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Review

Targeting Extracellular ROS Signaling of Tumor Cells

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Abstract. *Expression of membrane-associated NADPH oxidase (NOX1) represents a characteristic feature of malignant cells. NOX1-derived extracellular superoxide anions are the basis for autocrine stimulation of proliferation, but also drive the HOCl and the NO/peroxynitrite signaling pathways. This may cause the elimination of transformed cells. Tumor cells express membrane-associated catalase that efficiently protects the cells against apoptosis-inducing reactive oxygen species (ROS) signaling. Membrane-associated superoxide dismutase (SOD) plays a co-modulatory protective role that is functionally interrelated with the protective effect mediated by catalase. Due to the co-localization of NOX1, catalase and SOD on the outer membrane of tumor cells, specific inhibition of membrane-associated SOD causes superoxide anion-dependent inhibition of catalase. This establishes a strong apoptotic signaling through the NO/peroxynitrite pathway. In parallel, it causes a drastic decrease in the concentration of proliferation-stimulating H_2O_2. Knowledge of the biochemical network on the surface of tumor cells should, therefore, allow development of specific novel strategies for tumor therapy, based on the specific features of tumor cell-specific extracellular ROS interactions.*

Abbreviations: Dual oxidase (DUOX); NADPH oxidase1 (NOX1); nitric oxide (NO); peroxidase (POD); small interfering RNA (siRNA); rat sarcoma oncogene (RAS); *RAS-related C3 botulinum toxin substrate* (*RAC*); Reactive oxygen species (ROS); superoxide dismutase (SOD).

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This article summarizes the role of reactive oxygen species (ROS) during tumorigenesis, for the maintenance of the transformed state and as autocrine proliferation signal of malignant cells. Thereby, the central role of NOX1 expression and the resultant generation of extracellular superoxide anions are in special focus. The biochemical details of two major intercellular signaling pathways that are driven by extracellular superoxide anions, *i.e.* the HOCl and the NO/peroxynitrite pathway are discussed. These signaling pathways lead to the specific elimination of transformed cells through induction of apoptosis. The knowledge of intercellular ROS-mediated signaling allows to understand the biochemical basis of the protected state against ROS signaling that needs to be acquired by tumor cells during tumor progression. Despite remarkable NOX1 activity tumor cells are resistant to ROS signaling-dependent apoptosis induction as they express membrane-associated catalase. Recent data have provided evidence for additional comodulation of protection through membrane-associated SOD. Detailed analysis of the multiple functions of membrane-associated catalase and SOD and their specific interactions allows to draw a picture of the tight control of tumor cells against ROS signaling. Finally, the biochemical basis for strategies that utilize specific ROS-mediated apoptosis induction in tumor cells as a rational approach for future tumor therapy are presented. These strategies aim at the optimization of ROS-mediated effects directed specifically against tumor cells and the parallel minimization of stimulating effects of ROS for the proliferation of tumor cells.

Reactive Oxygen Species (ROS) and Tumorigenesis

ROS may be involved in multistep oncogenesis at many distinct and partially adverse steps (1). These comprise i) mutagenic effects of ROS related to tumor initiation and tumor progression (2, 3); ii) ROS-related mechanisms during tumor promotion (3, 4); iii) establishment and maintenance of the transformed state through extracellular superoxide anion generation by membrane-associated NADPH oxidase (NOX1) (5-29); iv) specific removal of transformed cells

through intercellular ROS signaling, mainly based on the superoxide anion-controlled HOCl and NO/peroxynitrite signaling pathways (1, 10, 12, 13, 15, 30-47); v) acquisition of the H_2O_2-catabolizing phenotype (48-52); and vi) resistance to intercellular ROS signaling through expression of membrane-associated catalase (1, 53-55). Thereby, resistance to intercellular ROS signaling through membrane-associated catalase seems to correlate mechanistically with the classical "H_2O_2-catabolizing phenotype" as defined by Deichman and co-workers (48-52).

An extensive analysis of tumor cell lines *in vitro* has shown that they are regularly characterized by i) extracellular superoxide anion generation and ii) expression of membrane-associated catalase that protects the cells against intercellular ROS signaling and apoptosis. This analysis included human tumor cells derived from glioblastoma, mammary carcinoma, carcinoma of the lung, thyroid carcinoma and sarcoma, melanoma, carcinoma of the kidney, stomach, colon, liver, pancreas and bladder, neuroblastoma, carcinoma of the prostate, ovarial carcinoma, human papilloma virus (HPV)-positive cervix carcinoma, osteogenic sarcoma, Ewing sarcoma, rhabdomyosarcoma, fibrosarcoma, chondrosarcoma, leukemia and lymphoma. Inhibition of catalase of these tumor cell lines cause ROS-dependent apoptosis induction through intercellular ROS signaling, whereas cells from normal tissue were not affected. Small interfering RNA (siRNA)-mediated knockdown of *NOX1* abrogated the sensitivity of tumor cells to apoptotic signaling after catalase inhibition, whereas knockdown of catalase activated ROS-mediated apoptosis signaling specifically of tumor cells (55). The combination of extracellular superoxide anion production with membrane-associated catalase expression therefore seems to define a highly specific phenotype of tumor cells that is required for the maintenance of the malignant properties. This phenotype has not been found in cells from normal tissue. Inhibition, inactivation or prevention of expression of membrane-associated catalase has therefore been suggested as a novel rational therapeutic approach for tumor therapy that utilizes the specific biochemical features of tumor cells for their selective apoptotic destruction (1).

Characteristics of the ROS-related Phenotype of Tumor Cells

Promalignant effects of ROS. Generation of extracellular superoxide anions through a membrane-associated NOX1 represents one of the hallmarks of the transformed state (5-29). Activated oncogenes such as *rat sarcoma oncogen* (*RAS*) and *RAS-related C3 botulinum toxin substrate* (*RAC*) (a member of the Rho family of small GTPases) play central roles in the activation of NOX1 (5, 56). Activated NADPH oxidase seems to be required for the control of proliferation and the maintenance of the transformed state (5, 7, 9, 17, 57), changes in the cytoskeleton of transformed cells (58) and induction of cell motility (59). A causal connection between oncogene activation, superoxide anion production and establishment of the malignant state (transformation) has also been shown to be relevant for the situtation *in vivo* (17, 19, 20, 22, 60). Specific overexpression of NOX1 has been demonstrated in human tumors (19, 60), and is dependent upon activation of RAS (19). Inhibition of NOX1 activity causes subsequent inhibition of tumor growth (17). Overexpression of *RAC1* in oral squamous cell carcinomas (61) and *RAC1* gene mutations in human brain tumors (62) point to the significance of the *RAS–RAC1–NOX1* network in tumor development. ROS have also been shown to be relevant for tumor angiogenesis (63, 64) and metastasis (65, 66). Together with induction of genomic instability (67), the effects of ROS on angiogenesis and metastasis, thus, contribute to tumor progression in a highly dynamic way, beyond the establishment and the control of the transformed state.

Destructive ROS effects directed specifically against transformed cells. Extracellular superoxide anions generated by transformed cells drive both the efficiency and selectivity of intercellular induction of apoptosis (1, 10, 12, 13, 15, 30-41, 43-47). As a consequence, transformed cells are selectively induced to die by apoptosis after a concerted action of transformed cell-derived ROS and signaling components, such as a novel peroxidase or NO released by surrounding non-transformed cells (classical intercellular induction of apoptotis), or the transformed cells themselves (autocrine, ROS-mediated apoptotic self-destruction) (1, 10, 12, 43-45, 53, 55). Four pathways involved in intercellular ROS-mediated signaling have been elucidated so far: i) the HOCl signaling pathway (10); ii) the NO/peroxynitrite signaling pathway (10, 15, 68); iii) the nitryl chloride signaling pathway (69); and iv) the metal ion catalyzed Haber–Weiss reaction (70). Thereby, the HOCl and the NO/peroxynitrite signaling pathways are of major and central importance. Their signaling chemistry was recently reviewed (1) and is also relevant to this article. Inter-cellular ROS signaling represents an example of highly specific and directed biochemical action of radical and non-radical reactive oxygen and nitrogen species (1, 44, 47).

