Increased Cytotoxicity of 3-Morpholinosydnonimine to HepG2 Cells in the Presence of Superoxide Dismutase

ROLE OF HYDROGEN PEROXIDE AND IRON*

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Nitric oxide (NO) and superoxide radical (O2·−) are known to be generated by a variety of mammalian cells. These agents may be important for host defense, but under certain conditions, NO· and NO2− may cause tissue damage by still to be clarified mechanisms. Both NO· and superoxide anion radical (O2·−) are known to be generated by macrophages, neutrophils, and endothelial cells (1–3). The production of these two radicals under pathological conditions can lead to the formation of peroxynitrite (ONOO−) and other reactive species that are cytotoxic (4–8). The cytotoxicity of ONOO− may be mediated by the ability of this compound to initiate lipid peroxidation (9, 10), cause oxidation of protein and non-protein sulfhydryls (11), produce nitration of tyrosine residues in proteins (5), or react with sugars (4) and DNA (12).

The active metabolite of 3-morpholinosydnonimine (SNAP) or DEA/NO (diethylamine/nitric oxide complex), the active metabolite of SIN-1, is the cause of toxicity, SOD will be protective since it is the toxic agent, SOD can potentiate NO· and NO2−, and other potent oxidants such as ONOO− and hydroxyl radical (OH) (16, 19, 20). These SIN-1-derived strong oxidants degrade deoxyribose (6), oxidize low density lipoproteins (21), mediate loss of microsomal α-tocopherol (22), damage surfactant protein A (23), and inhibit glyceraldehyde-3-phosphate dehydrogenase (24) and hepatic gluconeogenesis (25). Cytotoxic effects of SIN-1 have been demonstrated in a variety of cell lines (26–28). For example, the LD50 for SIN-1 cytotoxicity against Escherichia coli was 0.5 mM (29), and 1 mM SIN-1 was very toxic to neurons (30). SOD had a protective effect against SIN-1 toxicity to cultured neurons (30) and to E. coli (29), but had no protective effect with rat hepatoma cells (28), and SOD actually increased SIN-1 toxicity to Leishmania major (31). The effect of SOD on the toxicity of SIN-1 is complex, e.g. if ONOO− is the cause of toxicity, SOD will be protective since dismutation of O2·− will prevent formation of ONOO−. However, if NO· itself is the toxic agent, SOD can potentiate NO· toxicity

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The abbreviations used are: NO·, nitric oxide; NO2−, nitrogen oxida-

3-Morpholinosydnonimine (SN-1) is widely used to generate nitric oxide (NO·) and superoxide radical (O2·−). The effect of SOD on the toxicity of SIN-1 is complex, depending on what is the ultimate species responsible for toxicity. SIN-1 (<1 mM) was only slightly toxic to HepG2 cells. Copper, zinc superoxide dismutase (Cu,Zn-SOD) or manganese superoxide dismutase (Mn-SOD) increased the toxicity of SIN-1. Catalase abolished, while mannitol had no protective effect, iron chelators, thiourea and urate protected the cells against the SIN-1 plus Cu,Zn-SOD-mediated cytotoxicity. The cytotoxic effect of Cu,Zn-SOD but not Mn-SOD, showed a biphasic dose response being most pronounced at concentrations (10–100 units/ml). In the presence of SIN-1, Mn-SOD increased accumulation of H2O2 in a concentration-dependent manner. In contrast, Cu,Zn-SOD increased H2O2 accumulation from SIN-1 at low but not high concentrations of the enzyme, suggesting that high concentrations of the Cu,Zn-SOD interacted with the H2O2. EPR spin trapping studies demonstrated the formation of hydroxyl radical from the decomposition of H2O2 by high concentrations of the Cu,Zn-SOD. The cytotoxic effect of the NO donors SNAP and DEA/NO was only slightly enhanced by SOD; catalase had no effect. Thus, the oxidants responsible for the toxicity of SIN-1 and SNAP or DEA/NO to HepG2 cells under these conditions are different, with H2O2 derived from O2·− dismutation playing a major role with SIN-1. These results suggest that the potentiation of SIN-1 toxicity by SOD is due to enhanced production of H2O2, followed by site-specific damage of critical cellular sites by a transition metal-catalyzed reaction. These results also emphasize that the role of SOD as a protector against oxidant damage is complex and dependent, in part, on the subsequent fate and reactivity of the generated H2O2.

