Cytotoxicity of nitric oxide in Fu5 rat hepatoma cells: evidence for co-operative action with hydrogen peroxide

Iosif IOANNIDIS and Herbert de GROOT*
Institut für Physiologische Chemie, Universitätsklinikum, Hufelandstrasse 55, 45122 Essen, Federal Republic of Germany

The NO-releasing compounds 3-morpholinosydnonimine-*N*-ethylcarbamide (SIN-1), sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) mediated a rapid loss of viability of Fu5 rat hepatoma cells. SIN-1 in addition to NO also released the superoxide anion radical ($O_2^{-\cdot}$). Its cytotoxicity, however, was not affected by superoxide dismutase. In contrast, the H_2O_2-converting enzyme catalase significantly, but not completely, diminished cell damage, indicating participation of H_2O_2 in the tumoricidal activity of SIN-1. Glucose oxidase (5 m-units/ml), producing similar amounts of H_2O_2 to 5 mM SIN-1, had no effect on cell viability. When 5 m-units/ml glucose oxidase was added to incubations with 5 mM SNP, which alone initiated cell injury of about 40%, cell damage was significantly increased up to 95%. Similar results were observed with 1 mM SNAP and 20 m-units/ml xanthine oxidase, which mediated cytotoxicity of about 90% when both compounds were added together, compared with 35% and 55% cell injury, respectively, induced by the single compounds. The results indicate that a co-operative action with H_2O_2 enhances the tumoricidal activity of NO in Fu5 cells. No evidence for an interplay of NO with $O_2^{-\cdot}$ in cytotoxicity, e.g. via the peroxynitrite anion ($ONOO^-$), was found.

INTRODUCTION

NO conveys the cytotoxicity of activated macrophages against various pathogens, including tumour cells (Hibbs et al., 1988; Marletta et al., 1988; Nathan, 1992). One of the main targets of NO are thiol groups of proteins, frequently found in cell membranes. These reduced thiols react with NO to yield *S*-nitrosothiols (Ignarro et al., 1981). Due to its lipophilicity, or in connection with thiols, NO can pass easily into the intracellular area and affect cell metabolism. NO inhibits protein synthesis, the mitochondrial respiratory chain, DNA synthesis and aconitase, and may induce loss of intracellular iron (Hibbs et al., 1988; Marletta et al., 1988; Keller and Keist, 1989).

NO reacts rapidly with oxygen, whereby NO's half-life is limited to a few seconds. In addition, NO reacts readily with the superoxide anion radical ($O_2^{-\cdot}$) yielding the peroxynitrite anion ($ONOO^-$), which decays, once protonated, to the very reactive hydroxyl radical ($^\cdot OH$) and to nitrogen dioxide ($^\cdot NO_2$) (Blough and Zafiriou, 1985; Beckman et al., 1990). Formation of $ONOO^-$ has thus been suggested to increase cytotoxicity of both NO and $O_2^{-\cdot}$ by direct oxidation of tissue thiols (Radi et al., 1991). It has also been demonstrated that NO and $O_2^{-\cdot}$ can initiate lipid peroxidation in human low-density lipoproteins, possibly via the $ONOO^-$ pathway (Darley-Usmar et al., 1992). An interplay of NO and $O_2^{-\cdot}$ and thereby an increase in reactivity is suggested by a severalfold increase in luminol chemiluminescence, monitored in Kupffer cells as well as in chemical systems (Wang et al., 1991, 1993; Radi et al., 1993), and by a markedly enhanced decrease in α-tocopherol in the presence of both free radicals (de Groot et al., 1993).

In the present work we have studied injury of Fu5 rat hepatoma cells mediated by NO-releasing compounds and enzymes generating $O_2^{-\cdot}$ and H_2O_2. No evidence for an interaction of NO with $O_2^{-\cdot}$ in cytotoxicity is presented. However, we demonstrate that NO and H_2O_2 act co-operatively, significantly intensifying their tumoricidal activity.