Protection of tumor cells against ROS-driven apoptosis induction through expression of membrane-associated catalase. Whereas cells transformed *in vitro* are regularly and specifically sensitive to inter-cellular induction of aptoptosis and autocrine self-destruction, independent of the origin of tissue and the transforming principle, bona fide tumor cells established from tumors showed resistance against intercellular induction of apoptosis (53-55, 71, 72). This reflects the acquisition of the H_2O_2-catabolizing phenotype as defined by Deichman and co-workers (48-52) and is based

on the expression of membrane-associated catalase on the outer surface of the tumor cells (53, 55). Catalase-mediated protection of tumor cells against intercellular ROS signaling was found in all human and rodent tumor cell lines studied by our group far (Bauer *et al*., unpublished data). The impressive protective potential of membrane-associated catalase is based on the recently recognized multi-functionality of this enzyme (55). Its location on the cell membrane establishes a high local density of the enzyme and ensures for efficient interaction with intercellular ROS signaling. Decomposition of H_2O_2 by catalase (its classical activity) prevents HOCl synthesis and thus inhibits the HOCl signaling pathway. Oxidation of NO by compound I of catalase ($CATFe^{IV}=O^{+\bullet}$) (73) removes NO from the signaling system and thus interferes with NO/peroxynitrite signaling in cooperation with the recently discovered enzymatic decomposition of peroxynitrite by catalase (55, 74, 75).

The combination of these three functions of catalase ensures complete inhibition of ROS-related intercellular signaling driven by tumor cell-derived extracellular superoxide anions (Figure 1) and therefore fully-explains the protected state of tumor cells. Inhibition of membrane-associated catalase by 3-aminotriazole (3-AT) or neutralizing antibodies, its inactivation through exogenous or cell-derived, extracellular singlet oxygen or siRNA-mediated prevention of its expression were, therefore, suggested as a novel strategy for specific apoptosis induction in tumor cells (1). This approach utilizes the combination of two tumor cell-specific phenotypic features, *i.e*. expression of membrane-associated catalase and extracellular superoxide anion generation by NOX1 and is based on reactivation of intercellular ROS signaling (1). Extracellular superoxide anion production, thereby, is the key to direct apoptosis induction specifically of malignant cells, due to the relatively small free diffusion path length of superoxide anions. This ensures site-specific generation of hydroxyl radicals (either from superoxide anion/HOCl interaction or from spontaneous decomposition of peroxynitrite, generated through superoxide anion/NO interaction).

Co-modulatory Role of Extracellular SOD

Although tumor cell-associated catalase protects against HOCl signaling through decomposition of H_2O_2 and subsequent prevention of HOCl synthesis, it cannot protect cells against exogenously-added HOCl (76). In contrast, decomposition of tumor cell-derived H_2O_2 enhances apoptosis-induction by exogenous HOCl, as the consumption reaction between HOCl and H_2O_2 is abrogated (54, 76). In a recent study, addition of HOCl to MKN-45 gastric carcinoma cells caused superoxide anion-dependent apoptosis induction, mediated by hydroxyl radicals that were generated through the reaction between superoxide anions and HOCl ($HOCl + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + Cl^- + O_2$) (76). Inhibition of membrane-associated catalase had a limited negative effect on apoptosis induction by exogenous HOCl, as it allowed the consumption reaction between HOCl and H_2O_2. This effect was abrogated by an increase in the HOCl concentration applied. Unexpectedly, pre-treatment of the tumor cells with antibodies directed against SOD1 caused a strong enhancement of HOCl-mediated apoptosis induction (76).

This finding demonstrates that superoxide anions were rate-limiting under these conditions. This was obviously based on the activity of extracellular SOD. These results also demonstrate that tumor cell-associated SOD does not scavenge all free superoxide anions, as a substantial residual reaction with HOCl is still possible in the absence of antibodies directed against SOD. Importantly, the accessibility of the modulatory SOD by antibodies demonstrated that the enzyme was on the outside of the tumor cells. As the effect was demonstrated in freshly-suspended tumor cells, the tight association of SOD to the outer membrane of the tumor cells is clear. These findings have several interesting consequences for our understanding of the control of ROS signaling by tumor cells. As summarized in Figure 2, our picture of the situation on the surface of tumor cells has to be extended according to these findings. The superoxide anion source NOX1 is in direct proximity to two enzymes involved in the control of ROS signaling, namely catalase and SOD. Catalase has a dominant inhibitory effect on HOCl synthesis through decomposition of the peroxidase substrate H_2O_2 and a double inhibitory effect on NO/peroxynitrite signalling through oxidation of NO and decomposition of peroxynitrite. SOD partially inhibits HOCl/superoxide anion and NO/superoxide anion interactions and thus has a co-modulatory negative effect on both pathways. The increased production of H_2O_2 through the dismutation reaction of SOD will be compensated through the parallel action of catalase. Thus, it may be assumed that the interaction between catalase and SOD tightens the control on ROS signaling of tumor cells. The inhibitory effect of anti-SOD1 may either indicate that SOD1 is in fact found on the outside of the cells, analogous to reports in other cell systems (77). Alternatively, extracellular SOD3 may be responsible for the partial inhibitory effect. The neutralizing effect of anti-SOD1 might then be a consequence of sequence homology reported for SOD1 and SOD3 (78).

Enzymatic Details of the Protective Effects Against ROS Signaling Mediated by Membrane-associated Catalase and SOD

Membrane-associated catalase and SOD of tumor cells exhibit inter-connected and additive protective effects directed against inter-cellular ROS signaling by the HOCl and the NO/peroxynitrite signaling pathway. NOX1 is the source of superoxide anions that are the basis for the establishment of