Nitric oxide (NO·) and other reactive nitrogen oxidative metabolites (NO2−) are produced by a variety of mammalian cells. These agents may be important for host defense, but under certain conditions, NO· and NO2− may cause tissue damage by still to be clarified mechanisms. Both NO· and superoxide anion radical (O2·−) are known to be generated by macrophages, neutrophils, and endothelial cells (1–3). The production of these two radicals under physiological conditions can lead to the formation of peroxynitrite (ONOO−) and other reactive species that are cytotoxic (4–8). The cytotoxicity of ONOO− may be mediated by the ability of this compound to initiate lipid peroxidation (9, 10), cause oxidation of protein and non-protein sulfhydryls (11), produce nitration of tyrosine residues in proteins (5), or react with sugars (4) and DNA (12). Superoxide dismutase (SOD) present in the cytoplasm and mitochondria within cells and in the extracellular space dismutates two O2·− into H2O2 and O2 (13). The protective effect of SOD against oxygen-derived free radicals in vivo, in intact cells and in vitro studies is well documented (14, 15). However, in some studies a bell-shaped dose-response curve for the protective effect of SOD was observed. At low concentrations, SOD was usually protective, but at very high concentrations, its protective effect decreased or was reversed such that SOD potentiated toxicity (16–18).

SIN-1 (3-morpholinosydnonimine), the active metabolite of the vasodilatory drug molsidomine, is frequently used as a model compound for a continuous release of O2·− NO· and/or NO2−, and other potent oxidants such as ONOO− and hydroxyl radical (OH) (16, 19, 20). These SIN-1-derived strong oxidants degrade deoxyribose (6), oxidize low density lipoproteins (21), mediate loss of microsomal α-tocopherol (22), damage surfactant protein A (23), and inhibit glyceraldehyde-3-phosphate dehydrogenase (24) and hepatic gluconeogenesis (25). Cytotoxic effects of SIN-1 have been demonstrated in a variety of cell lines (26–28). For example, the LD50 for SIN-1 cytotoxicity against Escherichia coli was 0.5 mM (29), and 1 mM SIN-1 was very toxic to neurons (30). SOD had a protective effect against SIN-1 toxicity to cultured neurons (30) and to E. coli (29), but had no protective effect with rat hepatoma cells (28), and SOD actually increased SIN-1 toxicity to Leishmania major (31). The effect of SOD on the toxicity of SIN-1 is complex, e.g. if ONOO− is the cause of toxicity, SOD will be protective since dismutation of O2·− will prevent formation of ONOO−. However, if NO· itself is the toxic agent, SOD can potentiate NO· toxicity
by preventing its reaction with $O_2^-$, thereby elevating steady state concentrations of NO. The role of $H_2O_2$, the product of $O_2^-$ dismutation by SOD, has generally not been considered as important as nitrogenous metabolites in SIN-1 toxicity. It was of interest that the Cu,Zn-SOD did not provide more than 50% protection against SIN-1 toxicity to E. coli even when added in excess (29), perhaps due to the toxicity of $H_2O_2$ produced from $O_2^-$ dismutation. In the current report, the effect of SOD on SIN-1 cytotoxicity in a human hepatoma liver cell line (HepG2) was determined. It was observed that Cu,Zn-SOD and Mn-SOD enhanced SIN-1-mediated cytotoxicity to HepG2 cells, and that this potentiation was due to an increase in $H_2O_2$ formation caused by SOD-catalyzed dismutation of the $O_2^-$ released from SIN-1. The toxicity of SIN-1 under these reaction conditions and concentrations appeared to be independent of NO or $O_2^-$.  

EXPERIMENTAL PROCEDURES

Materials—SIN-1 and SNAP were from Biomol (Plymouth Meeting, PA). DEA/NO was from Research Biochemical (Natic, MA). Cu,Zn-superoxide dismutase from bovine erythrocytes and catalase were obtained from Boehringer Mannheim. Mn-superoxide dismutase from E. coli, benzene, Me$_2$SO, formate, thiourea, BSO, defereroxamine mesylate, 2,2'-dipyridyl, $H_2O_2$, EDTA, and DTPA were from Sigma. Sodium azide was from Aldrich. 5,5'-Dimethylpyrroline-N-oxide (DMPO) was from Sigma and was purified before use by charcoal treatment to remove paramagnetic impurities.

