. Neutrophils phagocytose bacteria into the intracellular phagosome, where an eruption of reactive species results in bacterial destruction. During successful conditions the bacteria is eliminated and there is no extracellular oxidant generation[5].

However *H. pylori* persist in the gastric mucosa, causing a chronic infection that increases the risk for pathological changes such as adenocarcinoma. Therefore the mechanisms for *H. pylori* to interfere with the oxygen and nitrogen radical system is of great importance for understanding the persistence and pathogenesis of *H. pylori*.

We and others have pointed out the association between *H. pylori* infection and an increased mucosal expression of iNOS both in humans and in mongolian gerbils[6-8]. Despite what one could expect, the juxtamucosal level of nitric oxide (NO) is lower in the infected than in the uninfected stomach[7,8]. We have shown that there is an inhibition of nitrotyrosine expression, being a reflection of the formation of peroxynitrite, in *H. pylori* infected Mongolian gerbils despite upregulated formation of both myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS)[6]. Results from *in vivo* registration of NO and hydrogen peroxide (H_2O_2) on Mongolian gerbils substantiates the fact infection with *H. pylori* reduces levels of NO[9]. It is recently suggested that specific proteins contained by *H. pylori* enables the pathogen to cope with the damaging effects of NO. These systems are suggested to be a part in the microbial protection against nitrosative stress[10]. Several traditional anti-inflammatory drugs have been shown to have an effect on epithelial cells infected by *H. pylori* by inhibiting the induction of iNOS by suppressing the activation of NADPH oxidase[11] .

The present project was performed to further compare a possible relation between *H. pylori* and the oxygen- and nitrogen radical system in humans.

Special interest was on the suspected upregulation on the enzymatic oxy- and nitro radical systems, and if this would result in an increased radical formation. To evaluate activity of peroxynitrite, expression of nitrotyrosine was used as an indicator of radical formation.

MATERIALS AND METHODS

Ethics approval

Study groups

Gastric biopsies were obtained from the antral wall of 21 individuals. The individuals were divided in a *H. pylori* neg. [$n = 13$, m/f = 7/6, age (mean) = 39] and a *H. pylori* pos. group [$n = 8$, m/f = 5/3, age (mean) = 53]. Gastro-esophageal reflux (GER) was diagnosed in one subject in the *H. pylori* pos group and in four subjects in the *H. pylori* neg group. Ulcer in the duodenum was found in two individuals in the *H. pylori* pos group.

Diagnostic procedures

Ongoing infection with *H. pylori* was detected using direct analyze from the biopsies using campylobacter-like organism test (CLO-test) and/or by using ^{14}C-urea breath test[12].

Western blot

Biopsies were collected during gastroscopy. The samples were snap-frozen in nitrogenum liquidum and kept for further analysis at -70 ℃. Sonication (Sonifier 450/250, Branson Ultrasonics Co. Danbury, United States) or homogenization (Polytron, PT-MR 2100, Kinematica) was performed of all samples at 2 ℃ in a PE-buffer (10

