**Helicobacter pylori** protects oncocenegenically transformed cells from reactive oxygen species-mediated intercellular induction of apoptosis

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Malignant transformation of gastric epithelial cells by chronic *Helicobacter pylori* infection is caused by several mechanisms including attraction of reactive oxygen species (ROS)-producing neutrophils and cytotoxicity-associated antigen A-mediated dysplastic alterations. Here we show that *H. pylori* protects transformed cells from ROS-mediated intercellular induction of apoptosis. This potential control step in oncogenesis depends on the HOCl and NO/peroxynitrite (PON) signaling pathways. *Helicobacter pylori*-associated catalase and superoxide dismutase (SOD) efficiently cooperate in the inhibition of HOCl and the NO/PON signaling pathways. *Helicobacter pylori* catalase prevents HOCl synthesis through decomposition of hydrogen peroxide. *Helicobacter pylori*-associated SOD interferes with the crucial interactions between superoxide anions and HOCl, as well as superoxide anions and NO. The ratio of bacteria to malignant cells is critical for sufficient protection of transformed cells. Low concentrations of *H. pylori* more efficiently inhibited ROS-mediated destruction of transformed cells when compared with high concentrations of bacteria. Our data demonstrate the critical role of *H. pylori* antioxidant enzymes in the survival of transformed cells, modulating an early step of oncogenesis that is distinct from the transformation process per se.

Introduction

*Helicobacter pylori* and oncogenesis

*Helicobacter pylori* colonizes the stomach of more than half of the world’s population and causes chronic gastritis which can progress to peptic ulcer disease, mucosa-associated lymphoid tissue-lymphoma or gastric cancer (reviewed in ref. 1). Plentiful of mechanisms on how *H. pylori* infections result in the development of gastric cancer have been described including cytokinetic rearrangements by the infection of the cytoxin-associated antigen A protein into gastric epithelial cells via a type IV secretion system (2–5), the disruption of the gastric epithelium (6) and the regulation of apoptosis, cell proliferation and the cell cycle. A further theory in *H. pylori*-mediated carcinogenesis is the immune response-mediated chronic inflammation and tissue regeneration initiated by the release of reactive oxygen species (ROS) and reactive nitrogen species by neutrophils and macrophages (7). *Helicobacter pylori* has been shown to activate the RAC1 GTPase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in gastric mucosal cells (8,9), resulting in superoxide anion and hydrogen peroxide (H$_2$O$_2$) generation. This mimics the condition of oncogene-controlled RAC-NOX-dependent extracellular superoxide anion generation (ref. 10; reviewed in ref. 11), contributes to increased ROS levels and stimulates cellular proliferation and mutagenesis. To fend off ROS and reactive nitrogen species, *H. pylori* is equipped with superoxide dismutase (SOD) and catalase. In contrast to SOD, which is located on the cell surface, the catalase is located in the cytoplasm and the periplasmic space (12,13).

Adverse effects of ROS during oncogenesis

In addition to their mutagenic and tumor-initiating potential, ROS play further central and partially adverse roles during oncogenesis (reviewed in ref. 11). Oncogenic transformation is functionally linked to the generation of extracellular superoxide anions by activated membrane-associated NADPH oxidase (NOX-1) (10,14–18). NOX-1-derived superoxide anions and their dismutation product, H$_2$O$_2$, control the proliferation and maintenance of the transformed state (10,14,15,18,19). However, superoxide anions also govern the efficiency and selectivity of a recently described control system that is capable of eliminating transformed cells by ROS-mediated intercellular induction of apoptosis (16,20–25). The interaction between non-transformed and transformed cells and the autocrine/paracrine interaction between transformed cells establish ROS signaling pathways that cause selective apoptosis in transformed target cells (11,22,23). The HOCl- and NO/peroxynitrite (PON) signaling pathways (16,17) are the predominant pathways (Figure 1A). The HOCl signaling pathway depends on the generation of H$_2$O$_2$, through dismutation of extracellular superoxide anions (2 O$_2^-$ + 2 H$^+$ → H$_2$O$_2$, O$_2$), utilization of H$_2$O$_2$ by peroxidase as substrate for the generation of HOCl (H$_2$O$_2$ + PODFe$_{3}^{II}$ → PODFe$_{3}^{III}=O$$^+$ + H$_2$O; PODFe$_{3}^{III}=O$$^+$ + Cl$^-$ + H$^+$ → PODFe$_{3}^{III}$$+$$^{+} +$ HCl + HOCl). The subsequent interaction of HOCl with superoxide anions according to the formula HOCl + O$_2$ → OH$^-$ + O$_2$ + Cl$^-$ results in the generation of hydroxyl radicals that trigger apoptosis induction through lipid peroxidation. The NO/PON signaling pathway depends on the following reactions: (i) NO + O$_2$ → ONOO$^-$; (ii) ONOO$^-$ + H$^+$ → ONOOH and (iii) ONOOH → NO$_2$, OH$^-$. Hydroxyl radicals are the final apoptosis inducers generated in the NO/PON signaling pathway. The HOCl and the NO/PON signaling pathways can be dissected through the use of specific inhibitors, as outlined in Supplementary Figure 1, available at Carcinogenesis Online.