Cell Cultures—The human hepatoma cell line HepG2 (American Type Culture Collection, HB8069) was used for these studies. Cells were cultured in minimum essential medium (MEM, Life Technologies, Inc.) supplemented with 1% PSN antibiotic mixture (Life Technologies, Inc.), and 10% fetal calf serum (Sigma) in 25-cm$^2$ plastic flasks (Corning) at 37 °C in a humidified atmosphere of 95% air and 5% CO$_2$. For cytotoxicity experiments, cells were plated at $4 \times 10^4$ cells/well/0.5 ml of tissue culture medium on 24-well tissue culture plates. After 2 h of incubation and attachment, SIN-1, SOD, as well as other compounds were added, and the cells were incubated for the indicated time periods at 37 °C. Stock solutions of all compounds were prepared in MEM medium immediately before use. The preparation of solutions and incubation with the HepG2 cells were carried out under protection from light. Cell viability (cytotoxicity) was determined using the Cell Titer 96 non-radioactive cell proliferation/cytotoxicity assay kit (Promega), which is based on the cellular conversion of a tetrazolium salt into a formazan product that can be detected spectrophotometrically (32). GSH levels were determined using the GSH-400 kit assay (Cayman Chemical, Ann Arbor, MI).

Determination of $H_2O_2$—Generation at different concentrations of SIN-1, in the absence or presence of SOD, was determined by two techniques after a 24-h incubation in tissue culture medium at 37 °C in 95% air and 5% CO$_2$ in the absence of cells. In the first method, the $H_2O_2$ content was measured by formation of formaldehyde from the oxidation of methanol by the catalase-$H_2O_2$ compound I complex (33). Incubations contained 100 mM methanol, 1200 units/ml catalase, SIN-1, and tissue culture medium. Reactions were terminated after a 1-h incubation at 37 °C by the addition of 20% trichloroacetic acid. The generation of formaldehyde was determined by the Nash reaction (34), and calculation of $H_2O_2$ concentration was carried out as described previously (35). In the second method, the formation of $H_2O_2$ was determined by the horseradish peroxidase-catalyzed reaction of $H_2O_2$ with 4-amino-antipyrine plus 3,5-dichloro-2-hydroxybenzenesulfonic acid. After a 1-h incubation of SIN-1 with the above compounds in tissue culture medium at 37 °C, the resulting product was measured spectrophotometrically at 546 nm (28, 36).

EPR Measurements—The production of $OH$ from the SOD-catalyzed decomposition of $H_2O_2$ was determined by spin trapping with DMPO. MEM containing 100 or 1000 units/ml Cu,Zn-SOD or Mn-SOD and 100 mM DMPO was incubated with 80 $\mu$m $H_2O_2$. The samples were filled in a Varian flat cell and measured within 1 min after addition of $H_2O_2$ using a Varian EPR 200 spectrometer operating in the X-band mode. Instrument parameters are indicated in the legends to figures.

In separate experiments evaluating iron-catalyzed decomposition of $H_2O_2$, 1 mM SIN-1 was co-incubated with either Cu,Zn-SOD (100 or 1000 units/ml) or Mn-SOD (100 or 1000 units/ml) for 24 h at 37 °C in the absence of cells. The samples of the incubate were added to the same concentration of FeSO$_4$ and incubated in the presence of manganese superoxide dismutase. $H_2O_2$ and 600 mM Me$_2$SO were added and decomposition of $H_2O_2$ was initiated by the addition of 40 $\mu$m FeSO$_4$. In the presence of Me$_2$SO, $OH$ radicals are

![Fig. 1. The effect of SIN-1, in the absence and presence of Cu,Zn-SOD, on the viability of HepG2 cells. HepG2 cells were incubated with the following concentrations of SIN-1 for the indicated time periods: A, 0.1 mM SIN-1; B, 0.316 mM SIN-1; C, 1 mM SIN-1; D, 3 mM SIN-1. The concentration of Cu,Zn-SOD added to the medium was as follows: no addition (●), 10 units/ml (○), 100 units/ml (△), or 1000 units/ml (●). Results are mean ± S.E. from three experiments.]
SOD Increases SIN-1 Toxicity

