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 cytotoxic-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 catalse 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 inhibit 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* 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). Plenty of mechanisms on how *H. pylori* infections result in the development of gastric cancer have been described including cytoskeletal rearrangements by the injection 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 metastasis. 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).

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$_2$O$_2$ $→$ H$_2$O + O$_2$). Utilization of H$_2$O$_2$ by peroxidase as substrate for the generation of HOCl (H$_2$O$_2$ + PODFe$^{II}$ $→$ PODFe$^{III}$ + O$_2$ + H$_2$O; PODFe$^{III}$ = O$^-$ + Cl$^-$ + H$^+$ $→$ PODFe$^{II}$ + 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 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

Erythrocytes and 10 vol% heat-inactivated horse serum under microaerobic conditions at 37°C for 44–70 h. Grown bacteria were identified as 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 cat by megaprimer PCR using a modified protocol described previously (32,33). In brief, 5'- and 3'-end
flanking regions of the \textit{H. pylori} catalase gene were amplified with primer pairs 
(306Fw: 5'-CAG GGA GGT GAG AGG-3', 672Rv: 5'-CTC CTG AAA TCG GAC TTA AGC CCT TGC ATG TGA A-3'; 683Fw: 5'-TGG CAG GGC GGG TAA ACG AAG ACG CCG CAG AAG TTA-3', 1138Rv: 5'-AAG AGC CTT AGT AGT CCT TTT G-3') carrying 5’ extensions complementary to the 5’ and 3’ ends of cat cassette, purified and then mixed with the cat amiplcok. Megaprimmer PCR was carried out using the above flanking primers 306Fw and 1138Rv, generating a PCR product carrying the catalase gene with inserted cat cassette. \textit{Helicobacter pylori} 26605 was then mutagenized with this PCR product by natural transformation and successful inactivation of the catalase was confirmed by a negative catalase test.

\textbf{Materials}

The NOX-1 inhibitor 4-(2-aminoethyl-benzenesulfonyl) fluoride (AEBSF), the fast decaying NO donor Diethylamino NONOate (DEA NONOate), the SOD inhibitor diethyldithiocarbamate (DCC), the catalase inhibitor 3-aminotriazole (3-AT), NaClO, mannitol (a specific hydroxyl radical scavenger), iron-containing superoxide dismutase (FeSOD) from \textit{Escherichia coli}, manganese-containing superoxide dismutase (MnSOD) from \textit{E. coli}, \textit{O}-omega-nitro- 

\textbf{Methods}

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 (10μM) and glutamine (280 μg/ml). Cells were grown in plastic tissue culture flasks and passaged once or twice weekly.

Cells

Non-transformed 208F rat fibroblasts and 208Fsrc3 cells, i.e. 208F rat fibroblasts transformed through constitutive expression of \textit{v-src} (34), were a generous gift by Drs C.Sers and R.Schäfer, 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 criss-cross morphology in monolayer, colony formation in soft agar and selectivity of intercellular ROS signaling (35). They were mutagenized with this PCR product by natural transformation and successful inactivation of the catalase was confirmed by a negative catalase test (35). However, it allowed a quantitative measurement of protection through \textit{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 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 morphology features ‘nuclear condensation/fragmentation’ as determined by inverted phase contrast microscopy correlated with DNA condensation and to DNA strand breaks, detectable by the terminal deoxynucleo-
dy tranferase-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 bisbenzimide) and TUNEL-positive cells in assays with substantial apoptosis induction (Supplementary Figure 3D and F; 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 clear 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 figure. 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 3 times, all others at least twice (with several assays). The Yates continuity corrected chi-square test was used for the statistical determination of significances (P > 0.01 = significant; P < 0.001 = highly significant).

\textbf{Strategy of analysis}

Inhibition of ROS-mediated apoptosis induction was used as specific functional assay that allowed to test interference of \textit{H. pylori}-associated catalase and SOD with ROS signaling. This approach is characterized by high sensitivity.
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Interference of Helicobacter pylori catalase and SOD with ROS-mediated apoptosis signaling. (A and B) Catalase interferes with apoptosis induction by H₂O₂-generating GOX and PON. 6×10⁷ 208Fsrc3 cells in 100 μl complete medium were treated with the indicated concentrations of GOX (A) or PON (B), in the absence or presence of 100 U/ml catalase. The percentages of apoptotic cells were determined in duplicate assays after 1.5 and 2.5 h. (C and D) Helicobacter pylori-associated catalase protects against apoptosis induction by H₂O₂ and PON. 6×10⁷ 208Fsrc3 cells received the indicated concentrations of either H.pylori wt or a catalase-negative strain of H.pylori. The assays received either 3 mlU/ml GOX (C) or 25 μM PON (D). Controls (shown under C) received no enzyme. The percentages of apoptotic cells were determined in duplicate assays after 1.5 h. (E-G) Helicobacter pylori interferes with specific HOCl- and NO/PON-dependent apoptosis signaling in transformed cells. 12.5×10⁷ 208Fsrc3 cells in 100 μl complete medium received 20 μg/ml transforming growth factor-beta1. The indicated concentrations of H.pylori wt and catalase-negative H.pylori were added. HOCl signaling was induced through addition of 200 μIU/ml catalase. The percentages of apoptotic cells were determined in duplicate assays after 1.5 and 2.5 h. Inhibition by catalase was highly significant at all concentrations of the inducers. C and D: Inhibition of GOX-mediated apoptosis induction by H.pylori wt was highly significant at all concentrations of H.pylori wt. Inhibition of PON-mediated apoptosis induction by H.pylori was highly significant for 1×10⁻⁵ H.pylori/ml and higher concentrations. There was no significant inhibition of GOX-mediated apoptosis induction by the catalase-negative strain of H.pylori and significant inhibition of PON-dependent apoptosis by 1.2×10⁻⁵ catalase-negative H.pylori/ml and higher concentrations. The differences between the inhibitory effects of H.pylori wt and the catalase-negative H.pylori strain were highly significant for both inducers and at all concentrations of catalase-negative H.pylori. E and F: All concentrations of H.pylori wt applied caused highly significant (P < 0.001) inhibition of HOCl–dependent apoptosis induction. 1×10⁻⁵/ml and 5×10⁻⁵/ml of the catalase-negative strain of H.pylori caused highly significant inhibition of HOCl-dependent apoptosis, whereas higher concentrations of catalase-negative H.pylori caused highly significant increase in apoptosis, compared with controls. Inhibition of MPO-driven, HOCl-dependent apoptosis induction was inhibited by taurine and AEBSF with high significance. G and H: Inhibition of NO-mediated apoptosis induction was significant (P < 0.01) for 2×10⁻⁵/ml catalase-negative H.pylori and highly significant (P < 0.001) for 1×10⁻⁵/ml and 5×10⁻⁵/ml of both H.pylori strains. Resumption of apoptosis induction (compared with control) at concentrations higher than 5×10⁻⁵/ml bacteria was highly significant for both strains of H.pylori. There was no statistical significant difference between the bell-shaped inhibition curves mediated by H.pylori wt and catalase-negative H.pylori. Inhibition of NO-mediated apoptosis induction by FeTPPS was highly significant.