mmol/L potassium Phosphate buffer, pH 6.8, and 1 mmol/L EDTA) containing CHAPS {3-[(3-cholamido-propyl) dimethyl-ammanio] 1-propanesulfonate}, aprotinin (1 μg/mL), leupeptin (10 μg/ mL), pepstatin (10 μg/mL) and Pefablock (1 mg/mL) (Boeringer Mannheim, Mannheim, Germany). All samples were centrifugated at 10.000 *g* for 10 min at 4 ℃. Analysation was performed of the supernatant for protein content using the method of Bradford[12] and then kept at -70 ℃ for future analysis. Samples were diluted in SDS-buffer and heated at 70 ℃ for 10 min before they were loaded on a NuPage 10% BisTris gel (Invitrogen, Carlsbad, CA, United States). One lane of each gel was loaded with prestained molecular weight standards (SeeBlue™, Invitrogen, Carlsbad, CA, United States). Following the electrophoresis the proteins were transferred to a polyvinyldifluoride membrane (Amersham, Buckinghamshire, United Kingdom) which was incubated with antibodies directed against iNOS, MPO, NADPH-oxidase or nitrotyrosine containing proteins. For identifying iNOS the NOS2 (H-174) sc-8310 (Santa Cruz Biotechnology inc) antibody was used. It is a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 2-175 mapping at the amino terminus of iNOS of human origin. Lack of cross-reaction with nNOS or eNOS was reported by manufacturer. Antibody Anti-myeloperoxidase 07-496 lot 24587 (Upstate, Lake Placid, NY, United States) was used for detecting MPO. This is a rabbit antibody that recognizes MPO subunits at 12 and 60 kDa. In the present study the 60 kDa band was used for quantification of the protein. Anti-nitrotyrosine rabbit immunoaffinity purified IgG catalog 06-284, lot 26427 (Upstate, Lake Placid, NY, United States) was used to assess nitrated proteins. For identifying NADPH-oxidase the p47-phox (H-195) sc 14015 (Santa Cruz Biotechnology inc.) was used. This is a rabbit polyclonal antibody raised against amino acids 196-390 of p47-phox of human origin. P47-phox is required for activation of NADPH-oxidase in neutrophils and other phagocytic cells. During activation of NADPH-oxidase, p47-phox migrate to the plasma membrane where it associates with other subunits to form the active complex. Goat anti-rabbit antibodies were used to identify immunoreactive proteins by chemiluminescence [iNOS, NADPH-oxidase (p47-phox) and nitrotyrosine; goat-anti rabbit sc 2007(Santa Cruz, CA, United States)] [MPO; IgG 12-448 (Upstate Lake Placid, NY, United States)]. CDP-Star (Tropix, Bedford, MA, United States) was used as a substrate. Images were captured by a LAS-100 cooled CCD-camera (Fujifilm, Tokyo, Japan) and semi-quantification was performed using the soft ware Gauge 3.3 (Fujifilm, Tokyo, Japan). As positive controls, to confirm the identity of the protein, RAW 264.7 (sc 2212, Santa Cruz Biotech) was used for iNOS, HL60 was used for MPO and NADPH-oxidase (p47-phox).For nitrotyrosine the immunoblotting control (12-354, Upstate) was used.

Statistical analysis

Statistical analysis was performed using non parametric Mann-Whitney *U*-test. *P*-values of < 0.05 were regarded as being of statistical significance.

RESULTS

Inducible nitric oxide synthase

Using western blot analysis iNOS was detected as a 130 kDa band. The iNOS expression was upregulated in the antrum of *H. pylori* infected individuals in comparison to the control group without infection as shown in Figure 1A, mean ± SD being 12.6 ± 2.4 *vs* 8.3 ± 3.1, *P* < 0.01. Western blot detecting iNOS with a band at 130 kDa in RAW 264.7 (pos contr.), and in human antral mucosa retrieved from *H. pylori* pos. and *H. pylori* neg. volunteers during endoscopy is shown in Figure 2.

Myeloperoxidase

As shown in Figure 1B, MPO expression was markedly upregulated in the antrum of the *H. pylori* infected individuals in comparison to the control group without infection, mean ± SD being 5.1 ± 3.4 *vs* 2.1 ± 1.9, *P* < 0.05. In several of the non-infected controls it was not possible to detect any MPO expression at all, whereas the expression was high in all the infected subjects. Western blot of the MPO positive 60 kDa band in the positive HL60 control and in gastric mucosal specimens of *H. pylori* pos. and *H. pylori* neg. volunteers is shown in Figure 2.

NADPH-oxidase

The expression of NADPH-oxidase was analysed by detecting the NADPH-oxidase subunit p47-phox expression. P47-phox was detected as a 47 kDa band using Western blot. Figure 1C shows a significantly higher expression of p47-phox in the the antrum of *H. pylori* infected individuals in comparison to the control group without infection, mean ± SD being 3.1 ± 2.2 *vs* 0.3 ± 0.2, *P* < 0.01. P47-phox was low in all non-infected controls. In the *H. pylori* infected subjects there was a large spreading of the p47-phox expression. A typical Western blot result is shown in Figure 2.

Nitrotyrosine

Western blot analysis did not show any significant increase nor decrease in nitrotyrosine expression the antrum of *H. pylori* infected individuals in comparison to the control group without infection, 7.0 ± 0.9 *vs* 6.9 ± 1.1, not significant (Figure 3). Regarding Western blot representing Nitrotyrosine, several bands of Nitrated proteins could be analysed. Shown in the Figure 2 is a typical 66 kDa band in the positive control and in *H.pylori* pos. and *H. pylori* neg. subjects.

