The Journal of Microbiology (2011) Vol. 49, No. 2, pp. 251-256
DOI 10.1007/s12275-011-0262-7

Identification of S-Nitrosylation of Proteins of *Helicobacter pylori* in Response to Nitric Oxide Stress

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(Received July 21, 2010 / Accepted November 10, 2010)

Innate and adaptive immune responses are activated in humans when *Helicobacter pylori* invades the gastric mucosa. Nitric oxide (NO) and reactive nitrogen species are important immune effectors, which can exert their functions through oxidation and S-nitrosylation of proteins. S-nitrosoglutathione and sodium nitroprusside were used as NO donors and *H. pylori* cells were incubated with these compounds to analyze the inhibitory effect of NO. The suppressing effect of NO on *H. pylori* has been shown *in vitro*. Furthermore, the proteins modified by S-nitrosylation in *H. pylori* were identified through the biotin switch method in association with matrix-assisted laser desorption ionization/time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS). Five S-nitrosylated proteins identified were a chaperone and heat-shock protein (GroEL), alkyl hydroperoxide reductase (TsaA), urease alpha subunit (UreA), HP0721, and HP0129. Importantly, S-nitrosylation of TsaA and UreA were confirmed using purified recombinant proteins. Considering the importance of these enzymes in antioxidant defenses, adherence, and colonization, NO may exert its antibacterial actions by targeting enzymes through S-nitrosylation. Identification of protein S-nitrosylation may contribute to an understanding of the antibacterial actions of NO. Our findings provide an insight into potential targets for the development of novel therapeutic agents against *H. pylori* infection.

***Keywords*:** *Helicobacter pylori*, S-nitrosylation, nitric oxide, biotin switch

Helicobacter pylori (*H. pylori*) is a Gram-negative, spiral, and microaerophilic bacterium that selectively colonizes the gastric mucosa, causing gastritis, ulcers, and adenocarcinomas (McGee and Mobley, 2000). By colonizing the gastric mucosa, *H. pylori* can stimulate the human immune system, which releases various antimicrobial factors. Nitric oxide (NO) is an important effector possessing antimicrobial activity and immunomodulatory function (Cole *et al*., 1999; Zaki *et al*., 2005). Current data imply that NO and reactive nitrogen species (RNSs) could exert strong antibacterial activity on *H. pylori in vitro*; moreover, the response of *H. pylori* proteins to NO stress has been previously described (Qu *et al*., 2009). However, the mechanisms by which RNSs suppress or kill *H. pylori* are not well defined.

Protein S-nitrosylation is a process by which a NO moiety irreversibly attaches to selected cysteine residues of specific proteins. It can produce a labile S-nitrosothiol structure and some functional alterations, involving a broad range of physiological and pathological cellular events, such as smooth muscle relaxation, neurotransmission, and immune defense (Rhee *et al*., 2005; Torta *et al*., 2008). The antimicrobial action of RNSs against HIV, *Leishmania*, *Plasmodium*, *Salmonella,* and *Mycobacterium* is mediated by the binding of NO to important cysteine groups (Persichini *et al*., 1998; Venturini *et al*., 2000; Salvati *et al*., 2001; Schapiro *et al*., 2003). In spite of the important role of RNSs as antimicrobial and regulatory molecules, no S-nitrosylated proteins have been identified in *H. pylori*.

Until now, the most commonly used method in the analysis of S-nitrosylated proteins has been the biotin switch method (BSM). It was developed by Jaffrey and Snyder (2001), through which the nitrosylated cysteines can be specially converted into biotinylated cysteines (Forrester *et al*., 2009). Later, they can be easily detected using streptavidin or any specific antibody. NO targets involved in a broad range of cellular processes, such as signaling, stress responses, protein synthesis, and so on, have been identified using this approach (Martínez-Ruiz and Lamas, 2004; Torta *et al*., 2008). In this study, we adopted the BSM to characterize the S-nitrosylated proteins in *H. pylori in vitro*.

Materials and Methods

Chemicals

Methyl methanethiosulfonate (MMTS), streptavidin-agarose, L-glutathione (reduced), neocuproine (crystalline), sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), sodium ascorbate, glutathione (GSH), dimethylformamide, dimethylsulfoxide, 4-(2-hydroxyethyl)-1-

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piperazineethanesulfonic acid (Hepes), 2-mercaptoethanol (2-ME), and sodium dodecyl sulfate (SDS) were obtained from Sigma, USA. N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide (Biotin-HPDP) was purchased from Pierce, USA.