Whereas in vitro transformed cells are usually sensitive to intercellular ROS signaling, *ex vivo* tumor cells are resistant to this process (11,24–26). This resistance is based on the expression of extracellular, membrane-bound catalase (Figure 1B) that decomposes H$_2$O$_2$, and, thus prevents HOCl synthesis. In addition, membrane-bound catalase inhibits NO/PON signaling through oxidation of NO and degradation of PON (25). Development of resistance to ROS-mediated signaling, as it is typical for later tumor stages and is regularly found in *bona fide* tumors, is considered a pivotal step in tumor progression (11,27–29). Mathematical modeling, however, has shown that ROS-dependent induction of apoptosis and elimination of transformed cells has the potential to overcome ROS-driven proliferation of transformed cells (30).

We show here that catalase and SOD of the gastric pathogen *H. pylori* have the capacity of protecting transformed gastric epithelial cells against intercellular ROS-mediated apoptosis. This might be a critical step in the stabilization of malignant cells and might substantially contribute to the development of gastric cancer.

Materials and methods

*Helicobacter pylori* culture and generation of an isogenic catalase-negative mutant

*Helicobacter pylori* wild-type 26695 and isogenic mutants were grown on Columbia-Agar-based culture media containing 10 vol% washed human
Helicobacter pylori protects transformed cells

erthrocytes and 10 vol% heat-inactivated horse serum under microaerobic conditions at 37°C for 44–70 h. Grown bacteria were identified as \textit{H. pylori} by typical morphology, biochemical reactions and Gram staining (31).

Catalase of the wild-type strain 26695 was inactivated by insertion of the chloramphenicol-acetyl-transferase gene \textit{cat} by megaprimer PCR using a modified protocol described previously (32,33). In brief, 5'- and 3'-end...
flanking regions of the H. pylori catalase gene were amplified with primer pairs (306Fw: 5'-CAG TGG GGA TGT TGG AG-3', 672Rv: 5'-CTG CTA AAA ATC TGG GAC TTA AGC CCT TGC ATG GTG TGA A-3'; 683Fw: 5'-TGG CAG GCC GGC TAA ACG AAG CCG CAG AAG TTA-3', 1138Rv: 5'-AAG AAC GCT AGT ATG CTT GGT-3') carrying 5' extensions complementary to the 5' and 3' ends of cat cassette, purified and then mixed with the cat amplon. Megaprimer PCR was carried out using the above flanking primers 306Fw and 1138Rv, generating a PCR product carrying the catalase gene with inserted cat cassette. Helicobacter pylori 26695 was then mutagenized with this PCR product by natural transformation and successful inactivation of the catalase gene was confirmed by a negative catalase test.

**Materials**

The NOX-1 inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), the fast decaying NO donor Diethylthlamino NONOate (DEA NONOate), the SOD inhibitor diethylidithiocarbamate (DDC), the catalase inhibitor 3-aminotriazole (3-AT), NaOCl, mannnitol (a specific hydroxyl radical scavenger), iron-containing superoxide dismutase (FeSOD) from Escherichia coli, manganese-containing superoxide dismutase (MnSOD) from E. coli, N-omega-nitro-l-arginine methylester hydrochloride (l-TNAME), taurine, catalase, glucose oxidase (GOX) and myeloperoxidase (MPO) were obtained from Sigma (Schneidelfeld, Germany).