EXPERIMENTAL

Materials

Catalase (from bovine liver; 65000 units/mg), glucose oxidase (GOD; grade II), nitrate reductase, peroxidase (grade I), superoxide dismutase (SOD; 5000 units/mg) and xanthine oxidase (XO) were purchased from Boehringer (Mannheim, Germany). Catalase (from human erythrocytes; 160000 units/mg) was from ICN (Meckenheim, Germany). Catalase (from *Aspergillus niger*; 7080 units/mg), cytochrome *c* (type III), haemoglobin (bovine) and sodium nitroprusside (SNP) were from Sigma (Deisenhofen, Germany). 3-Morpholinosydnonimine-*N*-ethylcarbamide (SIN-1) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) were kindly given by Cassella A.G. (Frankfurt, Germany) and by Dr. G. Kojdla (University of Düsseldorf, Germany) respectively

Cell line and incubations

The Fu5 rat hepatoma cell line (Schneider and Weiss, 1971) was a gift from Dr. N. Sies (Berkeley, CA, U.S.A.). The cells were maintained and harvested with small modifications as described elsewhere (Hugo-Wissemann et al., 1991). For the experiments cells were seeded in Dulbecco's Modified Eagle Medium/Nutrient Mix F12 (1:1; Gibco, Eggenstein, Germany), supplemented with L-glutamine (2 mM), gentamycin (50 μg/ml) and 10% fetal-calf serum (Biospa, Wedel, Germany), in 4-well culture plates (2 cm^2; Falcon, Heidelberg, Germany) containing 400 μl of medium/well (7.5×10^4 cells/cm^2). After culture at 37 °C (air/CO_2, 19:1) for 24 h, cell number was about $(1–2) \times 10^5$ cells/cm^2. Experiments were started by exposing the cells to SIN-1 (20 μM–20 mM), SNP (1–20 mM), SNAP (1 mM), XO (1–50 m-units/ml), GOD (0.2–20 m-units/ml), SOD (5–250 units/ml) and/or bovine liver catalase (1.3–500 units/ml). In those experiments where the enzymes XO and GOD were added, 1 mM hypoxanthine and 10 mM glucose, respectively, were included in the incubations. Stock solutions of all compounds were prepared in Hanks

Abbreviations used: GOD, glucose oxidase; LDH_i, intracellular lactate dehydrogenase; SIN-1, 3-morpholinosydnonimine-*N*-ethylcarbamide; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside; SOD, superoxide dismutase; XO, xanthine oxidase; Hb, haemoglobin.

* To whom correspondence should be addressed.

balanced salt solution (Gibco), except for SNAP, which was dissolved in 100% ethanol. The effect of bovine liver catalase on SIN-1 and GOD-induced cytotoxicity was also studied in an atmosphere of O_2/CO_2 (19:1). Likewise, additional experiments were performed with human erythrocyte catalase (50 units/ml), *Aspergillus niger* catalase (50 units/ml), boiled bovine liver catalase (10 min at 95 °C, initial activity 2×10^3 units/ml) and bovine liver catalase (50 units/ml) dialysed against Hanks balanced salt solution for 12 h at 4 °C.

Cell viability

Cell viability was determined by measuring intracellular lactate dehydrogenase (LDH_i) activity. Cells were washed with Hanks balanced salt solution to remove extracellular lactate dehydrogenase. Attached cells were solubilized with 400 μl of Triton X-100 (1% in Hanks balanced salt solution). Lactate dehydrogenase activity was monitored spectrophotometrically at 334 nm by an enzymatic assay (Bergmeyer and Bernt, 1974). Cell damage was estimated from the loss of LDH_i activity. In addition, viability of cells was examined by counting the number of cells taking up the vital dye Trypan Blue (Hugo-Wissemann et al., 1991). LDH_i activity and Trypan Blue uptake gave the same results for cell viability; therefore only one parameter (LDH_i activity) will be shown.

Measurement of $O_2^{-\cdot}$ and H_2O_2

$O_2^{-\cdot}$ release from SIN-1, SNP and SNAP was determined by monitoring SOD-inhibitable reduction of cytochrome *c* at 550 nm at 37 °C (Fridovich, 1970). After the addition of 80 μM cytochrome *c* and 7 μM oxyhaemoglobin (oxyHb) to 1 ml of Hanks balanced salt solution, SIN-1, SNP or SNAP was added to start the measurements. The absorption coefficient $\epsilon_{550} = 21\ mM^{-1} \cdot cm^{-1}$ was used to quantify $O_2^{-\cdot}$ formation (Massey, 1959).