Protein lysate preparation and *in vitro* S-nitrosylation

H. pylori 26695 was provided by Dr. Zhang Jianzhong of the Chinese Disease Control and Prevention Center. Bacterial cells were grown microaerobically (5% O_2, 10% CO_2, and 85% N_2) in Brucella broth medium containing 10% fetal bovine serum at 37°C. *H. pylori* cells at logarithmic phase were exposed to various concentrations (0.0, 0.5 mM, and 1.0 mM) of GSNO for different durations (0, 3, 6, 9, and 12 h). *H. pylori* was inoculated to obtain an initial absorbance (OD_{600}) value of 0.17, and triplicate cultures were treated with SNP (100 μM), GSNO (100 μM), or neither; the growth rate was monitored spectrophotometrically. *H. pylori* was harvested at the logarithmic phase by centrifugation, washed three times in sterilized ice-cold phosphate-buffered saline (0.01 M, pH 7.4), and then resuspended in lysis buffer. The lysis buffer contained 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, commonly known as CHAPS, 1% dithiothreitol (DTT), 4 mM Tris, 1% Pharmalyte™ [(pH 3-10) GE Healthcare, USA], 10 μl/ml protease inhibitor cocktail (GE Healthcare), 10 μg/ml RNase, and 10 μg/ml DNase. After sonication, the solution was centrifuged at 20,000×g for 60 min at 4°C to remove cell debris. The protein concentration was determined using the Bradford method and was adjusted to 0.6 μg/μl. The protein was stored at -80°C until further analysis.

To induce S-nitrosylation, the protein was incubated with GSNO (100 μM) in the dark for 20 min. As a control, the duplicate protein was reacted with GSH (100 μM) similarly. Then, 10 volumes of prechilled acetone (-20°C) were added to each sample. After incubation for 20 min at -20°C, the mixture was centrifuged at 2,000×g for 10 min at 4°C to remove the residual NO donor. The precipitate contained the S-nitrosylated proteins. These were then resuspended in a buffer containing 250 mM Hepes, 1 mM EDTA, and 0.1 mM Neocuproine (HEN).

Purification of the S-nitrosylated proteins by BSM

The BSM was carried out in the dark, as described before (Torta *et al*., 2008; Forrester *et al*., 2009). The initial sample of protein (1 mg) was blocked with 4 volumes of HEN buffer containing 2.5% SDS and 20 mM MMTS and incubated at 50°C for 20 min. Acetone precipitation was used to remove the remaining MMTS. The pellet thus formed was resuspended in HEN buffer containing 1% SDS. Then, the mixture was incubated with ascorbate (2 mM) and biotin-HPDP (4 mM) for 1 h at room temperature. Subsequently, the proteins were once again precipitated with acetone, and the pellet was resuspended once more. In the next step, 15 μl of packed streptavidin-agarose were added, and the mixture was incubated for 1 h at room temperature with agitation. The beads were washed five times with 10 volumes of washing buffer (20 mM Hepes; pH 7.7, 600 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) and incubated with the elution buffer (20 mM Hepes; pH 7.7, 100 mM NaCl, 1 mM EDTA, and 100 mM 2-ME) for 20 min at 37°C with gentle agitation. Finally, the supernatant containing the purified S-nitrosylated proteins was collected without disturbing the pelleted resin.

Separation of the S-nitrosylated proteins and identification by matrix-assisted laser desorption ionization/time-of-flight tandem mass spectrometry

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 10% gels. The electrophoresis was conducted at 80 mV per gel for 30 min, and then 120 mV per gel until the bromophenol blue front reached the bottom of the gel. The gels were silver-stained and scanned using an Image Scanner II (Amersham Biosciences, USA) at 256-grayscale and 300 dpi-degree levels. The protein bands were excised and digested with trypsin (5 mg/ml) overnight at 30°C. The digested peptides were lyophilized and desalted by passage through a C18 ZipTip (Millipore, USA). The resulting peptides were eluted with the matrix (5 mg/ml α-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid and 50% acetonitrile) and spotted onto matrix-assisted laser desorption ionization (MALDI) target plates. The mass spectra were then collected for peptide-mass fingerprinting (PMF). PMF was acquired on a MALDI/time-of-flight (TOF)-tandem mass spectrometer (MALDI-TOF-MS/MS spectrometer, Applied Biosystems, USA). The mass spectra (MS) and tandem mass spectra (MS/MS) were analyzed with a 50-ppm tolerance in mass using the software GPS Explorer V2.0.1 and Mascot V1.9 based on the SWISSPROT database of the National Center for Biotechnology Information and local *H. pylori* databases (April 2006, updated). The search parameters in the program allowed for an error of 100 ppm (0.01%) in the molecular mass.