DISCUSSION

The findings of the present investigation can confirm that *H. pylori* infection in humans is related to an up regulation of the expression of MPO, iNOS and NADPH-
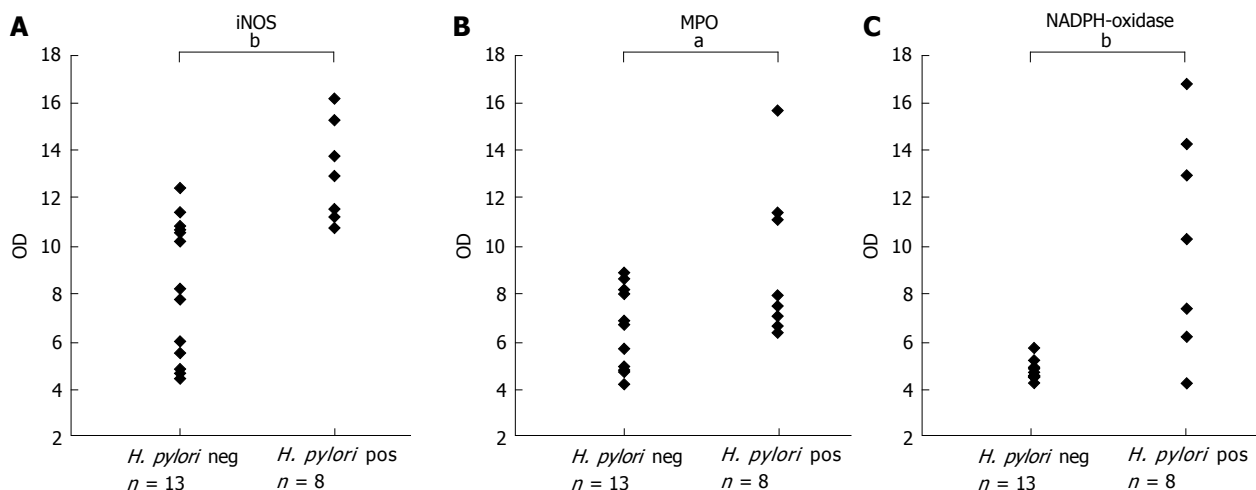
Figure 1 Scatter-plot demonstrating the result of Western blot inducible nitric oxide synthase, myeloperoxidase and nicotinamide adenine dinucleotide phosphate-oxidase protein expression in biopsies from the anrum of the *Helicobacter pylori* neg (*n* = 13) and *Helicobacter pylori* pos (*n* = 8) groups. A: Inducible nitric oxide synthase (iNOS), [b]$P < 0.01$; B: Myeloperoxidase (MPO), [a]$P < 0.05$; C: Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase subunit p47-phox in biopsies from the antrum of the *H. pylori* neg. (*n* = 13) and *H. pylori* pos. (*n* = 8) groups, [b]$P < 0.01$, OD: Optical density.
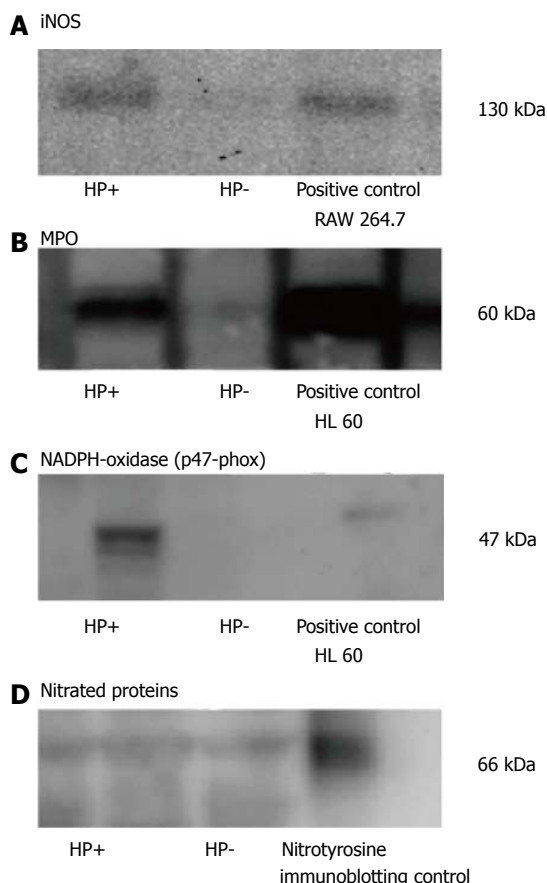