Fig. 2. Effect of SOD on the cytotoxicity of SIN-1 to HepG2 cells. Panel A, effect of catalase and azide on the potentiation of SIN-1 cytotoxicity by Cu,Zn-SOD. Viability of the HepG2 cells was determined after a 24-h incubation with 0.316 mM SIN-1 in the presence of the indicated concentrations of Cu,Zn-SOD. Additions were as follows: SIN-1 (●), SIN-1 plus 0.316 mM azide (○), SIN-1 plus catalase (200 units/ml) (■), SIN-1 plus boiled Cu,Zn-SOD (□), Cu,Zn-SOD in the absence of SIN-1 (▲). Results are mean ± S.E. from three experiments. Panel B, comparison of the effects of Cu,Zn-SOD (●) and Mn-SOD (▲) on the cytotoxicity of SIN-1 to HepG2 cells. The cytotoxic effect of 0.316 mM SIN-1 was determined in the presence of the indicated concentrations of Cu,Zn-SOD or Mn-SOD. Viability was determined after a 24-h incubation period. Results are mean ± S.E. from three experiments.

The protective effects of catalase and azide on the SIN-1 plus SOD toxicity was determined. Catalase (200 units/ml) abolished the SOD-mediated cytotoxic effect of SIN-1 at all Cu,Zn-SOD concentrations tested (Fig. 2A). The possible involvement of intracellular H$_2$O$_2$ and catalase in the cytotoxic effect of SOD plus SIN-1 was studied using sodium azide, an inhibitor of the intracellular catalase. Sodium azide slightly increased the toxicity of SIN-1 in the absence of SOD and further potentiated the cytotoxic effect produced by the combination of SIN-1 plus Cu,Zn-SOD (Fig. 2A).

Since H$_2$O$_2$ can be a precursor for formation of 'OH, the possible involvement of 'OH-like species in the SOD-mediated cytotoxic effect of SIN-1 was evaluated using 'OH scavengers. Viability of the HepG2 cells was lowered by 10% by 0.632 mM SIN-1 and by 90% in the presence of SIN-1 plus 100 units/ml Cu,Zn-SOD (Fig. 3). Thiourea and uric acid were very effective in protecting the HepG2 cells against this SOD-mediated cytotoxicity. However, other 'OH scavengers tested, including Me$_2$SO, sodium formate, and mannitol, had no protective effect.
The formation of $\text{H}_2\text{O}_2$ from $0.316 \text{ m} \text{M}$ SIN-1 was determined by assessing the oxidation of methanol by the catalase-$\text{H}_2\text{O}_2$ compound I complex as described under “Experimental Procedures.” Experiments were carried out in the absence (black bar) or presence of either 100 units/ml Cu,Zn-SOD or Mn-SOD (empty bars) or 1000 units/ml Cu,Zn-SOD or Mn-SOD (hatched bars). Results are mean ± S.E. from three experiments.

Since SIN-1 generates $\text{O}_2^\cdot$ and NO/NO$\cdot$ simultaneously, it was of interest to evaluate the effect of SOD on the cytotoxicity of other systems which produce only NO/NO$\cdot$. Therefore, SNAP and DEA/NO, which do not generate $\text{O}_2^\cdot$ were used as sources of NO. SNAP or DEA/NO alone (1 and 3 mM) displayed moderate cytotoxicity toward the HepG2 cells, and this toxicity, in contrast to the results with SIN-1, was only slightly potentiated in the presence of Cu,Zn-SOD (100 units/ml). Moreover, catalase did not protect cells against the cytotoxic effect of either SNAP or DEA/NO alone or SNAP or DEA/NO plus Cu,Zn-SOD (Fig. 10).
DISCUSSION

SIN-1 is often used as a model for the continuous release of $O_2^-$ and NO $z$ in order to mimic the release of these agents by macrophages, neutrophils, and endothelial cells (6, 19–22). The generation of these two radicals under physiological conditions can lead to the formation of ONOO$^-$. Superoxide dismutase (SOD), ubiquitously present in living aerobic organisms, generally has protective effects against oxygen-derived free radicals (14, 15). However, in some studies, a bell-shaped dose-response curve of the protective effect of SOD was observed (16–18). An increase in SOD activity has been postulated to enhance lipid peroxidation (40, 41) and cellular injury (42, 43). Mao et al. (44) attributed the toxicity of SOD to the production of $\cdot OH$ radicals as a result of an increased Fenton reaction. With respect to NO $z$ toxicity, the effects of SOD are dependent on whether NO $z$ itself or ONOO$^-$ is the actual species. Scavenging of $O_2^-$ by SOD has been shown to increase the half-life of NO $z$ (45). In the central nervous system, SOD increased the toxic effects of NO $z$ (46), perhaps by increasing the half-life of NO $z$.