Expression of recombinant alpha subunit of urease, UreA, and alkyl hydroperoxide reductase, TsaA

Recombinant His-urease and His-tsaA alkyl hydroperoxide reductase proteins were prepared from *E. coli* JM109 cells transfected with pTriEX-4-urease and pTriEX-4-tsaA recombined plasmids. These plasmids were constructed as follows. The genome of *H. pylori* 26695 was extracted to serve as the template. The primers used are listed in Table 1. The fragment containing the urease gene flanked by *Xho*I and *Nco*I sites was amplified by the polymerase chain reaction (PCR) using the primer pairs urea-1/urea-2. The fragment containing the urease gene flanked by *Xho*I and *Nco*I sites was amplified by PCR using the primer pairs tsaA-1/tsaA-2. Next, they were respectively ligated with the *Xho*I/*Nco*I-digested vector pTriEX-4, generating the plasmids pTriEX-4-urease and pTriEX-4-tsaA. Subsequently, the recombined vectors were transformed into *E. coli* JM109. Genomic DNA was extracted from the putative recombinants. The appropriateness of recombination was evaluated by PCR and DNA sequencing (Boshang Company, China). The fusion proteins His-urease and His-tsaA were purified by affinity chromatography through a Ni^{2+}-affinity column (Sigma, USA) after denaturation, as described previously (Marikar *et al*., 2008). Protein concentration was determined using the Bradford method and was adjusted to 0.6 μg/μl. The protein sample was stored at -80°C until further analysis.

Detection of protein S-nitrosylation in alpha subunit of urease, UreA, and alkyl hydroperoxide reductase, TsaA

To detect protein S-nitrosylation, the recombined proteins were incubated with GSNO (100 μM) and GSH (100 μM), respectively.

Table 1. Primer sets used in this study

Primers	Sequence (5′→3′)
ureA-1	*CTCGAG*CTCCTTAATTGTTTTTACATAG
ureA-2	*CCATGG*CAATGAAACTCACCCCAAAAGAGT
tsaA-1	*CTCGAG*AAGCTTAATGGAATTTTCTTTG
tsaA-2	*CCATGG*CAATGTTAGTTACAAAACTTGCCC

Italics indicate nucleotides that were added at the 5′ end to create a restriction site. Restriction sites for *Xho*I (ureA-1, tsaA-1) and *Nco*I (ureA-2, tsaA-2) are underlined.

Subsequently, the S-nitrosylated proteins were purified by the BSM and separated by 10% SDS-PAGE. The gels were silver-stained and scanned.

Assay of urease activity

The urease activity was assayed by a modified colorimetric method, as described previously (Krajewska and Zaborska, 2007). *H. pylori* cells at the logarithmic phase were exposed to various concentrations (0, 300, 600, and 900 μM) of GSNO in flasks, under microaerobic conditions at 37°C. To the duplicate cultures, 1 mM DTT was added to decompose GSNO. After 3 h, the absorbance at 600 nm (OD_{600}) was measured, 10 μl of the suspension was applied to a Skirrow agar plate with 5% (v/v) sheep blood under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C and incubated for 3-4 days before assessing the number of colony-forming units (CFU)/ml. An aliquot of the suspension (100 μl) was incubated with urease assay buffer (100 mM urea, 1 mM phenol red, 50 mM phosphate buffer, 2 mM EDTA, pH 7.8) for 20 min. The absorbance at 560 nm (A_{560nm}) was measured using a spectrophotometer (Bio-Rad, USA), with urease assay buffer as the negative control. The value A_{560nm}/Log_{10} (CFU/ml) was derived and used to evaluate the inhibitory effect of GSNO on urease activity.