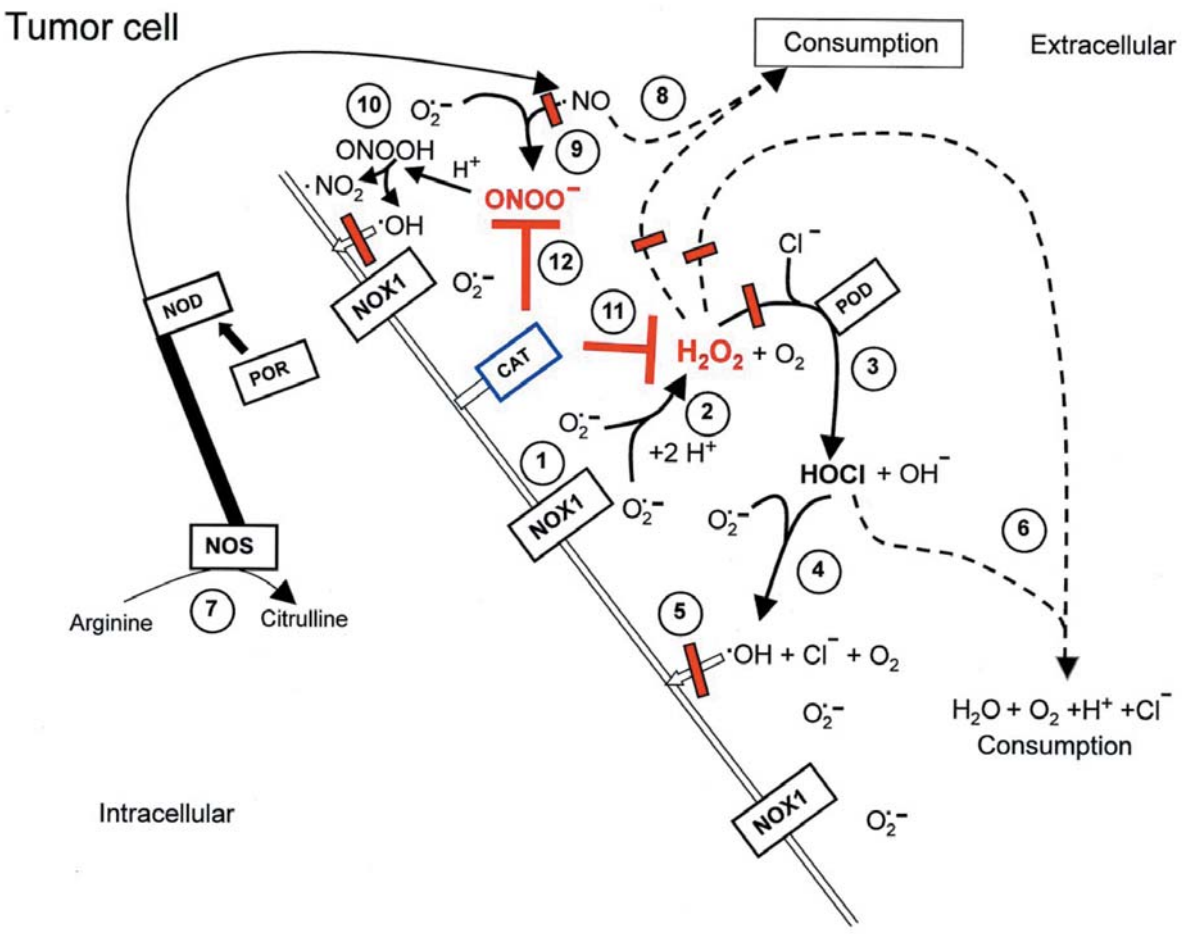

Figure 1. *Catalase protects tumor cells against intercellular ROS signaling. Membrane-associated NADPH oxidase (NOX1) generates extracellular superoxide anions (#1). These drive the HOCl pathway (steps 1-6) and the NO/peroxynitrite pathway (steps 7-10). HOCl and NO/peroxynitrite signaling lead to the selective elimination of transformed cells. Details of the signaling pathways. A: HOCl pathway: Superoxide anions (#1) dismutate and generate H_2O_2 (#2) that is utilized as substrate for the synthesis of HOCl by the dual oxidase (DUOX)-coded peroxidase domain (POD) (#3). The reaction between superoxide anions and HOCl (#4) leads to the generation of hydroxyl radicals (OH) close to the membrane of the malignant cells. Hydroxyl radicals cause lipid peroxidation and subsequent induction of apoptosis through the mitochondrial pathway of apoptosis (#5). Excess H_2O_2 compared to peroxidase leads to a consumption reaction between HOCl and H_2O_2 (#6) that blunts HOCl signaling. B: NO/peroxynitrite pathway: Nitric oxide is generated by NO synthase (NOS). A substantial amount of cellular NO is converted to nitrate by NO dioxygenase (NOD) that is connected to the activity of cytochrome P450 oxidoreductase (POR). NO passes the cell membrane and is either consumed in a complex reaction with H_2O_2 (#8) or reacts with superoxide anions to form peroxynitrite (ONOO-) (#9). After protonation of peroxynitrite, the resultant peroxynitrous acid (ONOOH) spontaneously decomposes and yields NO_2 and apoptosis-inducing hydroxyl radicals (#10). Resistance of tumor cells: Tumor cells express membrane-associated catalase on the outside of their membrane (53-55). Membrane-associated catalase interferes with HOCl signaling through decomposition of H_2O_2 (#11) and inhibits NO/peroxynitrite signaling through oxidation of NO (not directly shown in this figure) and decomposition of peroxynitrite (#12). This figure is from Reference 1.*

both signaling pathways. Superoxide anions are generated at high local density close to the membrane and the two antioxidative enzymes, whose enzymatic details are summarized in Figures 3 and 4. Catalase decomposes H_2O_2, as well as peroxynitrite, in analogous two-step reactions that involve the formation of compound I ($CATFe^{IV}=O^{+\bullet}$) and allow a catalytic cycle through re-establishment of native catalase (Figure 3) (1, 55). In addition, compound I of catalase oxidizes NO to NO_2 (73) and thus is in competition with peroxynitrite formation through NO/superoxide anion interaction. These coordinated actions of catalase have a dominant inhibitory effect on the HOCl and the NO/peroxynitrite signaling pathway that both are characterized by final generation of hydroxyl radicals. These trigger apoptosis induction through lipid peroxidation. Free superoxide anions are scavenged and dismutated to hydrogen peroxide through the classical activity of SOD1 in a two-step reaction, involving the reduced $SODCu^+$ intermediate (Figure 4). Removal of superoxide anions interferes with the hydroxyl

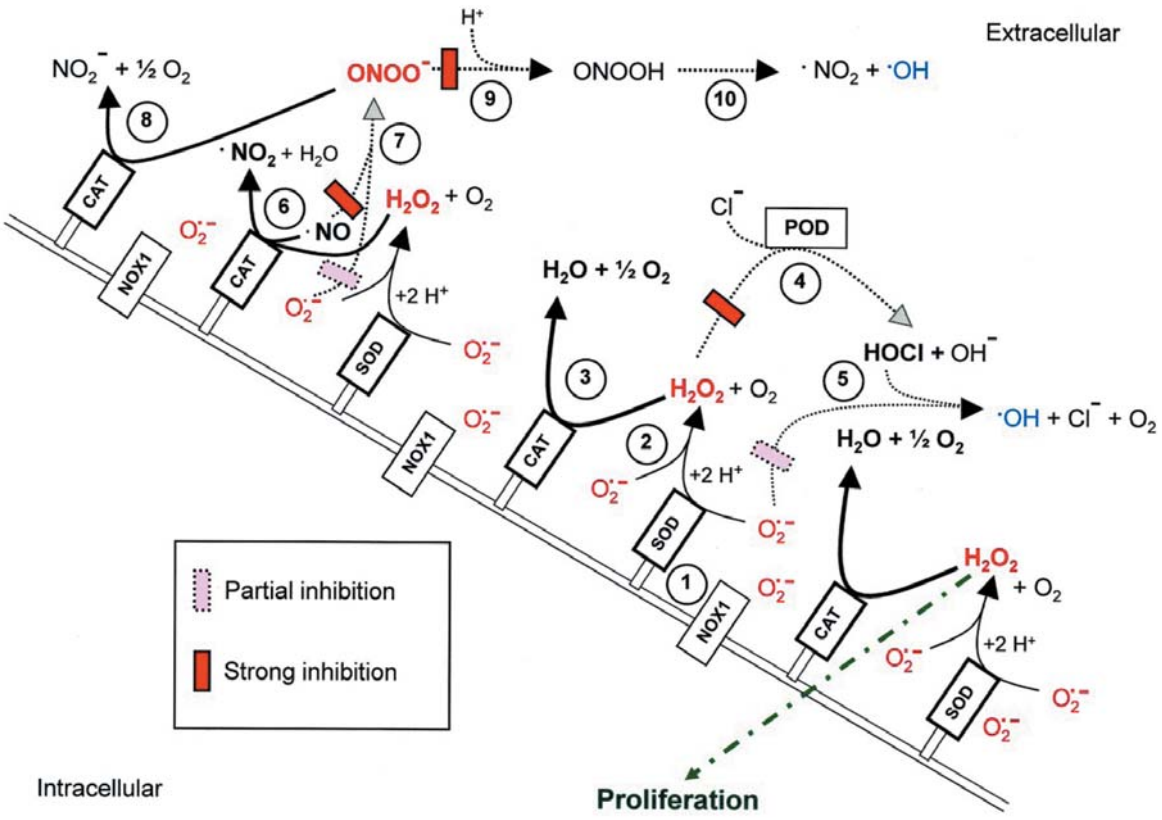

Figure 2. *Co-modulatory protective role of membrane-associated SOD of tumor cells. Recent findings show that in addition to catalase, an additional modulatory effect of SOD needs to be taken into account (76). The protective role of SOD is not as stringent as that of catalase, as basic superoxide anion/HOCl interaction is allowed even without inhibition of SOD (76). However, the modulatory potential of SOD is seen when it is inhibited and HOCl signaling is enhanced. The protective interaction between catalase and SOD is suggested to act in the following mode: NOX1 generates extracellular superoxide anions (#1) that dismutate to hydrogen peroxide, driven by SOD (#2), as well as spontaneously. The resultant H_2O_2 is efficiently decomposed by catalase (#3). Thus, HOCl formation through peroxidase (POD) (#4) is strongly inhibited. Due to the activity of SOD, eventually-generated HOCl only has a low probability to find a free superoxide anion as reaction partner for the generation of apoptosis-inducing hydroxyl radicals (#5). Catalase interacts with hydrogen peroxide to generate compound I which then can oxidize NO (#6) and thus counteract NO/superoxide anion interaction that would yield peroxynitrite (#7). Likewise, the reaction of SOD (#2) removes free superoxide anions and thus prevents peroxynitrite formation. Peroxynitrite formed despite the activity of SOD is decomposed by catalase (#8) and thus the formation of peroxynitrous acid (#9) and hydroxyl radicals (#10) is prevented. The interaction between SOD and catalase thus creates tight control of extracellular ROS signaling. H_2O_2 generated through the dismutation reaction catalyzed by SOD has a slight chance of not being decomposed by catalase, to enter the intracellular space and to drive proliferation.*

radical-generating interaction between HOCl and superoxide anions and with peroxynitrite formation through NO/superoxide interaction. In addition, in a reaction typical for metalloproteins, SOD decomposes peroxynitrite (79, 80). These partially redundant and overlapping protective effects of SOD and catalase seem to ensure maximal protection of the tumor cells.