SIN-1 has been found to be cytotoxic to different mammalian cells (26–28). SIN-1 induced neuronal cell death in a dose-dependent manner; however, the neurons were completely protected if SOD was present in the reaction system (30). Brunelli et al. (29) found that SIN-1 killed E. coli with an LD$_{50}$ of 0.5 mM. Cu,Zn-SOD (50–400 units/ml) provided substantial, but not complete, protection against this SIN-1 killing. Cu,Zn-SOD and catalase together completely protected E. coli against SIN-1 toxicity. Assreuy et al. (31) recently reported that SIN-1 (0.3 mM) was efficient in killing L. major, and this cytotoxicity was enhanced by the Cu,Zn-SOD (500 units/ml). The authors suggested that the killing of L. major by SIN-1 was dependent only on NO $z$, and the enhancement of toxicity by SOD was due to scavenging of $O_2^-$ thereby increasing the half-life of the NO $z$. Ioannidis and DeGroot (28) found that the cytotoxicity of SIN-1 to Fu5 hepatoma cells was not affected by SOD, but catalase diminished cell damage; the authors suggested a cooperative toxic action between $H_2O_2$ and the tumoricidal activity of NO $z$ in the Fu5 cells. Siegfried et al. (47) reported that SIN-1 had significant cardioprotective effects in a myocardial ischemia-reperfusion model, perhaps via quenching of $O_2^-$ by NO produced from the SIN-1.

![Figure 7](image-url)
We found that Cu,Zn-SOD did not protect against the cytotoxicity to human hepatoma HepG2 cells caused by a high concentration of (3 mM) SIN-1. Actually, both Cu,Zn-SOD and Mn-SOD potentiated SIN-1-mediated cytotoxicity against the HepG2 cells, resulting in toxicity at SIN-1 concentrations that alone had no, or only a minor, cytotoxic effect. Lack of potentiation by boiled Cu,Zn-SOD indicated that catalytically active enzyme was required for potentiating this cytotoxic effect of SIN-1. Catalase abolished the SIN-1 plus SOD-mediated cytotoxic effect at all Cu,Zn-SOD concentrations evaluated (Fig. 2), whereas boiled catalase had no effect. This suggests that \( \text{H}_2\text{O}_2 \) produced by a SOD-catalyzed dismutation of \( \text{O}_2^\cdot \) is involved in the overall cytotoxic effect of SIN-1 plus SOD. The further potentiation of the SIN-1 plus Cu,Zn-SOD cytotoxicity by sodium azide, an inhibitor of intracellular catalase, is in accord with this suggestion, as are assays of \( \text{H}_2\text{O}_2 \) accumulation produced by SIN-1 in the absence and presence of SOD. Alternatively, catalase may be protective by reaction with NO\(^\cdot\) thereby decreasing levels of NO\(^\cdot\) and ONOO\(^-\). In preliminary experiments, we found that catalase had no effect on assays of NO\(_x\) formation from SIN-1 as detected with the Griess reagent. It is likely that Cu,Zn-SOD and Mn-SOD are increasing dismutation of \( \text{O}_2^\cdot \) produced from SIN-1 to \( \text{H}_2\text{O}_2 \) in the culture medium.

**Fig. 8.** EPR spectra of DMPO/\( \text{OH}^- \) adducts produced by decomposition of \( \text{H}_2\text{O}_2 \) by SOD. Experiments were carried out as described under "Experimental Procedures," using DMPO as the \( \text{OH}^- \) spin trapping agent. The reaction system contained MEM medium, 80 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), 100 \( \mu \text{M} \) DMPO, and the indicated additions: 100 units/ml Cu,Zn-SOD (A), 1000 units/ml Cu,Zn-SOD (B), 100 units/ml Mn-SOD (C), or 1000 units/ml Mn-SOD (D). Each spectrum represents an average of two scans with the following instrumental settings: modulation amplitude, 1.25 G; time constant, 0.250 s; scan rate, 6.25 G/min; gain, \( 4 \times 10^4 \). No DMPO/\( \text{OH}^- \) adducts were observed in the presence of \( \text{H}_2\text{O}_2 \) or Cu,Zn-SOD alone.