Figure 2 Western blot. A: Western blot detecting inducible nitric oxide syntase (iNOS) with a band at 130 kDa in RAW 264.7 (pos contr.), and in human antral mucosa retrieved from *Helicobacter pylori* (*H. pylori*) pos. and *H. pylori* neg. volunteers during endoscopy; B: Western blot of the MPO positive 60 kDa band in the positive HL60 control and in gastric mucosal specimens of *H.pylori* pos. and *H.pylori* neg. volunteers; C: Western blot of p47-phox, representing NADPH-oxidase with a band at 47 kDa (pos. contr.) HL60, and in HP+ and HP-samples; D: Regarding Western blot representing Nitrotyrosine, several bands of Nitrated proteins could be analysed. Shown in the figure is a typical 66 kDa band in the pos. control and in *H.pylori* pos. and *H.pylori* neg. subjects.
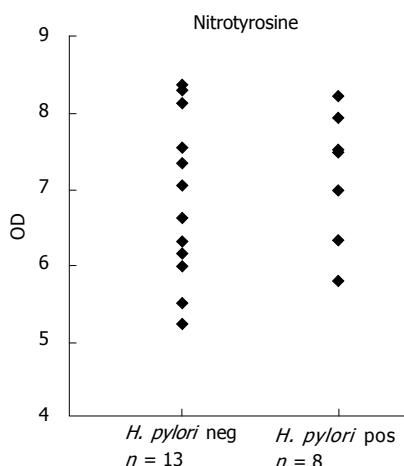

Figure 3 Nitrotyrosine. Scatter-plot demonstrating the result of Western blot analysis of nitrotyrosine formation in biopsies from the antrum of the *Helicobacter pylori* (*H. pylori*) neg. (*n* = 13) and *H. pylori* pos. (*n* = 8) groups. There were no significant changes between groups. OD: Optical density.

oxidase in the human gastric mucosa. Furthermore the study shows that there are no significant changes in levels of proteins containing nitrotyrosine compared to non-infected subjects following this up-regulation. This finding confirms the results from our studies on Mongolian gerbils, and supports the theory of an *H. pylori* related inhibition of radical formation[6,9].

Studying the early stages of *H. pylori* infecting the stomach is important for understanding the evolution of pathology such as carcinogenesis. Using an animal model makes it possible to assess different stages of pathological development in an experimental setting. However it is important to evaluate the experimental findings in a human population before making any conclusions regarding *H. pylori* infection in human gastric mucosa.

The initial host reaction to the *H. pylori* infection is

the same as to any bacterial infection: Phagocytic neutrophils and monocytes are recruited to the infected tissue and consume oxygen that is converted to O_2^- by NADPH-oxidase, and then dismutated to H_2O_2[13]. Activation of neutrophils results in the release of MPO, which catalyzes the oxidation of electron donors by H_2O_2[14]. A complex is formed that is responsible for the production of powerful oxidants with potential to react with a large variety of substances[15,16]. For example, MPO-H_2O_2 reacts with chloride to form hypochlorus acid and subsequently the oxidative chloramines are formed. The MPO-H_2O_2-chloride system is responsible for many biological effects, both beneficial and negative for the host[17].

In general, inflammation results in invading epithelial cells and macrophages leading to a marked expression of iNOS and resulting in generation of NO[17].

Several studies have described an increase in iNOS production following *H. pylori* infection in both humans and animal models[6-8,18-20]. Some have suggested that the up-regulated iNOS production following *H. pylori* infection would lead to an increase in NO production which could result in the increase of DNA damage and apoptosis[18-21]. It has been suggested that classification of iNOS expression in the gastric mucosa could be used clinically to identify patients with a high risk for gastric cancer[22]. The host will try to terminate the infection by activating the mucosal generation of the oxy- and nitroradical forming enzymes the resulting in formation of the cytotoxic peroxynitrite. In the extracellular space NO released from macrophages can eliminate *H. pylori*[23]. An effective increase of production of NO and oxy-radicals would lead to eradication of the bacteria. However *H. pylori* persist in the host, causing a chronic inflammatory reaction that instead in the long run may be deleterious to the host. The fact that *H. pylori* survives in this hostile environment despite up regulation of iNOS suggests that the pathogen has developed strategies to avoid NO-dependent eradication. An increasing number of studies have reported about the complexity of the *H. pylori* response to oxidative and nitrogen stress[24,25].

H. pylori may also have a direct effect on reduction in gastric mucosal blood flow by inhibiting NO production by iNOS and thereby reducing the vasodilatory and mast cell stabilizing effect of NO[26].