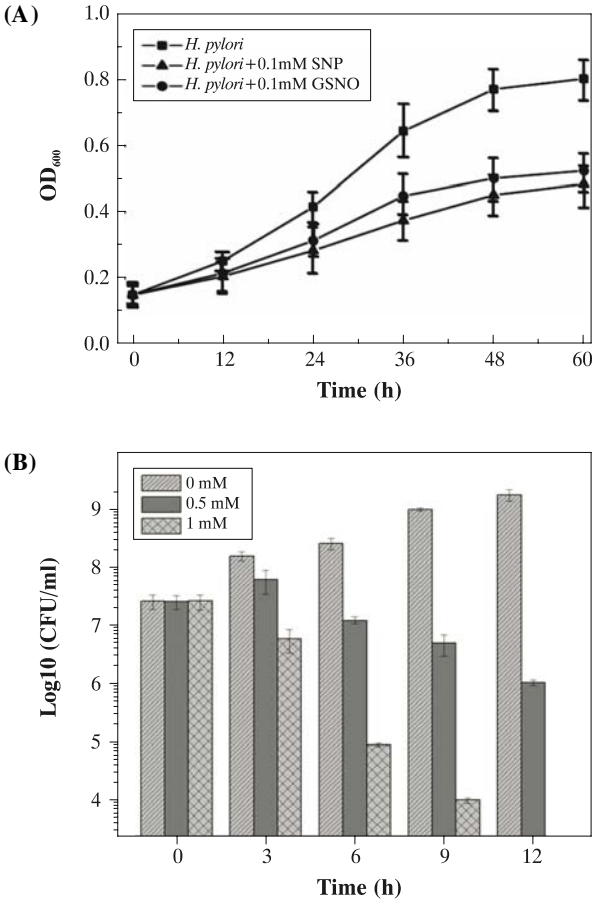

Fig. 1. (A) The growth curve of *H. pylori* in the presence of nitrosylation stress. *H. pylori* was inoculated to obtain an initial OD_{600} value of 0.17, and the triplicate cultures were treated with SNP (100 μM), GSNO (100 μM) or neither; the growth rate was monitored spectrophotometrically. The data represent the mean of three replicate assays. Application of Student's t-test showed that the differences between *H. pylori* with SNP or GSNO and the *H. pylori* without NO donor were significant ($p<0.05$). (B) GSNO has a suppressing effect on *H. pylori*. *H. pylori* cells were grown to logarithmic phase, and then exposed to various concentrations of GSNO for 0, 3 h, 6 h, 9 h, and 12 h. The data represents the mean of three replicate assays. Application of Student's t-test showed that the differences between *H. pylori* with 0.5 mM or 1 mM GSNO and the *H. pylori* without NO donor were significant ($p<0.05$).

Results

The inhibitory effect of NO donors on *H. pylori*

To investigate the effects of NO and RNSs on *H. pylori*, GSNO and SNP were used as NO donors and the bacterium was incubated with these compounds; the growth curves of the bacteria were recorded. As shown in Fig. 1A, the growth of *H. pylori* was significantly inhibited by GSNO or SNP treatment ($p<0.05$). We next examined the effects of GSNO at various concentrations (0.0, 0.5, and 1 mM) and treatment durations (0-12 h) on the viability of *H. pylori* cells. Figure 1B shows that GSNO decreased cell viability of the logarithmic-phase *H. pylori* in a time-dependent manner in this short period ($p<0.05$).

Multiple *H. pylori* proteins were S-nitrosylated after GSNO treatment

To elucidate the mechanisms by which RNSs suppress and kill *H. pylori*, protein S-nitrosylation was measured using BSM on *H. pylori* proteins treated with GSNO. More than 10 protein bands were detected in this assay, indicating that *H. pylori* proteins were S-nitrosylated after GSNO treatment (Fig. 2). Several bands were detected in the BSM assay when the *H. pylori* proteins were treated with GSH (Fig. 2). These might be endogenously biotinylated proteins or nonspecifically bound proteins, which were hard to avoid.

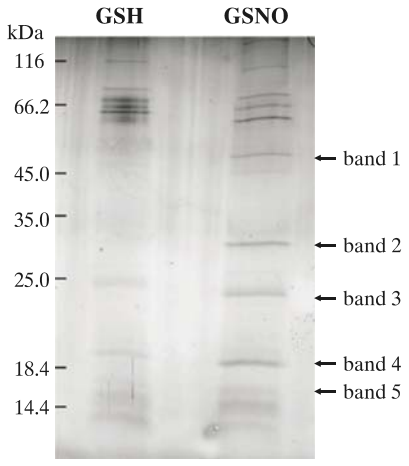

Fig. 2. Identification of S-nitrosylated proteins of *H. pylori* exposed to nitric oxide stress. *H. pylori* proteins were treated with 100 μM GSH as control and 100 μM GSNO respectively, then were subjected to the biotin switch method and purified by streptavidin-agarose. 10% SDS-PAGE was performed and the gels were silver stained and scanned.

Table 2. The S-nitrosylated proteins of *H. pylori* identified by exposure to nitric oxide stress

Band no.[a]	Proteins (genes)	TIGR ORF no.[b]	Gi	Mr (kDa)	Top score
1	chaperone and heat shock protein (*groEL*)	Hp0010	2313084	58.3	105
2	urease alpha subunit (*ureA*)	Hp0073	2313154	26.5	71
3	alkyl hydroperoxide reductase (*tsaA*)	Hp1563	2314747	22.2	73
4	*H. pylori* predicted coding region HP0721	Hp0721	2313852	17.6	103
5	*H. pylori* predicted coding region HP0129	Hp0129	2313213	16.3	75

[a] Band numbers refer to the proteins labeled in Fig. 2
[b] TIGR ORF no. follows the nomenclature of *H. pylori*

Five S-nitrosylated proteins were identified by MALDI-TOF-MS/MS

To reject the nonspecifically bound proteins or endogenously biotinylated proteins, five differentially expressed bands (Fig. 2) were excised and identified by MALDI-TOF-MS/MS. Five proteins were identified, including a chaperone and heat-shock protein (GroEL), urease alpha subunit (UreA), alkyl hydroperoxide reductase (TsaA), HP0721, and HP0129 (Table 2). It is noteworthy that all the five proteins we identified contained cysteine(s), showing that the BSM was highly specific.

Purified recombinant urease and TsaA were S-nitrosylated after GSNO treatment

To confirm the validity of the experimental system, two proteins (urease and TsaA) were expressed and their S-nitrosylation was detected by BSM. As shown in Fig. 3, in the protein samples treated with GSNO, urease and TsaA can be detected on silver-stained gels, suggesting that urease and TsaA indeed underwent S-nitrosylation after GSNO treatment.