The formation of H_2O_2 from SIN-1 and SNP was studied spectrophotometrically at 546 nm at 37 °C by the peroxidase-catalysed conversion of 4-aminophenazone to a pink-coloured substance (Fossati et al., 1980). Measurements were started after adding SIN-1 or SNP to 1 ml of reagent. This reagent consisted of 10 parts of solution A [4.2 mM 3,5-dichloro-2-hydroxybenzenesulphonic acid, dissolved in Mops buffer (50 mM Mops/50 mM KCl, pH 7.4)], 1 part of solution B (33 mM 4-aminophenazone) and 2.3 units/ml peroxidase (final activity).

Measurement of NO, nitrite and nitrate

NO formation from SIN-1, SNP and SNAP was determined by the spectrophotometric measurement of the conversion of oxyHb into methaemoglobin (metHb) at 578 nm versus 592 nm at 37 °C (Feelisch and Noack, 1987a). SIN-1, SNP or SNAP was added to 1 ml of Hanks balanced salt solution in the presence of 7 μM oxyHb, 200 units/ml SOD and 5.2×10^3 units/ml catalase. The absorption coefficient $\epsilon_{578} = 12.1\ mM^{-1} \cdot cm^{-1}$ was used in calculations (Wang et al., 1992).

In the presence of Fu5 cells, NO release by SIN-1 and SNP was estimated from the amounts of nitrite (NO_2^-) and nitrate (NO_3^-) anions formed. NO_2^- formation was monitored by a colorimetric assay based on the Griess reaction (Green et al., 1982). NO_3^- was estimated by first converting NO_3^- into NO_2^- by nitrate reductase (Beutler et al., 1986) and subsequently determining NO_2^- with the Griess reaction. In contrast with the haemoglobin method, pink-coloured culture medium did not interfere with these measurements.

RESULTS

Release of NO, NO_2^-, NO_3^-, $O_2^{-\cdot}$ and H_2O_2 from nitrovasodilators

SIN-1

After a lag period of about 10 min, NO and $O_2^{-\cdot}$ (H_2O_2) were released by SIN-1 at slightly different rates. At 1 mM SIN-1, NO was generated at a rate of 5.42 μM/min, and at 2 mM SIN-1 the rate of NO release increased to 7.35 μM/min. At 5 mM, SIN-1 produced 9.90 μM NO/min (Table 1). Thus NO formation dependent on SIN-1 concentration is non-linear, in accordance with previous reports (Feelisch and Noack, 1987a,b). The rate of $O_2^{-\cdot}$ formation from SIN-1 also increased with increasing concentration of the nitrovasodilator (Table 1); the rates determined, however, remained below the respective rates of NO release. Determination of $O_2^{-\cdot}$ required the presence of oxyHb in the assay mixture. In the absence of oxyHb no $O_2^{-\cdot}$ formation could be detected, possibly owing to the rapid reaction of NO with $O_2^{-\cdot}$ (Saran and Bors, 1989). Higher rates of $O_2^{-\cdot}$ formation by SIN-1 were indicated by determining H_2O_2, taking into account a 2:1 stoichiometry between $O_2^{-\cdot}$ and H_2O_2 (Table 1).

The values of NO formation given above were determined in the absence of cells. In the presence of cells, NO formation from SIN-1 was estimated from the amounts of NO_2^- and NO_3^- formed. At 5 mM SIN-1, the rate of NO_2^- release was 2.65 μM/min and that of NO_3^- release 1.72 μM/min. Thus even the sum of both rates was significantly below the rate of NO formation obtained in the absence of cells. Due to reactions of NO with cellular constituents, the detection of NO_2^- and NO_3^- may underestimate the true rate of NO formation.