The mechanism-based peroxidase inhibitor 4-aminobenzenyl hydrazide (ABH) was purchased from Acros Organics (Geel, Belgium); peroxynitrite and the reconstituted NO/PON signaling from Tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) was obtained from Calbiochem (Merck Biosciences GmbH, Schwalbach/Ts, Germany). Inhibitors of caspase-3 (Z-DEVDFMK), caspase-8 (Z-IEHD-FMK) and caspase-9 (Z-LEHD-FMK) were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany).

The sites of action of the signaling inhibitors AEBSF, ABH, catalase, SOD, taurine, mannnitol, l-TNAME and FeTPPS are illustrated in Supplementary Figure 1, available at Carcinogenesis Online. A detailed list of references related to the specificity of these inhibitors can be found in reference (24).

**Media for cell culture**

Cells were either kept in Eagle's Minimum Essential Medium, containing 5% inactivated fetal bovine serum (Biochrom, Berlin, Germany) or in RPMI 1640 medium, containing 10% inactivated fetal bovine serum, as indicated for the respective cell lines. Both media were supplemented with penicillin (40U/ml), streptomycin (50 μg/ml), neomycin (10 μg/ml), moronal (10U/ml) and glutamine (280 μg/ml). Cells were grown in plastic tissue culture flasks and passed once or twice weekly.

**Cells**

Non-transformed 208F rat fibroblasts and 208Fsrc3 cells, i.e. 208F rat fibroblasts transformed through constitutive expression of v-src (34), were a generous gift by Drs C.Sers and R.Schafer, Berlin, Germany. Despite their potential to induce tumorigenesis in vivo, 208Fsrc3 cells have not been under the selection pressure of natural antitumor mechanisms. Transformed cells show cross-cross morphology in monolayer, colony formation in soft agar and extracellular superoxide anion generation that drives both the efficiency and selectivity of intercellular ROS signaling (11,16,17). 208Fsrc3 cells were cultured in Eagle's Minimum Essential Medium, 5% inactivated fetal bovine serum and supplemented as indicated above.

The gastric carcinoma cell line MKN-45 was purchased from DSMZ (Braunschweig, Germany). The cells were cultured in RPMI 1640, 10% inactivated bovine serum and supplements. They show extracellular superoxide anion generation, but are resistant against intercellular ROS signaling through expression of a membrane-associated catalase (25).

The murine fibrosarcoma cell line L929 was obtained from Dr D.Adam, Kiell, Germany and were cultured in Eagle's Minimum Essential Medium, 5% fetal bovine serum plus supplements.

**Methods**

**Apoptosis induction.** Cells were seeded at the densities indicated in the respective figure legends in 96-well tissue culture clusters and 100 μl of complete medium (standard conditions). The modes of apoptosis induction are summarized below and are further specified in the respective figure legends.

In the experiment described in Figure 2A–D, apoptosis was induced either by H2O2 generated continuously by increasing concentrations of GOX or by addition of defined concentrations of PON. The use of a low density of cells (6 × 103 cells per 96-well plate) without addition of transforming growth factor-beta did not foster autocrine apoptosis induction within the time frame of the experiment and thus allowed to focus on GOX- and PON-mediated apoptosis induction. As recently shown, direct apoptosis induction by H2O2 or PON has no selectivity with respect to the transformed state of the target cells (35). However, it allowed a quantitative measurement of protection through H. pylori-associated catalase.

The subsequent experiments utilize specific ROS-mediated apoptosis induction in malignant cells. Specific HOCl signaling (Figure 2E and F) or NO/PON signaling (Figure 2G and H) were enhanced through addition of excess exogenous signaling components such as MPO (Figure 2E and F) (36) or for the fast decaying NO donor DEA NONOate (Figure 2G and H), respectively.

In the experiments described in Figures 3–5, apoptosis was induced by autocrine ROS-mediated intercellular signaling of the transformed cells in the presence of transforming growth factor-beta. Autocrine apoptosis induction is based on NOX-1-dependent extracellular superoxide anion generation and release of peroxidase (i.e. the peroxidase domain of DUOX) and NO. This results in the establishment of the HOCl signaling pathway as well as additional NO/PON signaling (11.16).