GSNO inhibited urease activity in a concentration-dependent manner

Five S-nitrosylated proteins have been identified. To further test whether *S*-nitrosylation could trigger functional changes, the inhibitory effect of GSNO on urease activity was evaluated. As shown in Fig. 4, GSNO inhibited urease activity in a concentration-dependent manner. DTT is a reagent that can effectively decompose GSNO. In the DTT-treated samples, the urease activity was almost unchanged, further supporting the proposal that *S*-nitrosylation has an important role in controlling urease activity.

Discussion

When NO diffuses into the bacterial cytoplasm, peroxynitrite is formed by its interaction with O_2^- (Raupach and Kaufmann, 2001). Subsequently, peroxynitrite can oxidize and S-nitrosylate microbial proteins, nucleic acids, and lipids, thereby exerting its toxic effect (Zaki *et al*., 2005). Moreover, NO and RNSs can react with thiols to form S-nitroso compounds (RSNO or RSOH), thus inhibiting cell respiration and metabolism of *H. pylori*. Recently, the death rate of inducible nitric oxide synthase (iNOS-/-) knockout mice was shown to be higher than that of wild-type mice after *H. pylori* infection (Nam *et al*., 2004). To determine the antibacterial mechanism of NO thoroughly, the identification of the potential targets for S-nitrosylation is of great importance.

SNP can release NO indirectly by nitrosylation of a thiolate group and the subsequent degradation of S-nitrosothiol. The positive attribute of NO donors is that, compared with authentic NO, they are more convenient and workable. The drawback is that this process will introduce some additional responses caused by the SNP-derived species, rather than the nitrosylating agent per se. Walter Florio *et al.* (2004) measured the concentration of peroxynitrite derived from SNP in culture supernatants of *Mycobacterium tuberculosis* exposed to SNP and found that its bactericidal effect in comparison to NO was negligible.

The S-nitrosothiols, RSNO, include GSNO (S-nitrosoglutathione), S-nitrosoacetylpenicillamine, S-nitrosocysteine and so on, usually undergoing homolysis to give RS and NO in some

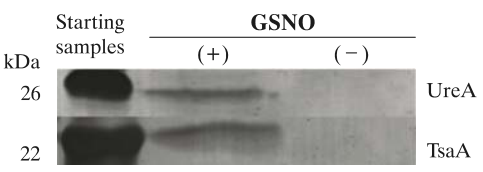

Fig. 3. Detection of S-nitrosylation in UreA and TsaA. The purified proteins (UreA and TsaA) were treated with 100 μM GSH as control and 100 μM GSNO respectively, then were subjected to the biotin switch method and purified by streptavidin-agarose. 10% SDS-PAGE was performed and the gels were silver stained and scanned.

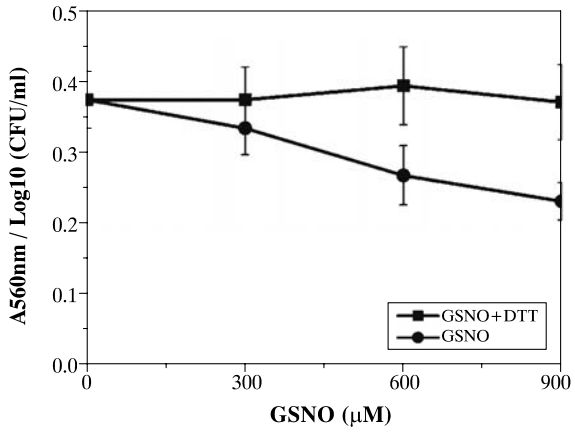

Fig. 4. *H. pylori* cells at logarithmic phase were incubated with GSNO at concentrations of 0, 300, 600, and 900 μM for 3 h, and then urease activity was assayed. As control, DTT (1 mM) was added to the same samples to decompose GSNO. The data represent the mean of three replicate assays. Application of Student's t-test showed that GSNO inhibited the urease activity ($p<0.05$).

cases, a metal-based catalysis is needed (Poole, 2005). GSNO is a widely used S-nitrosothiol with low molecular weight. It can nitrosylate thiolates readily, being a good nitrosylating agent. In this experiment, to avoid the nonspecific effect from the NO donors themselves as much as possible, two NO donors (GSNO and SNP) were chosen to analyze the inhibitory effect of NO on *H. pylori*.

BSM is the first and most commonly used method for the specific tagging of S-nitrosylated proteins (López-Sánchez *et al*., 2008; Torta *et al*., 2008). In this method, a thiol-blocking agent, MMTS was used to chemically block all the free thiols first, leaving the S-nitrosylated thiols unaffected. These unaffected S-NO bonds were then specifically reduced to free thiols by ascorbate. Free thiols were next tagged with biotin-HPDP, which could be enriched by affinity chromatography using streptavidin-agarose. The widely used NO donors are GSNO, S-nitroso-N-acetylpenicillamine, and diethylenetriamine-substituted diazen-1-ium-1,2-diolate (popularly known as DETA-NONOate). The recommended concentration range of the NO donor is 0.1-100 μM. In this experiment, we used 100 μM GSNO as the nitrosylating agent. All five proteins we identified contained cysteine(s); this result supports the notion that BSM can be highly specific if it is carried out carefully (Forrester *et al*., 2007). The S-nitroso proteome of *Mycobacterium tuberculosis* has been described. Several proteins of this proteome were essential enzymes of intermediary metabolism and antioxidant defense (Rhee *et al*., 2005). There is a similarity between them and our proteins. However, the sensitivity of BSM is relatively low, partly due to the requirement of sensitivity to ascorbate reduction (Huang and Chen, 2006).