SNP

In the cell-free system, SNP released only small amounts of NO (Table 1). In the presence of cells, NO formation by SNP appeared to be somewhat increased, since the rates of NO_2^- release (1.44 μM/min) and of NO_3^- release (0.94 μM/min) were comparable with the rate of NO release in the absence of cells (1.49 μM/min) at 20 mM SNP. An increased rate of NO release

Table 1 Formation of NO, $O_2^{-\cdot}$ and H_2O_2 from nitrovasodilators

NO, $O_2^{-\cdot}$ and H_2O_2 were determined spectrophotometrically: NO by monitoring NO-induced conversion of oxyHb into metHb at 578 nm, $O_2^{-\cdot}$ by observing the reduction of cytochrome *c* at 550 nm, and H_2O_2 by measurement of the A_{546} by using the peroxidase test. Reactions were performed at 37 °C. Assay conditions are described in detail in the Experimental section. Each value represents the mean ± S.E.M. from 6–9 experiments. Abbreviations: n.d., not detectable; *a*, not determined.

		Release (μM/min)		
Nitrovasodilator	(mM)	NO	$O_2^{-\cdot}$	H_2O_2
SIN-1	1	5.42 ± 0.40	0.79 ± 0.14	0.70 ± 0.10
	2	7.35 ± 1.24	3.97 ± 0.20	3.01 ± 0.18
	5	9.90 ± 1.16	6.68 ± 0.49	5.64 ± 0.82
SNP	1	0.22 ± 0.05	n.d.	n.d.
	5	0.31 ± 0.06	n.d.	n.d.
	20	1.49 ± 0.36	n.d.	n.d.
SNAP	1	2.71 ± 0.39	n.d.	*a*

from SNP in the presence of cells would be in line with the results obtained by Ignarro et al. (1981), who demonstrated an enhancement of the reductive formation of NO from SNP by various thiols. In contrast with SIN-1, SNP did not release detectable amounts of $O_2^{-\cdot}$ or H_2O_2.

SNAP

Significant amounts of NO, comparable with the amounts of NO released by SIN-1, were also generated by SNAP (Table 1). As with SNP, no $O_2^{-\cdot}$ was released by this nitrovasodilator, in line with recent reports (Field et al., 1978; Ignarro et al., 1981).

Cytotoxicity of nitrovasodilators, XO and GOD

SIN-1

In every control presented, LDH_i activity slightly increased during the first 8 h of incubation (Figures 1–5). No significant cytotoxicity was induced up to 1 mM SIN-1; 2 mM SIN-1 caused a decrease of about 35% in LDH_i activity after 8 h, whereas nearly 75% of the cells had lost their viability in incubations with 5 mM SIN-1 (Figure 1). In incubations with even higher SIN-1 concentrations (10 and 20 mM) LDH_i activity fell to zero after 8 h and 6 h respectively (results not shown). SOD at concentrations of up to 200 units/ml had no significant effect on the cytotoxicity of 5 mM SIN-1 (Figure 2). In contrast with SOD, catalase diminished the cytotoxicity of 5 mM SIN-1 in a concentration-dependent manner. At 50 units/ml, the protective effect of catalase was almost 80% (Figure 2). At 5 units/ml catalase, cell damage was diminished by about 50%, whereas 1.3 units/ml catalase hardly affected SIN-1-mediated injury (results not shown). Catalase also protected against cytotoxicity of 20 mM SIN-1. However, in this case higher concentrations of catalase were required. At 500 units/ml, catalase diminished cell injury by about 50%, and at 50 units/ml by about 25% (results not shown). In the presence of cytochrome *c* (40 and 80 μM) cell injury by 5 mM SIN-1 was decreased by about 80% (results not shown).

SNP

SNP (1 mM) had no detectable cytotoxic effect on the Fu5 cells. Upon incubation with 20 mM SNP, about 75% of the cells had lost their viability after 8 h (Figure 3). Catalase (5 and 50 units/ml) had no effect on cell damage induced by 20 mM SNP.

XO

Cell viability was not affected in incubations with up to 5 m-units/ml XO. A strong decrease in LDH_i activity was induced by 50 m-units/ml XO. Only 15% of the cells had survived after 8 h (Figure 4). Cell mortality was not affected by addition of SOD in various concentrations up to 250 units/ml, but was completely blocked by 65 units/ml catalase (Figure 4).

GOD

GOD activities up to 5-units/ml did not significantly affect cell viability. Pronounced damage was observed in incubations with 20 m-units/ml GOD. Almost 60% of the cells had lost their viability after 8 h (results not shown). In the presence of 500 units/ml catalase, cytotoxicity of 20 m-units/ml GOD was totally inhibited. Likewise, complete protection was observed at catalase concentrations down to 1.3 units/ml (results not shown).