Nevertheless, it was astonishing to find that protection by catalase alone seems to be dominant and complete, whereas the protective effect of SOD was only partial, as seen from the strong residual reaction with exogenously-added HOCl (76). A good explanation for this finding can be derived from the knowledge of specific side reactions of the $SODCu^+$ intermediate which is generated after reaction of $SODCu^{++}$ with a superoxide anion ($SODCu^{++} + O_2^{\bullet-} \rightarrow SODCu+ + O_2$) (97, 81, 82). We have recently shown that SOD shows protective activity at an optimal ratio of enzyme activity with respect to superoxide anion generation (97). If this ratio is shifted in favour of SOD, the $SODCu^+$ intermediate is relatively enriched and, lacking optimal superoxide anion concentration for completion of the SOD catalytic cycle, its protective function becomes a destructive one (Figure 5). This is mediated through a Fenton-type reaction of $SODCu^+$ with HOCl (81), leading to the formation of hydroxyl radicals. Alternatively, the $SODCu^+$ intermediate can reduce NO to the nitroxyl anion (NO^-) (83) that forms peroxynitrite after interaction with molecular oxygen. We have suggested that this

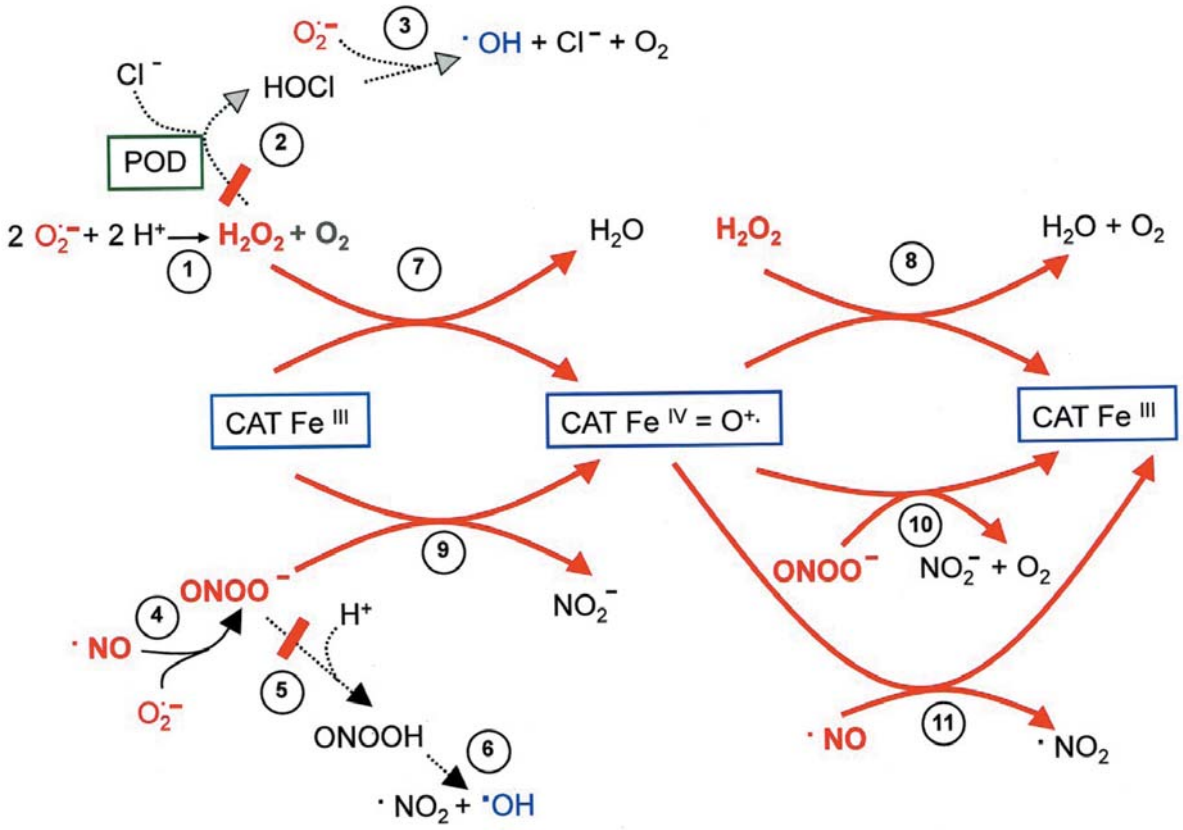

Figure 3. *Enzymatic details of the reaction of membrane-associated catalase of tumor cells. NOX1-derived superoxide anions drive the HOCl (#1-3) and the NO/peroxynitrite signaling pathway (#4-6). Membrane-associated catalase of tumor cells interferes efficiently with both signaling pathways. The native enzyme (ferricatalase; CATFe^{III}) reacts with a first molecule of H_2O_2 (#7). The resultant intermediate "compound I" (CATFe^{IV}=O$^{+\bullet}$) then reacts with a second molecule of H_2O_2 (#8) and the catalytic cycle is completed. This classical reaction of catalase inhibits the HOCl signaling pathway. Alternatively, catalase can decompose peroxynitrite ($ONOO^-$), utilizing a two-step reaction that is analogous to that involved in decomposition of H_2O_2 (#9, #10). This reaction inhibits NO/peroxynitrite signaling. Compound I can oxidize NO (#11) and thus interfere with NO/peroxynitrite signaling through an additional step.*

specific chemistry triggered by the $SODCu^+$ intermediate is the basis for the long known bell-shaped inhibition curves described for SOD (97), which characterizes a transition from a protective function of SOD to a destructive one at high enzyme concentrations. Therefore, tumor cells need to follow the strategy of not overexpressing membrane-associated SOD in order to prevent the destructive effects mediated by this enzyme at high concentrations. As the protective effect of catalase is dominant and very strong, the partial inhibition of the signaling pathways by SOD is compensated.

Advantages of Catalase and SOD Co-expression for Tumor Cells

The combination of catalase and SOD establishes at least three advantages for tumor cells. Firstly, as outlined above (Figure 2), the protective effects of both enzymes are complementary and partially redundant, thus ensuring optimal protection against ROS-mediated apoptosis signaling. Secondly, catalytic dismutation of superoxide anions by SOD ($k=2\times10^9$ M^{-1} s^{-1}), resulting in the formation of hydrogen peroxide occurs at much higher efficiency than the spontaneous dismutation reaction ($k=8\times10^4$ M^{-1} s^1). Hydrogen peroxide represents a central autocrine proliferation stimulus for tumor cells (1). Although catalase is likely to decompose most of the newly-generated H_2O_2, the strict fixation of catalase to the membrane allows a defined low concentration of H_2O_2 to pass the layer of immobile catalase and to enter the cell and trigger a proliferation stimulus. The specific partially impaired complete destruction of H_2O_2 by membrane-bound catalase has been confirmed through measurement of H_2O_2 in the vicinity of tumor cells. However, the concentration of H_2O_2 reached under these conditions is not sufficient to drive the peroxidase reaction while catalase is not inhibited. To ensure the generation of H_2O_2 as proliferation stimulus, tumor cells need to maintain expression of active NOX1 although it is at