In all experiments, assays were performed in duplicate. After the indicated time of incubation at 37°C and 5% CO2, the percentage of apoptotic cells was determined by inverted phase contrast microscopy based on the classical criteria for apoptosis, i.e. nuclear condensation/fragmentation or membrane blebbing (25,37,38). The characteristic morphological features of intact and apoptotic cells, as determined by inverted phase contrast microscopy are shown in Supplementary Figure 2, available at Carcinogenesis Online. At least 200 neighboring cells from randomly selected areas were scored for the percentage of apoptotic cells at each point of measurement. Control assays ensured that the morphological features 'nuclear condensation/fragmentation' as determined by inverted phase contrast microscopy could be related to intense DNA condensation and to DNA strand breaks, detectable by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction (17,20,39), as demonstrated in Supplementary Figure 3, available at Carcinogenesis Online. There is a remarkable coherence between the pattern of cells with condensed/fragmented nuclei (stained with bisbenzimid) and TUNEL-positive cells in assays with substantial apoptosis induction (Supplementary Figure 3D and E, available at Carcinogenesis Online), whereas there is no significant nuclear condensation/fragmentation in control assays (Supplementary Figure 3C and E, available at Carcinogenesis Online). The significance of this spatial coherence was confirmed through quantitation of cells with condensed/fragmented nuclei versus TUNEL-positive cells (Supplementary Figure 4A, available at Carcinogenesis Online). Though positivity in the TUNEL reaction represents one of the clearest hallmarks for apoptosis, we found that the TUNEL reaction is not suitable for routine quantitation in our cell culture system, as the preparation of the samples for the TUNEL reaction cause a marked loss preferentially of apoptotic cells. Therefore, quantitation of apoptosis induction was rather based on the direct determination of the percentage of cells with condensed/fragmented nuclei or membrane blebbing determined by inverted phase contrast microscopy. As shown in Supplementary Figure 4B, available at Carcinogenesis Online, this method is strictly quantitative when it is adequately performed and thus allows to quantitatively study the modulation of apoptosis induction. For further control of the significance of the method used, quantitation of apoptosis was performed with Annexin V staining and fluorescence microscopy, in parallel to determination of apoptosis induction in unstained cultures by phase contrast microscopy. As shown in Supplementary Figure 5, available at Carcinogenesis Online, the early apoptosis marker Annexin V positivity preceded the marker nuclear condensation/fragmentation (indicative of the completed apoptosis process). When apoptosis induction was inhibited by the NOX-1 inhibitor AEBSF, both markers remained at background level. This result demonstrates that both markers are suitable for quantitation of apoptosis, but focus on different sites within the kinetics of apoptosis induction. Finally, a comparison of the quantitation of Annexin V staining by fluorescence microscopy and by fluorescence-activated cell sorting analysis, in conjunction with phase contrast microscopy confirmed the validity of each one of these methods (Supplementary Figure 6, available at Carcinogenesis Online).

**Statistical analysis.** In all experiments, assays were performed in duplicate. The empirical standard deviation was calculated and is shown in the figures. Absence of standard deviation bars for certain points indicates that the standard deviation was too small to be reported by the graphic program. Empirical standard deviations were calculated merely to demonstrate how close the results were obtained in parallel assays within the same experiment and not with the intention of statistical analysis of variance, which would require larger numbers of parallel assays. The key experiments (Figures 3–5) have been repeated more than three times, all others at least twice (with duplicate assay). The Yates continuity corrected chi-square test was used for the statistical determination of significance (P < 0.01 = significant; P < 0.001 = highly significant).

**Strategy of our analysis**

Inhibition of ROS-mediated apoptosis induction was used as specific functional assay that allowed to test interference of H. pylori-associated catalase and SOD with ROS signaling. This approach is characterized by high sensitivity...
and specificity, its outcome is of direct relevance for the evaluation of potential protection of malignant cells against elimination through ROS signaling. The use of H.pylori wt (carrying catalase and SOD) and a catalase-negative strain of H.pylori (carrying only SOD) allowed to differentiate between the roles of the two enzymes. The experiments were controlled through the use of specific scavengers and the conclusions were challenged through the use of purified catalase and SOD in parallel reconstitution experiments.