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 H₂O₂- and PON-mediated apoptosis induction

H₂O₂ 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 concentration 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 H₂O₂ 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 taurine 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

Fig. 3. Autocrine, ROS-mediated apoptosis induction in src oncogene-transformed murine fibroblasts. 12.5 × 10⁴ 208Fsrc3 cells in 100 μl complete medium, 96-well tissue culture cluster, received 20 ng/ml transforming growth factor-beta and were further cultivated either in the absence of further additions or received 50 μM caspase-3 inhibitor, 25 μM caspase-9 inhibitor (A), 25 μM caspase-8 inhibitor (B), 100 U/ml manganese-containing SOD, 50 mM of the HOCl scavenger taurine, 2.4 mM of the NOS inhibitor t-NAME (C) or 100 U/ml bovine liver catalase (CAT) or a combination of catalase and t-NAME (D). For control, assays with 12.5 × 10⁴ parental non-transformed 208F cells in the presence of 20 ng/ml transforming growth factor-beta were cultivated in parallel under A. The percentages of apoptotic cells were determined kinetically in duplicate assays. Statistical analysis: (A and B) The difference between apoptosis induction in transformed 208Fsrc3 and non-transformed 208F cells was highly significant at all time points. Inhibition of apoptosis induction in 208Fsrc3 cells by inhibitors of caspase-3 and caspase-9 was highly significant throughout the experiment, whereas there was no significant inhibition by the inhibitor of caspase-8, except for 50 h. (C and D). Inhibition of autocrine apoptosis induction in 208Fsrc3 cells by SOD, taurine, catalase plus l-NAME was highly significant (P < 0.001) at all time points, as was inhibition by catalase during the first 26 h. After that time point, the effect of catalase on autocrine apoptosis induction remained significant (P < 0.01), whereas there was no significant effect of t-NAME.
not (Figure 3A). 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 µM of catalase, apoptosis induction was initially blocked; however, apoptosis resumed after 26 h (Figure 3D).

Aptosis induction at these time points and in the presence of catalase was inhibited by l-NAME, indicating that decomposition of H₂O₂ 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
A bell-shaped inhibition curve was observed instead of the linear inhibition curve observed at 24 h. At 30 h 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 $5 \times 10^3$ *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 4D). 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* was 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* in the presence of 3-AT, resulted in maximal inhibition of apoptosis induction after maximal inhibition (right side of the bell-shaped curve) were 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 on NO (lack of inhibition by l-NAME) or PON (no inhibition by FeTPPS) (Figure 5B and D). As SOD can interfere with the HOCl signaling pathway by preventing the critical HOCl/superoxide anion interaction, and as SOD is known to cause bell-shaped inhibition curves (42–44), SOD seemed to be a likely candidate to explain the specific effects shown in Figure 5. A more detailed study of the protective effects of H. pylori SOD and its destructive side reactions at high concentrations of the enzyme, as well as control study with purified SOD is presented under Supplementary Information, 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 H. pylori catalase and SOD for protection against apoptotic ROS signaling. As H. pylori-associated catalase is shown to protect against the apoptosis-inducing effects of high concentrations of H₂O₂ and the recently described alternative substrate PON (25,40), the functional homology of H. pylori-associated catalase with mammalian catalase is proven. As verified for MPO-driven HOCl synthesis (36) and for autocrine HOCl 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 24h. Later, this cooperative protective effect is restricted to low H. pylori concentrations; at higher bacterial concentrations apoptosis resumes 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 nitroxy 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 curves is most likely due to the potential of the SODFe²⁺ 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, cytokotoxin-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 ex vivo tumor cell lines studied so far) (11). Eradication of H. pylori by antimicrobial chemotherapy of infected individuals may thus not only prevent the development of complications such as mucosa-associated lymphoid tissue-lymphoma or peptic ulcer disease but may also stop H. pylori-mediated cancer cell protective mechanisms and tumor progression.

Supplementary material

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

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