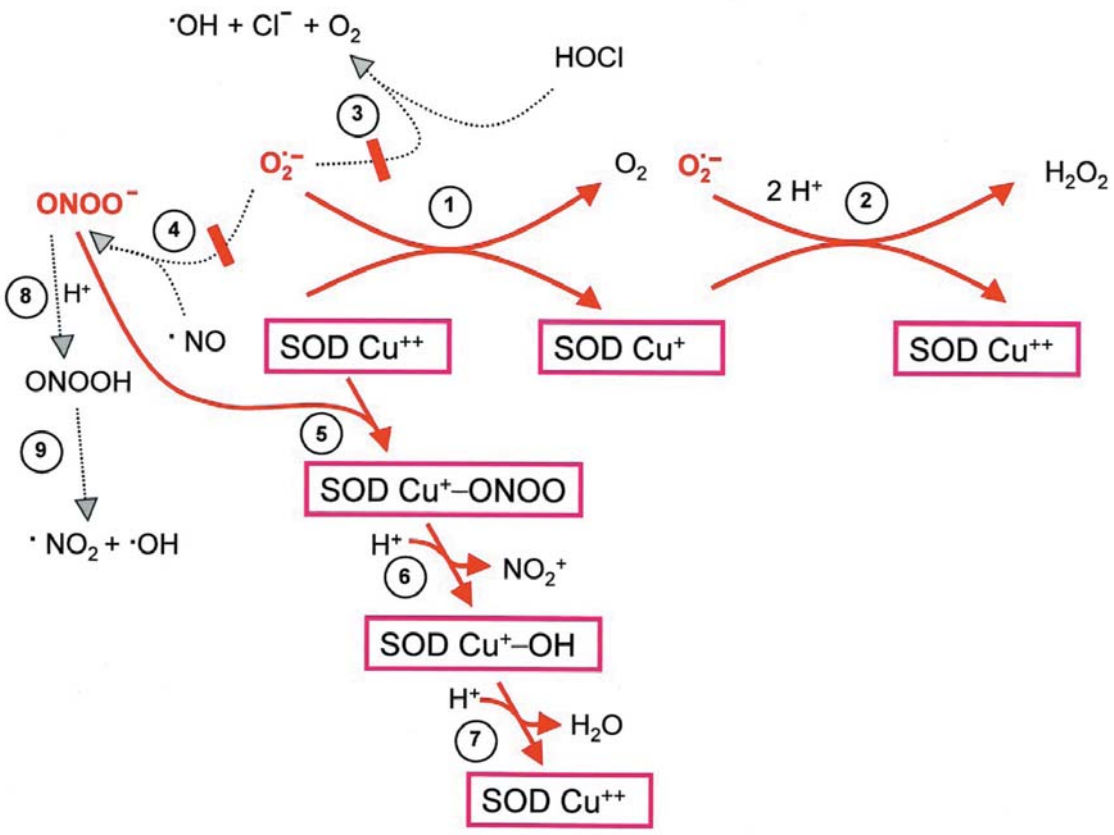

Figure 4. *Enzymatic details of the reaction of membrane-associated SOD of tumor cells. The native form of CuSOD ($SODCu^{++}$) reacts with a first superoxide anion (#1), resulting in the formation of O_2 and the reduced intermediate of SOD ($SODCu^{+}$). This intermediate reacts with a second superoxide anion (#2), resulting in the formation of H_2O_2 and re-generation of the native enzyme. This catalytic reaction cycle of SOD interferes with superoxide anion/HOCl interaction (#3) and peroxynitrite formation through superoxide anion/NO interaction (#4). In addition, peroxynitrite is decomposed by CuSOD (#5-7), thus preventing reaction steps #8-9. The resulting nitrosium ion (NO_2^{+}) may lead to nitration of the enzyme without causing an inhibitory effect (79).*

the same time endangering the tumor cell through its central role in ROS signaling.

The third advantage of tumor cells derived from SOD/catalase co-expression is based on the interaction of superoxide anions with catalase (Figure 6). The localization of NOX1 and catalase in the cell membrane causes a relatively high local generation of superoxide anions at the site of their generation and in the ultimate neighbourhood of catalase. As superoxide anions inhibit catalase through formation of inactive compound III ($CATFe^{III}\ O_2^{\bullet -}$) (84-89) and through conversion of compound I ($CATFe^{IV}{=}O^{+\bullet}$) to the inactive compound II ($CATFe^{IV}{=}O$) (84-89), catalase has no opportunity to exhibit its enzymatic activity when it is located close to active NOX1 (Figure 6). The inactive compound II can be converted to the inactive compound III by H_2O_2 (89). A one-electron transfer from a superoxide anion to compound II has been suggested to re-generate native, active ferricatalase (84). However, in the presence of high local concentrations of superoxide anions, this re-generated enzyme would form inactive compound III readily. Therefore, it is obvious that a protective function against ROS signaling by membrane-associated catalase alone would not be possible in NOX1-expressing tumor cells. Partial removal of superoxide anions by SOD beyond an inhibitory concentration for catalase would, however, restore and optimize the protective function of catalase (Figure 7). This crucial interaction between tumor cell-specific SOD and catalase has been directly shown in recent experiments (Bauer *et al.* unpublished data). In this experimental approach, apoptosis induction by exogenous peroxynitrite was used as test system. Protection against extracellular peroxynitrite can only be achieved through protective enzymes on the outside of the tumor cell membrane, whereas intracellular catalase has no protective effect against extracellular peroxynitrite. This is due to the reaction of peroxynitrite (or hydroxyl radicals derived from peroxynitrous acid) with the cell membrane, a reaction that obviously cannot be counteracted by catalase located

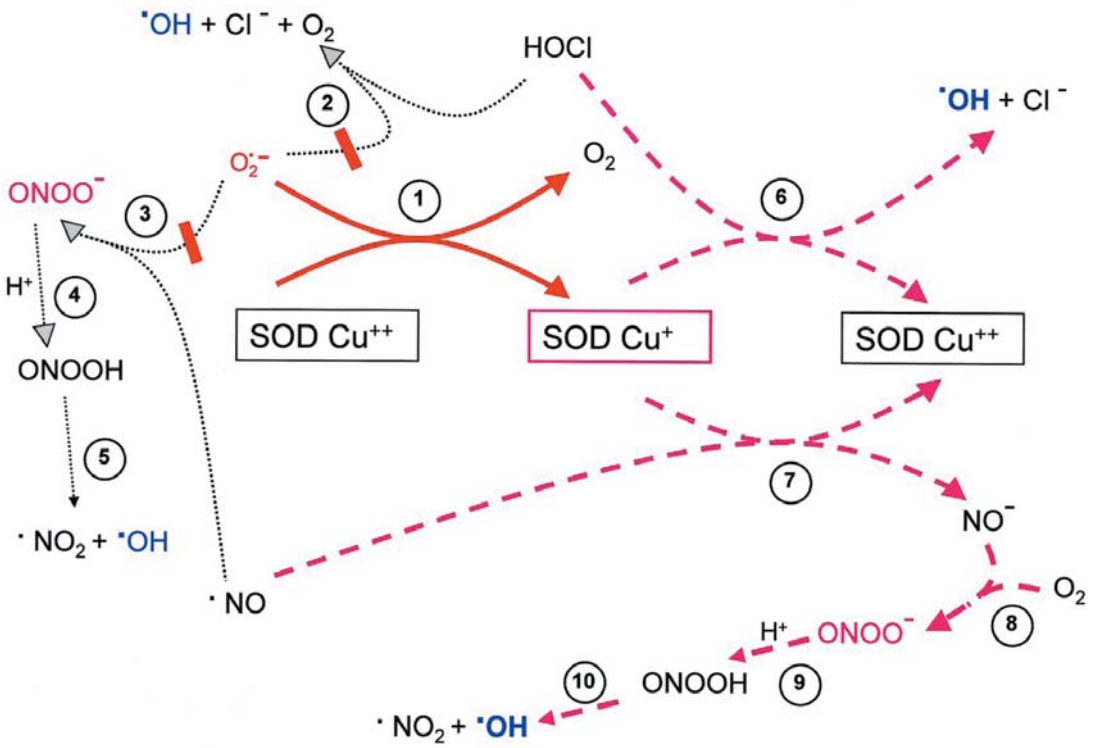

Figure 5. *Destructive effects of high concentrations of SOD. When the concentration of CuSOD is relatively high in relationship to available superoxide anions, its reaction with superoxide anions (#1) efficiently prevents superoxide anion/HOCl interaction (#2), as well as peroxynitrite formation (#3) and the subsequent reactions of peroxynitrite (#4, 5). However, under these conditions, the intermediate $SODCu^{+}$ rarely finds a second superoxide anion for the completion of the catalytic cycle. As a consequence, $SODCu^{+}$ may react with HOCl in a Fenton-type reaction, yielding apoptosis-inducing hydroxyl radicals (#6) or with NO, leading to the formation of nitroxyl anions (NO^{-}) (#7). The reaction between nitroxyl anions with molecular oxygen then leads to the formation of peroxnitrite, peroxynitrous acid, NO_2 and hydroxyl radicals (#8-10). These complex side-reactions of the $SODCu^{+}$ intermediate are the reason for the bell-shaped inhibition curves that are characteristic of SOD. As tumor cells show incomplete inhibition of superoxide anion-dependent processes (76), they seem to express a concentration of SOD that is far from the point where the protective function of the enzyme turns into a destructive one.*

inside the cell. When tumor cells were treated with neutralizing antibodies directed against catalase, they were no longer protected against apoptosis induction by exogenous peroxynitrite. This abrogation of protection did not require for on-going superoxide anion generation. Addition of antibodies directed against SOD1 sensitized the tumor cells for apoptosis induction by peroxynitrite with similar efficiency. However, the abrogation of protection by anti-SOD was strictly dependent on ongoing superoxide anion synthesis, indicative for the inhibitory effect of superoxide anions on catalase. Direct decomposition of peroxynitrite by membrane-associated SOD was found to be much less than decomposition by membrane-associated catalase.