Results

Helicobacter pylori catalase protects against H\textsubscript{2}O\textsubscript{2}- and PON-mediated apoptosis induction

H\textsubscript{2}O\textsubscript{2} generated by GOX as well as exogenous PON caused apoptosis induction in src-oncogene-transformed 208Fsrc3 fibroblasts, dependent on the concentration applied and on the time of incubation (Figure 2A and B). In line with recent published work (25,40), purified catalase completely inhibited apoptosis induction by both compounds. Helicobacter pylori wt efficiently protected against GOX- and PON-mediated apoptosis induction, dependent on the concentration of H.pylori wt applied (Figure 2C and D). In contrast, a catalase-negative strain of H.pylori showed no protection against GOX-mediated apoptosis induction and only a minor protective effect against PON-dependent apoptosis induction. This finding demonstrates that H.pylori wt carries a substantial contribution of active catalase. The residual protective effect of the catalase-negative strain of H.pylori against PON is explained by the potential of bacterial SOD to decompose PON (41). When tumor cells, rather than transformed cells, were tested for apoptosis induction by exogenous H\textsubscript{2}O\textsubscript{2} and PON, much...
higher concentrations of both compounds had to be applied to induce cell death, compared with the transformed cells studied in Figure 2 (Supplementary Figure 7, available at Carcinogenesis Online). This resistance was due to the protective effect of tumor cell catalase (25), as it was abrogated by the catalase inhibitor 3-AT. When very high concentrations of GOX or PON exceeded the protective potential of the tumor cells by their own catalase, either addition of exogenous catalase (Supplementary Figure 7A and B, available at Carcinogenesis Online) or H. pylori wt (Supplementary Figure 7C and D, available at Carcinogenesis Online) restored efficient protection of the tumor cells against apoptosis induction by H₂O₂ and PON. These findings demonstrate that purified catalase, tumor cell-associated catalase and H. pylori-associated catalase decompose the same substrates.

Helicobacter pylori interferes with apoptosis induction through the HOCl and the NO/PON signaling pathway

Specific HOCl or NO/PON signaling was established and enhanced through addition of either MPO or the fast decaying NO donor DEA NONOate to dense cultures of transformed 208Fsrc3 cells (Figure 2E–H). Establishment of fast HOCl signaling through the addition of MPO (36) was demonstrated by the complete inhibition through the HOCl scavenger taureine and the NOX-1 inhibitor AEBSF (Figure 2E and F). In contrast, the addition of the fast decaying NO donor DEA NONOate established apoptosis induction through NO/PON signaling, as shown by the complete inhibition through the PON decomposition catalyst FeTPPS (Figure 2G and H). MPO-driven HOCl signaling was completely inhibited by H. pylori wt at a concentration of 10³ bacteria/ml and higher, whereas 5 × 10³ bacteria/ml were required for complete inhibition by the catalase-negative mutant of H. pylori, which caused a bell-shaped inhibition curve. This finding demonstrates the strong protective effect of H. pylori catalase against HOCl signaling and also indicates the potential of H. pylori SOD to interfere with HOCl signaling, at distinctly higher concentrations of H. pylori.

Helicobacter pylori wt and the catalase-negative strain of H. pylori inhibited NO/PON signaling with the same efficiency, resulting in characteristic bell-shaped inhibition curves (Figure 2G and H). This finding demonstrates that H. pylori catalase is not involved in protection against PON that is generated in this experimental context through the interaction of NO with superoxide anions close to the membrane of the transformed cells. In contrast, H. pylori SOD seems to be solely responsible for the H. pylori-mediated protective effect at the left side of the bell-shaped inhibition curve and its destructive effect at the right side of the curve.