In the results, GroEL is a homotetradecamer each containing seven radially arranged subunits. Each GroEL subunit folds into three domains and serves to prevent irreversible misfolding and aggregation (Horwich *et al*., 2006). GroEL has also been detected in *H. pylori* in humans under bile or acidic stress (Shao *et al*., 2008a, 2008b). In addition, the expression of GroEL was dependent on the growth phase (Choi *et al*., 2008). GroEL contains one cysteine, which may be one of the targets of NO attack on *H. pylori*. Considering the importance of GroEL in stress adaptation and maintenance, S-nitrosylation of GroEL would have some indirect or secondary suppressing effect on *H. pylori*.

In *H. pylori*, TsaA, Tpx, and Bcp belong to the peroxiredoxin family, which can use reduced thioredoxin as an electron donor to reduce their respective substrates (Baker *et al*., 2001). The reduced TsaA of *Salmonella typhimurium* can be transformed to the oxidized protein after converting the hydroperoxides to alcohols. Then, the oxidized protein can link the two subunits through an intersubunit disulfide bond with the N-terminal cysteine (Ellis and Poole, 1997). The thioredoxin system can reduce the disulfide bond to restore the activity (Schröder and Ponting, 1998). The enzyme TsaA of *H. pylori* contains two cysteines; therefore, it can be inferred that this protein acts in a similar manner. TsaA not only acts as a peroxide reductase in reducing organic hydroperoxides, but also prevents protein misfolding under oxidative stress by acting as a molecular chaperone; it can be induced under oxidative stress (Seaver and Imlay, 2001; Chuang *et al*., 2005). The TsaA of *S. typhimurium* and *H. pylori* can protect bacterial and human cells against RNSs. Furthermore, the deletion of *tsaA* made *H. pylori* hypersusceptible to RNSs (Bryk *et al*., 2000).

H. pylori contains plentiful urease, accounting for 5% of the total proteins. The genome corresponding to urease can be divided into two categories, ureAB and ureIEFGH. Tests on animals showed that deletion of ureA, ureB, urgE, or ureI can harm bacterial colonization (Tsuda *et al*., 1994). *H. pylori* can survive the acidic stress in the human stomach partly due to the urease system. It can elevate the pH by converting urea to ammonia and carbon dioxide (Scott *et al*., 2000). In addition, urease helps *H. pylori* to migrate in a viscous environment (Nakamura *et al*., 1998). $ONOO^-$ can reduce the viability of *H. pylori* in a time-dependent manner, whereas urease helps produce CO_2/HCO_3^-, which protects the bacteria from $ONOO^-$ cytotoxicity (Kuwahara *et al*., 2000). Urease plays an important role in the colonization of *H. pylori* in the acidic environment and in NO-stress adaptation. Modification of the cysteines by any chemical reagent could inhibit the urease activity of *Klebsiella aerogenes*, and cysteine319 of urease was identified as an essential catalytic residue based on site-directed mutagenesis studies (Todd and Hausinger, 1991; Martin and Hausinger, 1992). Bismuth compounds also were shown to exert their antibacterial activity and binding to the cysteine of the active site (Cys319), which is the main mechanism of enzyme inactivation (Zhang *et al*., 2006). It can be speculated that S-nitrosylation of urease might also lead to enzymatic inhibition. Indeed, our test showed that the urease activity of *H. pylori* was inhibited by GSNO; this inhibition effect could be reversed when DTT was added, which could decompose GSNO. In short, our results supported the view that *S*-nitrosylation might play a role in controlling urease activity. HP0721 is a sialic acid-specific adhesion protein of *H. pylori*, which can bind to fetuin-agarose and sialic acid–containing glycosphingolipids on thin-layer plates. Sialic acid can be detected mainly at inflamed sites in humans. It can be inferred that the interaction of sialic acids with the host may be important in the long-term pathology of *H. pylori* infections (Voland *et al*., 2002). Moreover, HP0721 is a cysteine protein present both on the cell surface and in the supernatant fraction, indicating an important role of HP0721 in mediating bacterial/host interactions (Sabarth *et al*., 2002; Bennett and Roberts, 2005). HP0129 also is present in the bulk proteins released by *H. pylori in vitro* (Kim *et al*., 2002); however, its function remains unknown so far.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Nos: 30770118, 30972775, 30800406, 30800037, and 30971151), National Basic Research Program of China (973 Program 2007CB512001) and the Science Foundation of Shandong Province, P. R. China (Nos: ZR2009CZ001 and ZR2009CM002).