**Fig. 9.** Concentration dependence of Cu,Zn-SOD-catalyzed decomposition of \( \text{H}_2\text{O}_2 \). The data shown are amplitudes of the second peak of DMPO/\( \text{OH}^- \) EPR signals obtained as described in the legend to Fig. 8. Each point represents mean \( \pm \) S.D. from three experiments.

**Fig. 10.** The effect of SNAP or DEA/NO on the viability of HepG2 cells. The cytotoxic effect of either 1 \( \text{mM} \) (open bars) or 3 \( \text{mM} \) (hatched bars) SNAP (upper panel) or DEA/NO (lower panel) on HepG2 cells was determined after a 24-h incubation in the presence of the indicated additions; 200 units/ml catalase (CAT), 100 units/ml Cu,Zn-SOD (SOD), control (absence of SNAP or DEA/NO) (C). Results are mean \( \pm \) S.E. from four experiments.
rather than in the HepG2 cells themselves. For example, the conditioned medium containing SIN-1 plus Cu,Zn-SOD was cytotoxic when added later to HepG2 cells indicating formation of a stable toxic product, probably H$_2$O$_2$. This raises the question as to whether the SIN-1 plus SOD, i.e. H$_2$O$_2$-mediated, toxicity is occurring extra- or intracellularly.

The toxicity of H$_2$O$_2$ produced by the SIN-1/SOD system appears to be intracellular and mediated by transition metal-catalyzed decomposition of H$_2$O$_2$ at or near critical cellular sites, based upon the following results. 1) The presence of sodium azide, an inhibitor of intracellular catalase, increases cell killing by the SIN-1/SOD combination (Fig. 2); 2) depletion of intracellular glutathione by BSO also enhanced the observed toxicity of SIN-1/SOD (Fig. 5); 3) penetrable metal chelators such as deferoxamine and 2,2’-dipyridyl were very effective in reducing the SIN-1/SOD-mediated cytotoxicity, while EDTA and DTPA, which do not readily cross cellular membranes and hence act to chelate iron extracellularly (48), provided only a small protection. The inhibition of SIN-1/SOD toxicity by metal chelators would argue against a significant role for ONOO$^-$ or singlet oxygen, which may be produced from the interaction of H$_2$O$_2$ with NO$^-$ (49) or H$_2$O$_2$ with ONOO$^-$ (50) in the mechanism of SIN-1 toxicity. The possibility that the cytotoxicity was mediated by the reaction of NO$^-$ with H$_2$O$_2$ is not likely since no reaction between H$_2$O$_2$ and NO$^-$ was found at physiological pH (51). The requirement for H$_2$O$_2$ and iron in the overall mechanism by which SIN-1/SOD causes toxicity to the HepG2 cells would suggest that ‘OH-like species formed via a Fenton-type reaction may be the oxidants responsible for the cytotoxicity. Indeed, thiourea and urate, potent ‘OH scavengers, were effective in protecting the HepG2 cells against SIN-1/SOD toxicity. However, other ‘OH scavengers such as M$_6$SO, formate, mannitol, benzoate, and ethanol did not afford any protection; M$_6$SO, ethanol, and formate are permeable agents and should be available to scavenge ‘OH that is accessible. The inability of ‘OH scavengers to provide protection against oxidant injury has sometimes been explained by site-specific damage produced when H$_2$O$_2$ is decomposed by transition metals bound to critical cellular targets (52–53). The ability of thiourea and urate to protect against the SIN-1/SOD cytotoxicity may reflect the ability of these agents to chelate metals (54) or react with H$_2$O$_2$ (55, 56).