We have by use of electrochemical microelectrodes *in vivo* confirmed reduction of intraluminal NO in Mongolian gerbils following infection with *H.pylori*[9]. Reduced levels of NO could be explained by inhibition of iNOS activity[27]. Helicobacter produced arginase has been proposed as one of the ways for *H. pylori* to inhibit NO production[23,25]. *H. pylori* may also produce asymmetrical dimethyl arginine (ADMA) that can block iNOS by competitive inhibition. ADMA is a methylated form of arginine that has been found to be significantly up-regulated in the human antrum of *H. pylori* positive individuals[8,28]. Another explanation for reduced NO levels could be scavenge of NO by reacting with reactive oxygen species (ROS)[7,18]. A result of this reaction would be an increase

in the production of peroxynitrite, and resulting in increased levels of nitrotyrosine. Thus nitrotyrosine can be used to indicate peroxinitrite activity over time. The present investigation as well as previous studies on *H. pylori* infection in Mongolian gerbil demonstrates a significant up regulation of the formation of iNOS and MPO, but no significant changes in the levels of nitrotyrosine[6]. These findings strongly support the theory supports the theory of an *H. pylori* related inhibition of radical formation at an enzymatic level of NO generation.

The present study does not provide data on if *H. pylori* also inhibit the oxy-radical forming enzymes. Oxidative stress could potentially have a negative effect on the capacity of *H. pylori* to infest the human stomach. However it is shown that *H. pylori* produces a number of antioxidative proteins, the most described ones being bacterial produced superoxide dismutase (SOD)[29]. SOD production is described as being of importance for *H. pylori* being able to grow and survive in a situation with oxidative stress, and is regarded as a factor being of importance for of the microbial colonization of the stomach. Catalase and arginase are other examples of antioxidant proteins produced by *H. pylori* that might contribute to bacterial survival under conditions of oxidative stress[23,30,31].

Taken together the present study illustrates the complex picture of the oxidative stress response to *H. pylori* infection. The nitro- and oxy-radical formation systems are up-regulated following infection and inflammation. This up-regulation is to be regarded as an attempt from the host to eradicate the bacteria. However, long standing up-regulation of the reactive oxygen- and nitrogen species will also lead to tissue damage and a risk of carcinogenesis. This study supports the theory supports the theory of an *H. pylori* related inhibition the. The mechanisms behind how the bacteria and the impotent host defence act to induce DNA- and tissue damaging effects need to be further explored.

The results suggest that there is a relationship between inhibition of formation of ROS and reactive nitrogen species and *H. pylori* being able to survive in the human gastric mucosa.

COMMENTS

Background

Helicobacter pylori (H. pylori) colonization of the mucosal space of the stomach causes a chronic infection resulting in the development of pathological changes such as adenocarcinoma. Mechanisms for *H. pylori* to interfere with the oxygen and nitrogen radical system is of great importance for understanding the persistence and pathogenesis of *H. pylori*.

Research frontiers

Several studies have described an increase in inducible nitric oxide syntase (iNOS) production following *H. pylori* infection in both humans and animal models. An effective increase of production of NO and oxy-radicals would lead to eradication of the bacteria. The fact that *H. pylori* survives in this hostile environment despite up regulation of iNOS suggests that the pathogen has developed strategies to avoid NO-dependent eradication. The findings of the present investigation can confirm that *H. pylori* infection in humans is related to an up regulation of the expression of MPO, iNOS and NADPH-oxidase in the human gastric mucosa. Furthermore the study shows that there are no significant

changes in levels of proteins containing nitrotyrosine compared to non-infected subjects following this up-regulation.

Breakthroughs and innovations

The investigation presented here illustrates the complex picture of the oxidative stress response to *H. pylori* infection. The nitro- and oxy-radical formation systems are up-regulated following infection and inflammation. This up-regulation is to be regarded as an attempt from the host to eradicate the bacteria. However, long standing up-regulation of the reactive oxygen- and nitrogen species will also lead to tissue damage and a risk of carcinogenesis. This study supports the theory of an *H.pylori* related inhibition of formation of reactive oxygen species (ROS) and reactive nitrogen species. The mechanisms behind how the bacteria and the impotent host defence act to induce DNA- and tissue damaging effects need to be further explored.

Applications

By understanding how *H. pylori* manages not to be extinguished in the hostile environment by hindering the formation of reactive oxygen species and reactive nitrogen intermediates we will gain a greater understanding of the mechanisms involved in *H. pylori* related disease.

Terminology

Myeloperoxidase (MPO) is an enzyme of importance in the microbicidal role of phagocytes. iNOS was first identified in macrophages. iNOS is involved in the production of NO, but has also many other functions. Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) is a transmembrane electron transport chain involved in the production of different ROS. Nitrotyrosine can be used as marking the activity of peroxynitrite.

Peer review

In this study the authors demonstrated, in human gastric mucosa of *H.pylori* positive patients, an increase of some enzymes belonging to oxidative stress pathway, while the amount of nitrotyrosine rich proteins did not differ from *H.pylori* negative tissues.

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