Based on the outlined biochemical situation, the combination of both enzymes can be predicted to occur in all tumor cells that generate superoxide anions and that are protected by membrane-associated catalase. Otherwise the membrane-associated catalase would be ineffective due to inhibition by free superoxide anions and therefore would not have the potential to interfere with apoptosis induction by intercellular ROS signaling.

Unravelling the Interaction Between SOD and Catalase: Novel Potential Opportunities for Tumor Therapy

At first glance, the co-inhibitory effect of tumor cell-associated SOD in addition to protective catalase might be misinterpreted as an additional obstacle to tumor therapy based on reactivation of extracellular ROS signaling by tumor cells. However, the understanding of the biochemical interactions in this complex signaling system opens new avenues and may be the key for improvements of the therapeutic concept and its application.

The tightly-protected state of tumor cells is summarized in Figure 8. Membrane-associated NOX1 represents the

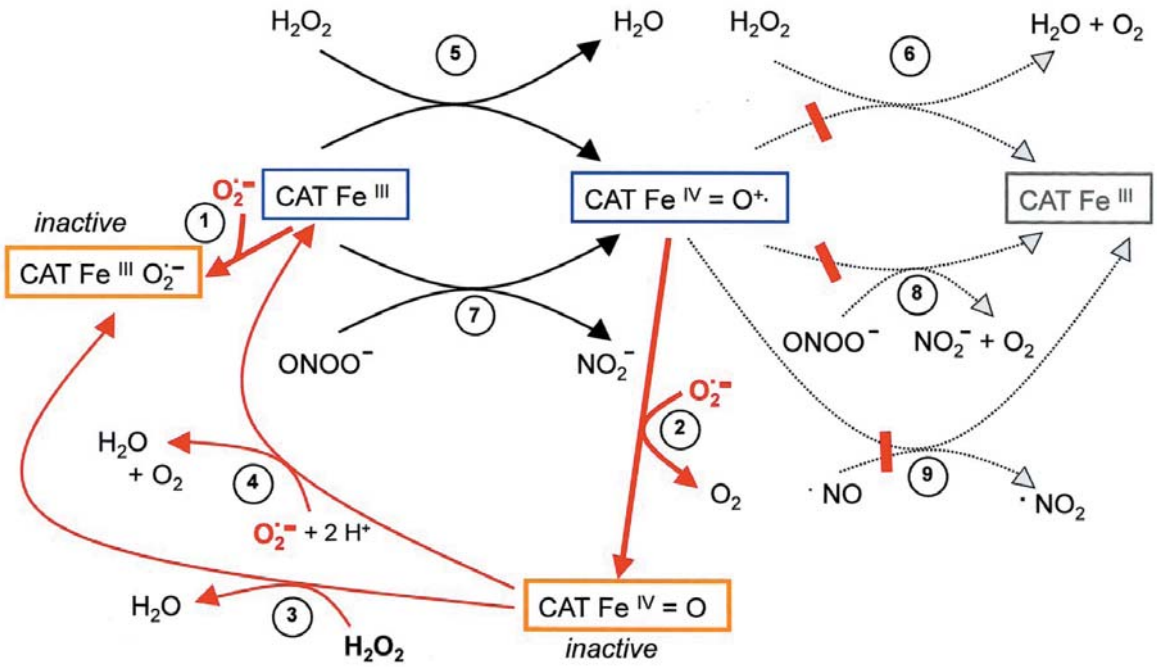

Figure 6. *Superoxide anions inhibit catalase. Superoxide anions react with native catalase and generate inactive compound III ($CATFeIIIO_2^{\bullet-}$) (#1). In addition, the one electron transfer from superoxide anions to compound I ($CATFe^{IV}=O^{+\bullet}$) leads to the formation of the inactive compound II ($CATFe^{IV}=O$) (#2). Inactive compound II may react with H_2O_2 and generate inactive compound III (#3). Alternatively, superoxide anions may transform inactive compound II into the native enzyme (#4), which is very likely to be immediately inhibited by superoxide anions according to reaction #1. Thus, superoxide anions have a tight negative control on catalase and prevent the reactions steps #5-9.*

source for intensive generation of extracellular superoxide anions. Scavenging of extracellular superoxide anions leads to formation of H_2O_2 and interferes with NO/superoxide and HOCl/superoxide anion interaction, two crucial steps in ROS signaling. However, removal of superoxide anions is not complete, due to limited expression of extracellular SOD, as recently determined (76). Nevertheless, intercellular ROS signaling is efficiently inhibited as decomposition of H_2O_2 by catalase, decomposition of peroxynitrite by catalase as well as SOD (with catalase representing the major activity (97) and oxidation of NO by compound I of catalase cause a strong block of both signaling pathways. Therefore, it is clear and also experimentally verified that inhibition or inactivation of membrane-associated catalase allows re-establishment of intercellular signaling and apoptosis induction in tumor cells, despite the remaining partial protective activity of SOD (Figure 9). The activity of SOD would be even enhancing HOCl signaling through generation of H_2O_2 that drives HOCl synthesis by peroxidase. At the same time, however, increased availability of H_2O_2 might increase the proliferation stimulus for tumor cells that survive ROS signaling. It is also conceivable that the lack of H_2O_2 consumption after catalase inhibition might have a negative effect on SOD activity (90).

Inactivation or inhibition of membrane-associated SOD leads to a much more complex but also more favourable signaling situation (Figure 10). As a primary result, superoxide anions are not scavenged and thus are available for unrestricted NO/superoxide interaction leading to peroxynitrite formation. Decomposition of peroxynitrite by SOD is abrogated through inhibition of SOD. Importantly, decomposition of peroxynitrite by catalase is also abrogated under the condition of direct inhibition of SOD, as the resultant free superoxide anions inhibit catalase. Inhibition of catalase by superoxide anions also prevents oxidation of NO and thus generates an additional enhancement to NO/peroxynitrite signaling. Inhibition of SOD causes a major decline in the generation of H_2O_2, which now depends solely on spontaneous dismution of superoxide anions at a 10,000-fold lower reaction rate. Therefore, HOCl synthesis occurs at a barely detectable level and the unlimited superoxide anion/HOCl interaction has no significant biological effect. The shift to dominating NO/peroxynitrite signaling under the condition of inhibition of membrane-associated SOD of tumor cells has been experimentally verified (Bauer *et al*., unpublished data). It bears the chance for further enhancement through additional modulation of NO metabolism, such as inhibition of arginase, increased

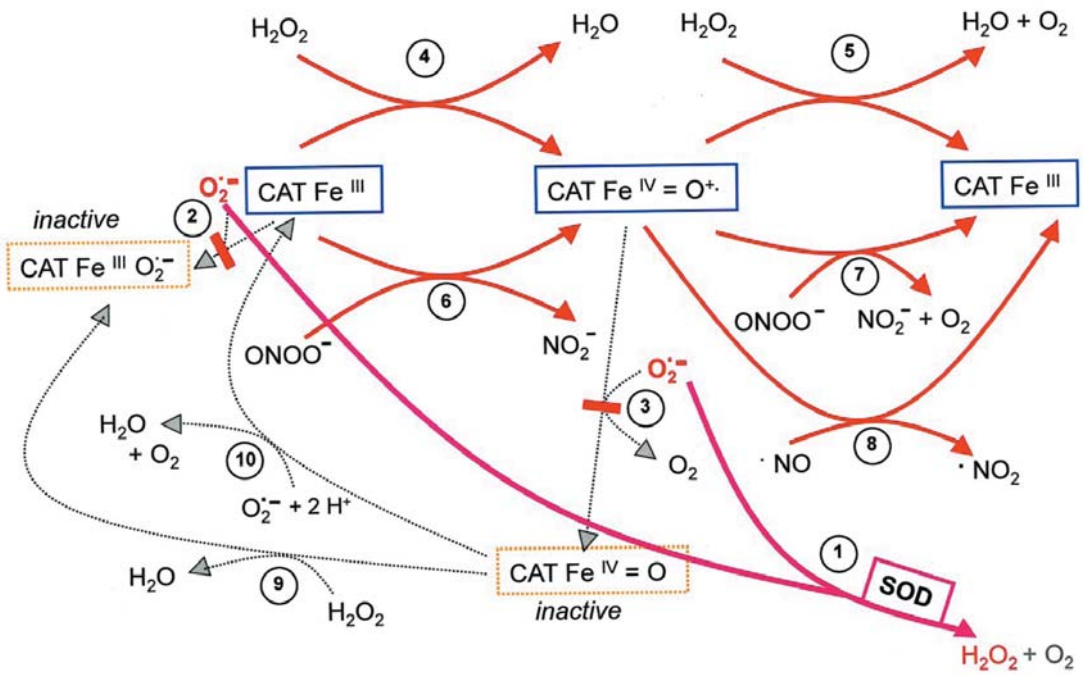

Figure 7. *SOD prevents superoxide anion-mediated inhibition of catalase. Co-expression of SOD in the membrane of tumor cells, in the neighbourhood of NOX1 and catalase causes partial removal of free superoxide anions through catalytic dismutation (#1). This reaction efficiently interferes with inhibition of catalase by free superoxide anions (#2 and 3) and thus ensures the protective effects of catalase that are mediated through its multiple enzymatic functions (#4-8). Native ferricatalase ($CATFe^{III}$) and compound I ($CATFe^{IV}=O^{+\bullet}$) are the essential forms of catalase, when SOD is co-expressed, whereas inactive compound II ($CATFe^{IV}=O$) and compound III ($CATFe^{III}O_2^{\bullet-}$) are not generated in significant concentrations.*