The effectiveness of Cu,Zn-SOD to potentiate SIN-1 toxicity displayed an unusual SOD concentration dependence in that lower concentrations of SOD enhanced the SIN-1 toxicity to a greater extent than higher concentrations. The Mn-SOD did not display this biphasic response. These effects can probably be related to the levels of H$_2$O$_2$ that accumulate in the reaction system, since H$_2$O$_2$ plays a central role in the cytotoxicity of SIN-1/SOD. Highest levels of H$_2$O$_2$ are found when SIN-1 is incubated with the lower concentration of the Cu,Zn-SOD or with low and high concentrations of the Mn-SOD. The lowest levels of H$_2$O$_2$ are found when SIN-1 is incubated with the high concentration of Cu,Zn-SOD; these levels are even lower than those produced by SIN-1 alone, in the absence of SOD. These results suggest that high concentrations of the Cu,Zn-SOD, but not the Mn-SOD, can decompose H$_2$O$_2$. The ability of Cu,Zn-SOD but not Mn-SOD to decompose H$_2$O$_2$ in a Fenton-type reaction was originally reported by Yim et al. (39). It should be kept in mind that because of the high rate constant for the SOD-catalyzed dismutation of O$_2^-$ (2 $\times$ 10$^9$ m$^{-1}$ s$^{-1}$), only small concentrations of SOD are usually necessary to scavenge the O$_2^-$ formed. Considerably higher concentrations of Cu,Zn-SOD are required to decompose substantial amounts of the formed H$_2$O$_2$, probably because of the expected low rate of this reaction (the reported rate constant of different Fenton catalysts is in the range 10$^8$ to 10$^9$ m$^{-1}$ s$^{-1}$; Refs. 57 and 58). The ability of high concentrations of the Cu,Zn-SOD, but not the Mn-SOD, to decompose H$_2$O$_2$, with the subsequent production of ‘OH (39) was confirmed by the experiments shown in Figs. 7–9. This raises the question as to why ‘OH formed by the decomposition of H$_2$O$_2$ by high concentrations of Cu,Zn-SOD did not contribute to the cytotoxicity of SIN-1/SOD, i.e. less toxicity is observed under conditions in which ‘OH is being produced, and Mn-SOD which does not decompose H$_2$O$_2$, to ‘OH was equally cytotoxic at concentrations of 100 units/ml and more cytotoxic at 1000 units/ml than Cu,Zn-SOD at the same concentrations. Most likely the ‘OH produced by this reaction is formed deep within the channel of the Cu,Zn-SOD (39) and reacts with the enzyme before escaping into the bulk solution to damage the cells. It must also be considered that although levels of H$_2$O$_2$ found in the presence of SIN-1 plus the high concentration of Cu,Zn-SOD are lower than that produced by SIN-1 alone, toxicity is observed in the former but not the latter case (Fig. 1, B and C), which may actually be a reflection of sufficient concentrations of H$_2$O$_2$ crossing the cellular membrane before decomposition by the extracellular Cu,Zn-SOD occurs or there is a contribution toward overall toxicity by ‘OH produced by the SIN-1 plus Cu,Zn-SOD combination.

SOD is generally thought to play a protective role in biological systems against oxidant action. It is now becoming apparent that the net effect of SOD is complex and often depends on the fate of the generated H$_2$O$_2$ and the availability of H$_2$O$_2$ degradation enzymes such as catalase and glutathione peroxidase. SIN-1 is the active metabolite of molsidomine, an anti-anginal drug (59), which is used for the treatment of ischemia reperfusion injury (60) and human erectile dysfunction (61). It is also used for the production of O$_2^-$ and NO$^-$ in a variety of experimental systems. The potentiation of SIN-1 cytotoxicity by SOD therefore requires caution in the use of this agent and in considering the therapeutic efficacy of SOD in systems that generate O$_2^-$ and NO$^-$: Our results indicate that the observed potentiation of the cytotoxicity of SIN-1 by SOD is linked to the formation of H$_2$O$_2$ by SOD-catalyzed dismutation of O$_2^-$: The largely extracellularly produced H$_2$O$_2$ then diffuses inside the cells, where it exerts its toxic effect via reaction with transition metals bound to critical cellular sites. The Cu,Zn-SOD did not significantly increase or decrease the cytotoxicity of SNAP or DEA/NO (sources of NO$^-$ but not O$_2^-$), nor did catalase protect HepG2 cells against the cytotoxicity of SNAP or DEA/NO (Fig. 10), which suggests no significant role for NO$^-$ in the observed cytotoxic effects of SIN-1 at the concentrations utilized (e.g. 0.316 or 1 mM) and under these reaction conditions. A role for NO$^-$ in SIN-1 toxicity may occur under different conditions or concentrations, e.g. in the potent toxic effects of higher concentrations of SIN-1 (e.g. 3 mM), found in the absence of SOD (Fig. 1D).

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