References

Baker, L.M., A. Raudonikiene, P.S. Hoffman, and L.B. Poole. 2001. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. *J. Bacteriol.* 183, 1961-1973.

Bennett, H.J. and I.S. Roberts. 2005. Identification of a new sialic acid-binding protein in *Helicobacter pylori*. *FEMS. Immunol. Med. Microbiol.* 44, 163-169.

Bryk, R., P. Griffin, and C. Nathan. 2000. Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* 407, 211-215.

Choi, Y.W., S.A. Park, H.W. Lee, D.S. Kim, and N.G. Lee. 2008. Analysis of growth phase-dependent proteome profiles reveals differential regulation of mRNA and protein in *Helicobacter pylori*. *Proteomics* 8, 2665-2675.

Chuang, M.H., M.S. Wu, J.T. Lin, and S.H. Chiou. 2005. Proteomic analysis of proteins expressed by *Helicobacter pylori* under oxidative stress. *Proteomics* 5, 3895-3901.

Cole, S.P., V.F. Kharitonov, and D.G. Guiney. 1999. Effect of nitric oxide on *Helicobacter pylori* morphology. *J. Infect. Dis*. 180, 1713-1717.

Ellis, H.R. and L.B. Poole. 1997. Roles for the two cysteine residues of AhpC in catalysis of peroxide reduction by alkyl hydroperoxide reductase from *Salmonella typhimurium*. *Biochemistry* 36, 13349-13356.

Florio, W., G. Batoni, S. Esin, D. Bottai, G. Maisetta, F. Favilli, F.L. Brancatisano, and M. Campa. 2004. Influence of culture medium on the resistance and response of *Mycobacterium bovis BCG* to reactive nitrogen intermediates. *Microbes Infect.* 8, 434-441.

Forrester, M.T., M.W. Foster, M. Benhar, and J.S. Stamler. 2009. Detection of protein S-nitrosylation with the biotin-switch technique. *Free Radic. Biol. Med.* 46, 119-126.

Forrester, M.T., M.W. Foster, and J.S. Stamler. 2007. Assessment and application of the biotin switch technique for examining protein S-nitrosylation under conditions of pharmacologically induced oxidative stress. *J. Biol. Chem.* 282, 13977-13983.

Horwich, A.L., G.W. Farr, and W.A. Fenton. 2006. GroEL-GroES-mediated protein folding. *Chem. Rev.* 106, 1917-1930.

Huang, B. and C. Chen. 2006. An ascorbate-dependent artifact that interferes with the interpretation of the biotin switch assay. *Free Radic Biol. Med.* 15, 562-567.

Jaffrey, S.R. and S.H. Snyder. 2001. The biotin switch method for the detection of S-nitrosylated proteins. *Sci. STKE*. 86, pl1.

Kim, N., D.L. Weeks, J.M. Shin, D.R. Scott, M.K. Young, and G. Sachs. 2002. Proteins released by *Helicobacter pylori in vitro*. *J. Bacteriol.* 184, 6155-6162.

Kuwahara, H., Y. Miyamoto, T. Akaike, T. Kubota, T. Sawa, S. Okamoto, and H. Maeda. 2000. *Helicobacter pylori* urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. *Infect. Immun.* 68, 4378-4383.

López-Sánchez, L.M., F.J. Corrales, M. De La Mata, J. Muntané, and A. Rodríguez-Ariza. 2008. Detection and proteomic identification of S-nitrosated proteins in human hepatocytes. *Methods Enzymol.* 440, 273-281.

Marikar, F.M., D. Ma, J. Ye, B. Tang, W. Zheng, J. Zhang, M. Lu, and Z. Hua. 2008. Expression of recombinant human FADD, preparation of its polyclonal antiserum and the application in immunoassays. *Cell. Mol. Immunol.* 5, 471-474.

Martin, P.R. and R.P. Hausinger. 1992. Site-directed mutagenesis of the active site cysteine in *Klebsiella aerogenes* urease. *J. Biol. Chem.* 267, 20024-20027.

Martínez-Ruiz, A. and S. Lamas. 2004. Detection and proteomic identification of S-nitrosylated proteins in endothelial cells. *Arch. Biochem. Biophys.* 423, 192-199.

McGee, D.J. and H.L. Mobley. 2000. Pathogenesis of *Helicobacter pylori* infection. *Curr. Opin. Gastroenterol.* 16, 24-31.

Nakamura, H., H. Yoshiyama, H. Takeuchi, T. Mizote, K. Okita, and T. Nakazawa. 1998. Urease plays an important role in the chemotactic motility of *Helicobacter pylori* in a viscous environment. *Infect. Immun.* 66, 4832-4837.