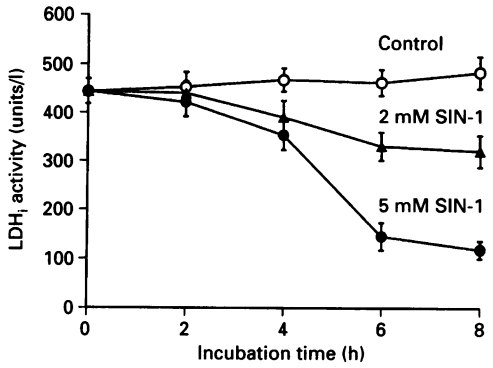

Figure 1 SIN-1-induced damage to Fu5 cells

Cells (7.5×10^4/cm^2) were incubated in 4-well culture plates at 37 °C for 24 h and then treated with various concentrations of SIN-1. At the times indicated, the LDH_i activity of the cells (expressed as units/l of incubation medium) was monitored. Data presented are means ± S.E.M. from 6 experiments.

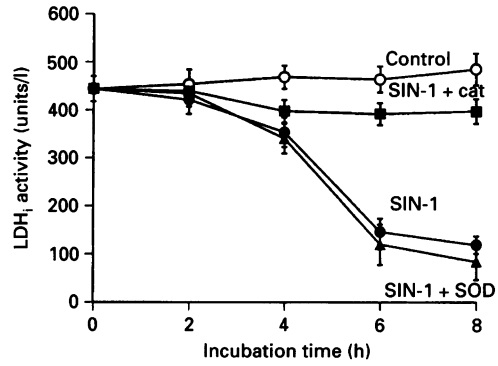

Figure 2 Effects of antioxidative enzymes on SIN-1-induced damage to Fu5 cells

Cells (7.5×10^4/cm^2) were incubated in 4-well culture plates at 37 °C for 24 h and then treated with 5 mM SIN-1; 5 units/ml SOD and 50 units/ml catalase (cat) were added immediately before the exposure to SIN-1. At the times indicated, the LDH_i activity of the cells (expressed as units/l of incubation medium) was monitored. Data presented are means ± S.E.M. from 7 experiments.

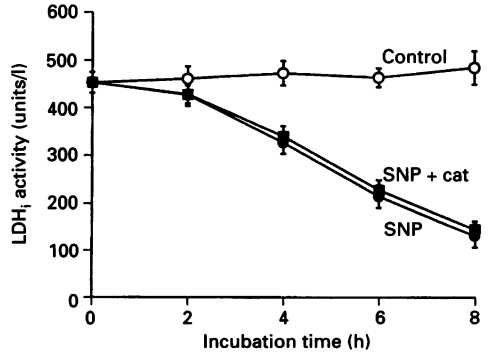

Figure 3 SNP-induced damage to Fu5 cells

Cells (7.5×10^4/cm^2) were incubated in 4-well culture plates at 37 °C for 24 h and then treated with 20 mM SNP; 50 units/ml catalase (cat) was added immediately before the exposure to SNP. At the times indicated, the LDH_i activity of the cells (expressed as units/l of incubation medium) was monitored. Data presented are means ± S.E.M. from 5 experiments.

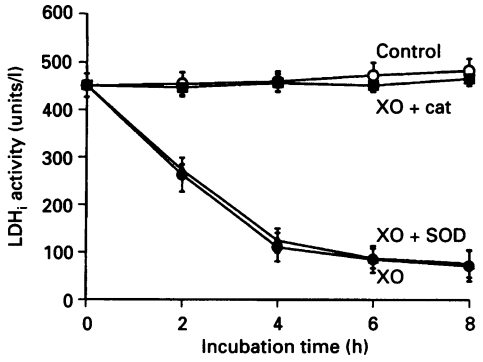

Figure 4 XO-induced damage to Fu5 cells

Cells ($7.5 \times 10^4/cm^2$) were incubated in 4-well culture plates at 37 °C for 24 h and then treated with 50 m-units/ml XO plus 1 mM hypoxanthine; 50 units/ml SOD and 65 units/ml catalase (cat) were added immediately before the exposure to XO. At the times indicated, the LDH_i activity of the cells (expressed as units/l of incubation medium) was monitored. Data presented are means ± S.E.M. from 5 experiments.