supply of arginine, induction of NO synthase, inhibition of NO dioxygenase, or application of defined NO donors. These applications may be useful in *in vivo* trials for the therapeutic efficacy of SOD inhibition. In contrast, the effect of positive modulation of NO metabolism might be problematic when catalase inhibition causes re-activation of HOCl signaling, due to the interference of NO with HOCl signaling, based on the complex consumption reaction between NO and H_2O_2 (55). These considerations, based on extensive studies *in vitro* that define the biochemical interactions during intercellular ROS signalling, favour the concept of utilizing inhibition of SOD rather than catalase in future experimental trials for tumor therapy. Whereas inhibition of tumor cell-associated catalase causes an increase in H_2O_2, inhibition of SOD leads to a drastic decrease in H_2O_2, as catalytic dismutation of superoxide anions is abrogated and spontaneous dismutation occurs with at a 10,000-fold lower reaction rate. Therefore, as a positive side-effect, inhibition of SOD diminishes the chance for proliferation stimulation of surviving tumor cells by H_2O_2. The tools for future studies *in vivo*, *i.e.* neutralizing Fab fragments directed against SOD, are being established. The study of the extracellular ROS-related signaling mechanisms seems to be nearly completed, as is the analysis of the intracellular reactions triggered by extracellular ROS signaling. However, one central question is still completely open: it needs to be clarified whether tumor stem cells exhibit NOX1 expression and protection by membrane-associated catalase and SOD.

Concluding Remarks

Many authors have pointed to the unique redox situation in tumor cells and suggested the use of ROS signaling for future therapeutic approaches. So far, however, intracellular ROS signaling has mainly been regarded as the primary target (91-95).

The focus on extracellular ROS-related signaling pathways of tumor cells, as suggested here, opens up the opportunity to utilize unique tumor cell-specific features to trigger their selective apoptotic self-destruction. The combination of active NOX1, catalase and SOD in the membrane of tumor cells represents a sophisticated mode for efficient and interrelated protection of the tumor cells against apoptosis induction by their own ROS. The reversal of this protective mechanism into a destructive one might bear great promise. The knowledge of the biochemical interactions between membrane-associated catalase and SOD, as well as of the biochemical consequences of their inhibition, should now allow for establishment of

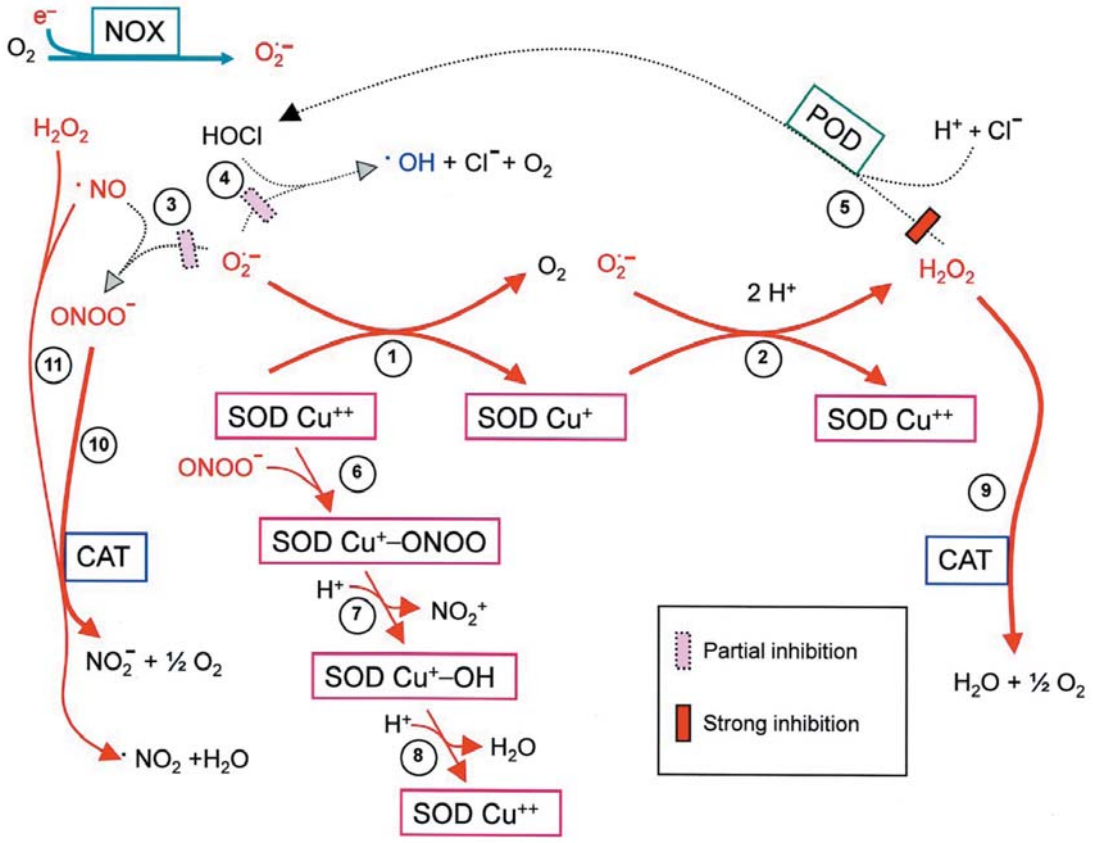

Figure 8. *Cooperation of catalase and SOD in the protection of tumor cells against ROS-mediated apoptosis signaling. Membrane-associated NADPH oxidase (NOX) generates extracellular superoxide anions. SOD reacts with a substantial concentration of free superoxide anions (#1 and #2) and thus prevents their reaction with NO (#3) or HOCl (#4). The reaction of SOD with superoxide anions results in the formation of H_2O_2 which is a potential substrate for peroxidase (POD) (#5). SOD also decomposes eventually generated peroxynitrite (#6-8). Catalase efficiently decomposes H_2O_2 (#9) and thus prevents synthesis of HOCl (#5) and the subsequent HOCl signaling pathway. Catalase efficiently interferes with NO/peroxynitrite signaling through decomposition of peroxynitrite (#10) and oxidation of NO (#11). Oxidation of NO requires the preceding formation of compound I through reaction of native catalase with H_2O_2. The cooperation between membrane-associated catalase and SOD thus results in a tight and interconnected control of inter-cellular ROS signaling and establishes the protection of the tumor cells against intercellular ROS signaling.*

novel strategies for selective apoptosis induction in tumors, as outlined here. As discussed above, targeting of membrane-associated SOD leads to parallel superoxide anion-dependent inhibition of catalase and thus to maximal abrogation of tumor cell-protective mechanisms. As inhibition of SOD causes a dramatic decrease in the generation of H_2O_2 through dismutation of superoxide anions, HOCl signaling has no chance of becoming re-activated, but the NO/peroxynitrite pathway is strongly favored. This should allow additional positive support through the application of NO donors or modulation of endogenous NO production or stabilization. In addition, the decrease in H_2O_2 has the positive side-effect of reducing the proliferation stimulus for surviving tumor cells.