Nam, K.T., S.Y. Oh, B. Ahn, Y.B. Kim, D.D. Jang, K.H. Yang, K.B. Hahm, and D.Y. Kim. 2004. Decreased *Helicobacter pylori* associated gastric carcinogenesis in mice lacking inducible nitric oxide synthase. *Gut* 53, 1250-1255.

Persichini, T., M. Colasanti, G.M. Lauro, and P. Ascenzi. 1998. Cysteine nitrosylation inactivates the HIV-1 protease. *Biochem. Biophys. Res. Commun.* 250, 575-576.

Poole, R.K. 2005. Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem. Soc. Trans.* 33, 176-180.

Qu, W., Y. Zhou, C. Shao, Y. Sun, Q. Zhang, C. Chen, and J. Jia. 2009. *Helicobacter pylori* protein response to nitric oxide stress. *J. Microbiol*. 47, 486-493.

Raupach, B. and S.H. Kaufmann. 2001. Immune responses to intracellular bacteria. *Curr. Opin. Immunol.* 13, 417-428.

Rhee, K.Y., H. Erdjument-Bromage, P. Tempst, and C.F. Nathan. 2005. S-nitroso proteome of *Mycobacterium tuberculosis* enzymes of intermediary metabolism and antioxidant defense. *Proc. Natl. Acad. Sci. USA* 102, 467-472.

Sabarth, N., S. Lamer, U. Zimny-Arndt, P.R. Jungblut, T.F. Meyer, and D. Bumann. 2002. Identification of surface proteins of *Helicobacter pylori* by selective biotinylation, affinity purification, and two-dimensional gel electrophoresis. *J. Biol. Chem.* 277, 27896-27902.

Salvati, L., M. Mattu, M. Colasanti, A. Scalone, G. Venturini, L. Gradoni, and P. Ascenzi. 2001. NO donors inhibit *Leishmania infantum* cysteine proteinase activity. *Biochim. Biophys. Acta.* 1545, 357-366.

Schapiro, J.M., S.J. Libby, and F.C. Fang. 2003. Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. *Proc. Natl. Acad. Sci. USA* 100, 8496-8501.

Schröder, E. and C.P. Ponting. 1998. Evidence that peroxiredoxins are novel members of the thioredoxin fold superfamily. *Protein Sci.* 7, 2465-2468.

Scott, D.R., E.A. Marcus, D.L. Weeks, A. Lee, K. Melchers, and G. Sachs. 2000. Expression of the *Helicobacter pylori* ureI gene is required for acidic pH activation of cytoplasmic urease. *Infect. Immun.* 68, 470-477.

Seaver, L.C. and J.A. Imlay. 2001. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J. Bacteriol.* 183, 7173-7181.

Shao, C., Q. Zhang, W. Tang, W. Qu, Y. Zhou, Y. Sun, H. Yu, and J. Jia. 2008a. The changes of proteomes components of *Helicobacter pylori* in response to acid stress without urea. *J. Microbiol.* 46, 331-337.

Shao, C., Q. Zhang, Y. Sun, Z. Liu, J. Zeng, Y. Zhou, X. Yu, and J. Jia. 2008b. *Helicobacter pylori* protein response to human bile stress. *J. Med. Microbiol.* 57, 151-158.

Todd, M.J. and R.P. Hausinger. 1991. Identification of the essential cysteine residue in *Klebsiella aerogenes* urease. *J. Biol. Chem.* 266, 24327-24331.

Torta, F., V. Usuelli, A. Malgaroli, and A. Bachi. 2008. Proteomic analysis of protein S-nitrosylation. *Proteomics* 8, 4484-4494.

Tsuda, M., M. Karita, T. Mizote, M.G. Morshed, K. Okita, and T. Nakazawa. 1994. Essential role of *Helicobacter pylori* urease in gastric colonization: definite proof using a urease-negative mutant constructed by gene replacement. *Eur. J. Gastroenterol. Hepatol.* 6, S49-52.

Venturini, G., M. Colasanti, L. Salvati, L. Gradoni, and P. Ascenzi. 2000. Nitric oxide inhibits falcipain, the *Plasmodium falciparum* trophozoite cysteine protease. *Biochem. Biophys. Res. Commun.* 267, 190-193.

Voland, P., D.L. Weeks, D. Vaira, C. Prinz, and G. Sachs. 2002. Specific identification of three low molecular weight membrane-associated antigens of *Helicobacter pylori*. *Aliment. Pharmacol. Ther.* 16, 533-544.

Zaki, M.H., T. Akuta, and T. Akaike. 2005. Nitric oxide-induced nitrative stress involved in microbial pathogenesis. *J. Pharmacol. Sci.* 98, 117-129.

Zhang, L., S.B. Mulrooney, A.F. Leung, Y. Zeng, B.B. Ko, R.P. Hausinger, and H. Sun. 2006. Inhibition of urease by bismuth (III): implications for the mechanism of action of bismuth drugs. *Biometals* 19, 503-511.