Helicobacter pylori catalase and SOD interfere with ROS-mediated autocrine apoptosis induction

Src oncogene-transformed fibroblasts showed autocrine apoptosis induction, whereas their non-transformed parental cells 208F did...
Selective apoptosis induction in 208Fsrc3 cells was blocked by the inhibitors of caspase-3 and caspase-9, but not by the inhibitor of caspase-8 (Figure 3A and B). The requirement for functional caspase-9 and caspase-3 points to the central role of the mitochondrial pathway of apoptosis for autocrine apoptosis induction in 208Fsrc3 cells, whereas there was no indication for an involvement of caspase-8-dependent receptor-mediated apoptosis induction. Apoptosis induction in 208Fsrc3 cells was primarily dependent on the HOCl pathway (Figure 3C), as it was inhibited by the HOCl scavenger taurine and the superoxide anion scavenger SOD. The NOS inhibitor l-NAME showed no inhibitory effect. Thus, the NO/PON pathway seemed not to be functional at the onset of apoptosis induction. In the presence of 100 U/ml of catalase, apoptosis induction was initially blocked; however, apoptosis resumed after 26 h (Figure 3D).

Apoptosis induction at these time points and in the presence of catalase was inhibited by l-NAME, indicating that decomposition of H_2O_2 by catalase had blocked HOCl signaling and had allowed the NO/PON pathway to become functional.

Addition of H. pylori wt to 208Fsrc3 cells caused concentration-dependent inhibition of autocrine apoptosis induction at 24 h (Figure 4A and B). As seen by the inhibition profile, autocrine apoptosis induction was due to the HOCl signaling pathway, as it was inhibited by the NOX-1 inhibitor AEBSF, the peroxidase inhibitor ABH, the HOCl scavenger taurine and the hydroxyl radical scavenger mannitol (Figure 4A). Inhibitors of the NO/PON pathway (the NOS inhibitor l-NAME and the PON decomposition catalyst FeTPPS) showed only marginal effects (Figure 4B). When inhibition of apoptosis induction by H. pylori wt was measured at 30 h as opposed to
24h, a bell-shaped inhibition curve was observed (Figure 4C and D) instead of the linear inhibition curve observed at 24h (Figure 4A and B). At 30h and in the absence of H.pylori, autocrine apoptosis induction was dependent on the HOCl pathway, as it was inhibited by the NOX-1 inhibitor AEBSF, the peroxidase inhibitor ABH and the HOCl scavenger taurine (Figure 4C). There was no indication for involvement of either NO or PON in the absence of H.pylori, as neither the NOS inhibitor l-NAME nor the PON decomposition catalyst FeTPPS showed an inhibitory effect (Figure 4D). A concentration of 5 × 10^3 H.pylori/ml resulted in maximal inhibition of autocrine apoptosis induction. Higher concentrations of the bacteria caused apoptosis induction to resume. This increase in apoptosis induction was no longer controlled by the HOCl signaling pathway, as it was not inhibited by ABH or taurine (Figure 4C). Rather, apoptosis in the presence of high concentrations of H.pylori was completely dependent on NO and PON, as l-NAME and FeTPPS caused complete inhibition (Figure 4D).

In order to study specifically the role of H.pylori SOD in the protection of transformed cells against autocrine ROS signaling, a catalase-negative mutant of H.pylori (Figure 5A and B) as well as H.pylori wt in the presence of the catalase inhibitor 3-AT (C and D) were tested for the protection of transformed cells against autocrine ROS-mediated apoptosis signaling. As shown in Figure 5A–D, low concentrations of the catalase-negative mutant of H.pylori, as well as H.pylori wt in the presence of 3-AT, showed a strong inhibition of apoptosis, with optimal inhibition at a concentration of 5 × 10^3 H.pylori/ml. However, a concentration-dependent increase in apoptosis induction resumed when the concentration of H.pylori was further increased. Apoptosis induction at the left and the right flank of these bell-shaped inhibition curves was completely dependent on superoxide anions (inhibition by AEBSF), peroxidase (inhibition by ABH), HOCl (inhibition by taurine) and hydroxyl radicals (inhibition by mannitol) (Figure 5A and C), but not dependent.