Novel strategies should preferentially target extracellular SOD and not the intracellular enzyme with its protective function for normal tissue. An immunotherapeutical approach seems to be feasible. However, this approach should be based on neutralizing Fab fragments and not on complete antibodies, as neutralizing Fab fragments would only trigger ROS-mediated apoptosis in tumor cells with NOX1-positive/membrane-associated catalase-positive/membrane-associated SOD-positive phenotype, whereas complete immunoglobulins might trigger unwanted toxic effects on SOD-carrying normal tissue (96) through induction of antibody-dependent cellular cytotoxic effects mediated by natural killer cells.

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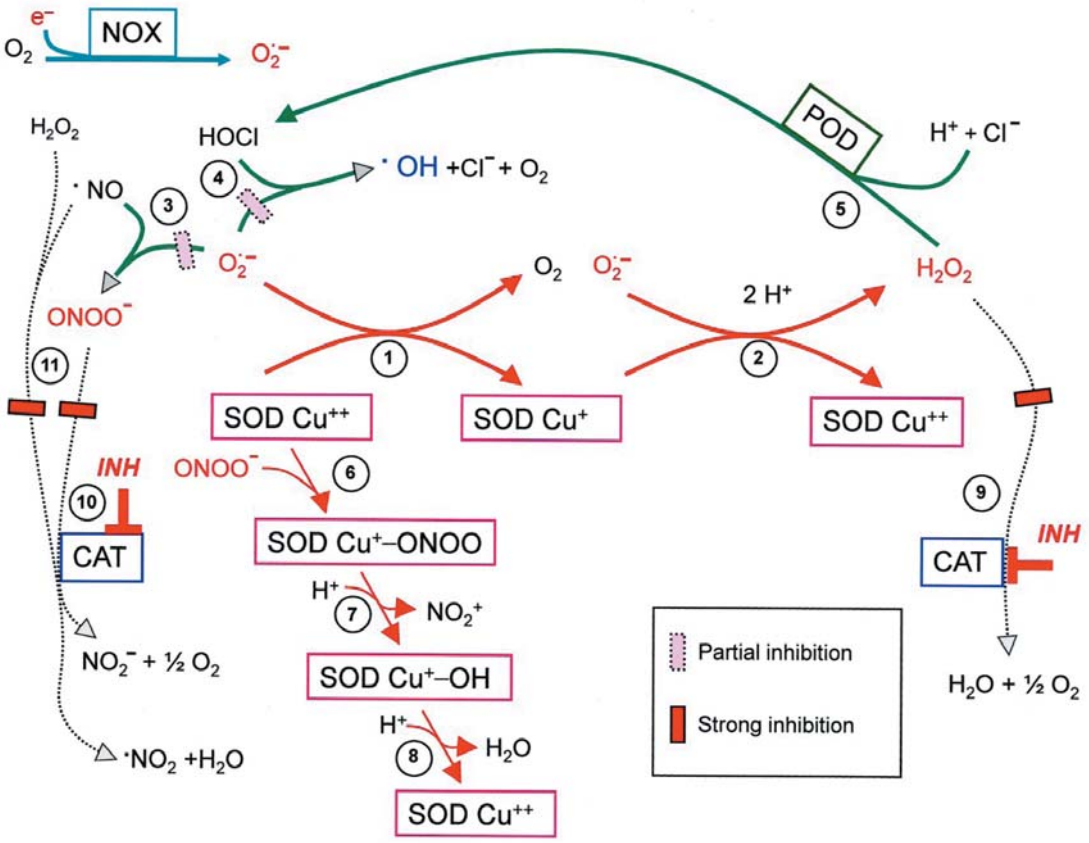

Figure 9. *Inhibition of catalase causes apoptosis induction in tumor cells. Inhibition of membrane-associated catalase abrogates the dominant inhibitory effects of catalase directed against HOCl synthesis (#5) and against NO/peroxynitrite signaling (#10, 11). As the concentration of SOD only allows for partial scavenging of free superoxide anions, the interaction between superoxide anions and NO (#3), as well as superoxide anions and HOCl (#4) is possible. Therefore, inhibition of catalase allows for ROS-mediated apoptosis induction in tumor cells through both signaling pathways.*

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Formulae

Superoxide anion ($O_2^{\bullet-}$); hydrogen peroxide (H_2O_2); hypochlorous acid (HOCl); hydroxyl radical ($^{\bullet}OH$); nitric oxide ($^{\bullet}NO$); nitrogen dioxide ($^{\bullet}NO_2$); peroxynitrite ($ONOO^-$), peroxynitrous acid (ONOOH); native catalase/ferricatalase ($CATFe^{III}$); compound I of catalase ($CATFe^{IV}=O^{+\bullet}$); compound II of catalase ($CATFe^{IV}=O$); Compound III of catalase ($CATFeIII\ O_2^{\bullet-}$).

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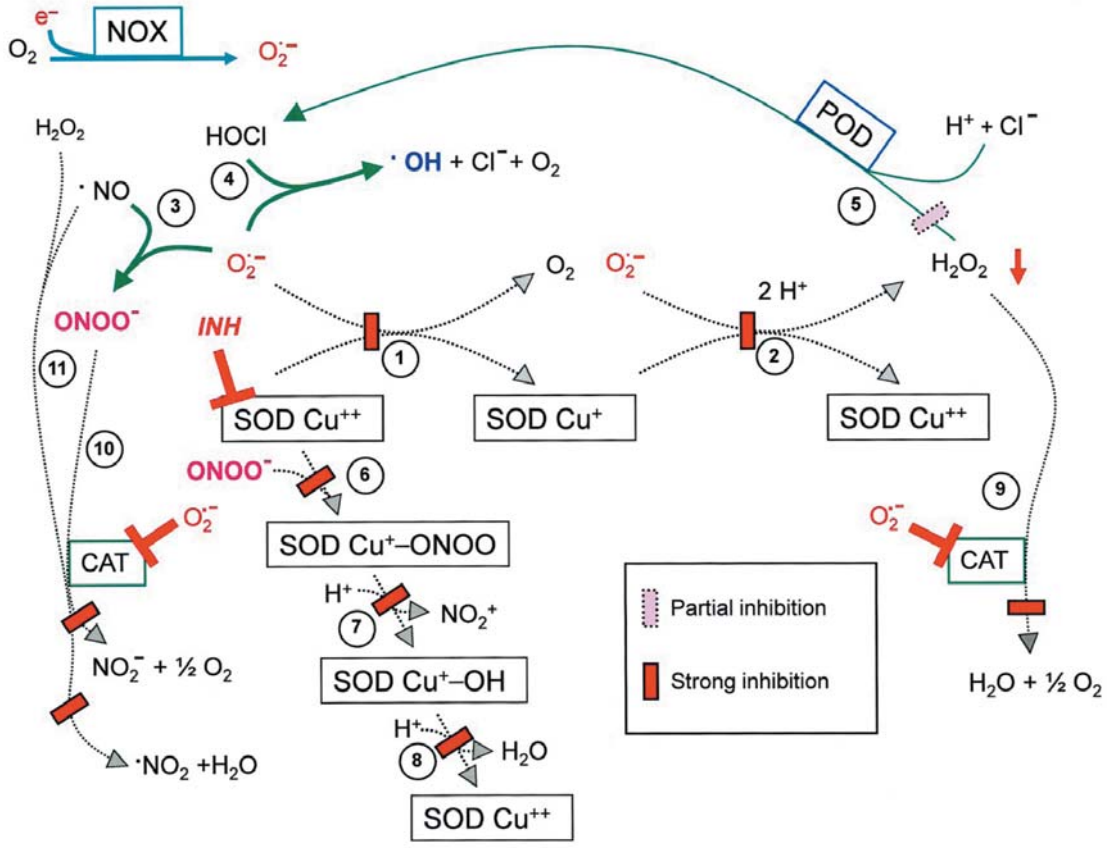

Figure 10. *Inhibition of SOD affects catalase activity and causes apoptosis induction in tumor cells. The inhibition of SOD (#1, 2) allows for peroxynitrite formation (#3) and HOCl/superoxide anion interaction (#4), but causes impaired HOCl synthesis (#5) as the generation of H_2O_2 depends solely on spontaneous dismutation. Inhibition of SOD also prevents SOD-mediated degradation of peroxynitrite (#6-8). As inhibition of SOD results in a high local concentration of free superoxide anions-catalase is efficiently inhibited as biochemical consequence (#9) (see Figure 6 for details). Therefore, the protective effects of catalase (#9-11) are also abrogated when SOD is inhibited. As a consequence, inhibition of SOD-alone causes efficient ROS-mediated apoptosis induction mainly through the NO/peroxynitrite signaling pathway, as HOCl synthesis is impaired due to the lack of SOD-dependent generation of H_2O_2.*

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