Fig. 5. Interference of catalase-negative Helicobacter pylori (A and B) and H.pylori wt in the presence of the catalase inhibitor 3-AT (C and D) with autocrine, ROS-mediated apoptosis induction in transformed fibroblasts. 12.5 × 10^3 208Fsrc3 cells in 100 μl complete medium, 96-well tissue culture cluster, received 20ng/ml transforming growth factor-beta and were further cultivated either in the absence of further additions or in the presence of the indicated inhibitors (100 μM of the NOX-1 inhibitor AEBSF, 150 μM of the mechanism-based peroxidase inhibitor ABH, 50 mM of the HOCl scavenger taurine, 10 mM of the hydroxyl radical scavenger mannitol, 2.4 mM of the NOS inhibitor l-NAME, 25 μM of the PON decomposition catalyst FeTPPS). Assays received the indicated concentrations of catalase-negative H.pylori (A and B) or H.pylori wt plus 50 mM 3-AT (C and D). The percentages of apoptotic cells were determined in duplicate assays after 24h. For the clarity of the presentation, the data obtained for inhibitors of HOCl signaling (A and C) were presented separately from the data obtained for inhibitors specific for NO/PON signaling (B and D). Statistical analysis: (A–D): Inhibition of apoptosis induction by catalase-negative H.pylori or H.pylori in the presence of 3-AT as well as resumption of apoptosis induction after maximal inhibition (right side of the bell-shaped curve) are statistically highly significant (P < 0.001). The effect of the inhibitors of HOCl signaling (ABH, taurine, mannitol) are highly significant (P < 0.001), whereas there was no significant effect of l-NAME or FeTPPS.
Helicobacter pylori protects transformed cells

Fig. 6. Helicobacter pylori-associated catalase and SOD protect transformed cells against ROS-mediated apoptosis induction. (A) Wt H.pylori catalase decomposes H$_2$O$_2$ and thus inhibits the HOCl signaling pathway (#1–5). Bacterial SOD prevents superoxide anion/HOCI and superoxide anion/NO interaction. It thus complements the protective effect of catalase for HOCl signaling and also prevents PON formation. Though bacterial catalase is shown to degrade PON that has been added to cells, its concentration reached in the experiments presented here is not sufficient to interfere with the effects of PON generated close to the cell membrane, as this protective effect requires high local concentrations of catalase for steric and kinetic reasons (25). (B) Catalase-negative H.pylori protect transformed cells from HOCl and NO/PON signaling by preventing the superoxide anion-driven steps in both pathways. Therefore, both wt H.pylori and its catalase-negative mutant can protect transformed cells from ROS-mediated apoptosis and elimination. Protection against ROS-mediated apoptosis signaling by wt H.pylori and its catalase-negative mutant depends on the concentration of H.pylori but is only effective at relatively low concentrations of bacteria. An increase of H.pylori above its optimal protective concentration causes an efficient and concentration-dependent destruction of the malignant cells. This specific effect is caused by the ability of the SODFe$^{4+}$ intermediate to transfer an electron either to HOCl or NO, instead of completing the SOD cycle. The specifically resulting signaling chemistry is outlined in the text and in Supplementary Discussion, available at Carcinogenesis Online.
Discussion

*Helicobacter pylori* catalase and SOD are key enzymes for the protection of *H. pylori* against oxidative stress-based attacks by neutrophils and macrophages. Now, we show here that these bacterial enzymes may also have the potential to protect malignantly transformed cells against ROS-mediated apoptosis signaling (Figure 6). This protection prevents the elimination of transformed cells and thus may enhance tumor progression. The protection of the transformed cells is likely to increase the chance that a few clones from the protected population establish their own protection against ROS signaling by expression of membrane-associated antioxidant enzymes (24,25) and thus have a chance to survive even in the absence of *H. pylori*. According to established concepts in tumor progression (27–29), the step of acquisition of resistance may be critical for the overall outcome of tumorigenesis and may add to the multiple potential roles of *H. pylori* during multistep oncogenesis.

The use of *H. pylori* wild-type bacteria and a catalase-negative mutant of *H. pylori*, was used to dissect the specific roles of -36 None declared., -associated Figure 6B against oxidative stress-based attacks by neutrophils, infection., 6775–6778. 47 Online, lipopolysaccharide activates 11 ++ catalase and SOD are key enzymes for the protection of *H. pylori* catalase and SOD for the generation of nitroxyl anions. (2007) Carcinogenesis of Helicobacter pylori. by antimicrobial chemotherapy 2 and associated catalase is shown to protect against the apoptotic signaling of transformed cells (12), decomposition of H2O2 by *H. pylori* catalase efficiently inhibits the HOCl signaling pathway through interference with HOCl synthesis, but was not sufficient to protect the cells against PON generated by the cells through the reaction between cell-derived NO and extracellular superoxide anions that are generated in close vicinity to the cell membrane. This failure may be explained by the specific steric situation (Figure 6A), where PON seems to have a higher chance to hit the nearby cell membrane rather than to be decomposed by catalase (25). Thus, the protective effect against NO/PON-mediated apoptosis solely depends on the prevention of superoxide anion/NO interaction by SOD.

Cooperative effects between *H. pylori*-associated catalase and SOD

The protection of *H. pylori* catalase against HOCl signaling is supported by SOD-dependent prevention of HOCl/superoxide anion interaction, the crucial step for the generation of apoptosis-inducing hydroxyl radicals. This cooperative protective effect is observed at the first 24 h. Later, this cooperative protective effect is restricted to low *H. pylori* concentrations; at higher bacterial concentrations apoptosis resumés and is induced by PON. As discussed below and in more detail under Supplementary Information, available at Carcinogenesis Online, this effect is due to inhibition of HOCl signaling by *H. pylori* catalase, subsequent increase in the concentration of NO and the utilization of NO by excess *H. pylori* SOD for the generation of nitroxyl anions.

*Helicobacter pylori*-associated SOD interacts with the HOCl and the NO/PON signaling pathway

Protection of transformed and tumor cells against ROS-mediated apoptosis induction in the presence of a catalase inhibitor or by a catalase-negative *H. pylori* mutant demonstrates that bacterial SOD alone is sufficient to protect against apoptosis induction by ROS signaling (Figure 6B). This potential is explicitly demonstrated for the inhibition of HOCl/superoxide anion interaction that leads to the formation of apoptosis-inducing hydroxyl radicals and the inhibition of NO-dependent PON formation. Partial or complete protection by bacterial SOD, however, is restricted to low concentrations of SOD-carrying bacteria. At higher concentrations of bacteria, i.e. at relatively high concentrations of SOD compared with available superoxide anions from the malignant cells, the protective function of SOD turns into a destructive one resulting in a bell-shaped inhibition curve (42–44). The destructive function of SOD at the right flank of the bell-shaped curve is most likely due to the potential of the SODFe++ intermediate to trigger Fenton chemistry-dependent hydroxyl radical formation from HOCl (47).

In the presence of catalase-negative *H. pylori* or wild-type *H. pylori* plus catalase inhibitor 3-AT, HOCl synthesis is optimal but HOCl/superoxide anion interaction is prevented by SOD. This situation is different from the scenario controlled by *H. pylori* wt (Figure 4C and D), where HOCl synthesis was prevented by *H. pylori*-associated catalase, the concentration of free NO was enhanced and excess SOD catalyzed nitroxyl anion formation that finally reacted with molecular oxygen and generated PON (48). The details of these complex side reactions of SOD are presented under Supplementary Information, available at Carcinogenesis Online.

Our data add a novel facet to the multiple potential causative roles of *H. pylori* in multistep carcinogenesis. In addition to establish the malignant state of gastric epithelial cells through induction of chronic inflammation, cytokinin-associated antigen A-mediated dysplastic alterations and induction of oncogene-controlled NOX-1 activity, *H. pylori* may also protect already transformed cells against elimination by complex ROS signaling chemistry. The protection by *H. pylori* catalase and SOD may stabilize the population of transformed cells and thus increase the likelihood that transformed cells set up their own resistance mechanism by expression of an own membrane-associated catalase (a feature found in all *H. pylori* mutant of *H. pylori*). Now, we show here that these bacterial enzymes may protect the cells against PON generated by the cells through the reaction between cell-derived NO and extracellular superoxide anions that are generated in close vicinity to the cell membrane. This failure may be explained by the specific steric situation (Figure 6A), where PON seems to have a higher chance to hit the nearby cell membrane rather than to be decomposed by catalase (25). Thus, the protective effect against NO/PON-mediated apoptosis solely depends on the prevention of superoxide anion/NO interaction by SOD.

Supplementary material

Supplementary Figures 1–12 can be found at http://carcin.oxfordjournals.org/

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