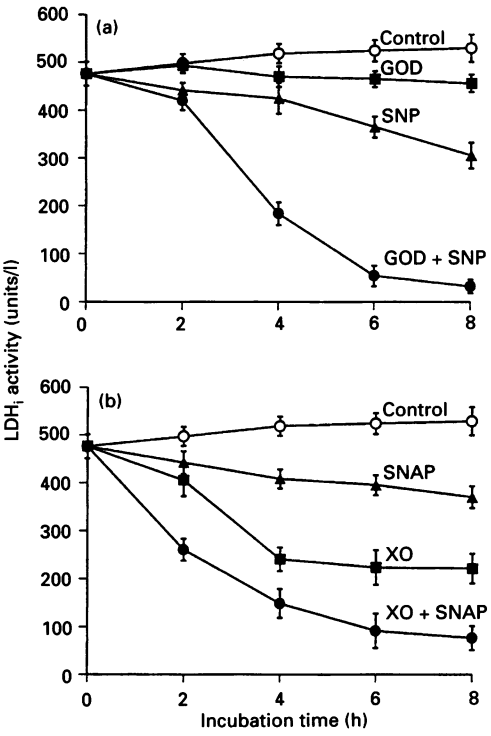

Figure 5 Toxicity in Fu5 cells after treatment with GOD–SNP (a) and XO–SNAP (b)

Cells ($7.5 \times 10^4/cm^2$) were incubated in 4-well culture plates at 37 °C for 24 h and then exposed to 5 m-units/ml GOD plus 10 mM glucose and 5 mM SNP (**a**) or 20 m-units/ml XO plus 1 mM hypoxanthine and 1 mM SNAP (**b**). At the times indicated, the LDH_i activity of the cells (expressed as units/l of incubation medium) was monitored. Data presented are means ± S.E.M. from 3–5 experiments.

GOD–SNP and XO–SNAP

In the presence of 5 m-units/ml GOD or 5 mM SNP alone, only slight losses of cell viability were observed (Figure 5a). In incubations with both compounds, however, a marked increase in cytotoxicity occurred, leading to almost complete cell damage after 6 h. Similar results were obtained with 20 m-units/ml XO and 1 mM SNAP (Figure 5b). In incubations with 1 mM SNAP or 20 m-units/ml XO alone, nearly 35% and 55% of the cells, respectively, had lost their viability after 8 h. Again, cell injury was significantly increased when both compounds were added together.

Catalase from distinct sources, O_2-saturated atmosphere

The experiments described above were performed in an air-saturated atmosphere (air/CO_2, 19:1) and bovine liver catalase was used. Incubations performed in an atmosphere of O_2/CO_2 (19:1) or with catalase from human erythrocytes, with catalase from *Aspergillus niger* or with dialysed bovine liver catalase provided basically similar results (not shown). Boiled bovine liver catalase had no protective effect.

DISCUSSION

In proportion to its concentration, the nitrovasodilator SIN-1 caused a rapid decrease in LDH_i activity in Fu5 rat hepatoma cells (Figure 1). It is known, and also confirmed here (Table 1), that NO and $O_2^{-\bullet}$ are generated during the decomposition of SIN-1 (Feelisch et al., 1989); hence some involvement of these radicals in the cytotoxic process must be supposed. However, the $O_2^{-\bullet}$ scavenger SOD did not affect SIN-1-mediated cell damage (Figure 2), suggesting that $O_2^{-\bullet}$ does not play a role in SIN-1 toxicity in Fu5 cells. In contrast, even at low concentrations catalase significantly diminished SIN-1 cytotoxicity. Likewise, cytochrome *c*, which reoxidizes $O_2^{-\bullet}$ to O_2, provided significant protection. Hence H_2O_2, but not $O_2^{-\bullet}$, appears to be involved in the cell-destructive mechanisms initiated by SIN-1.

Since catalase even at very high concentrations did not completely protect the cells from the toxic activity of SIN-1 (Figure 2), the remaining cell injury in the incubations with both SIN-1 and catalase must be due to NO alone. Thus both NO and H_2O_2 contribute to SIN-1-mediated cell injury.

Co-operative action between NO and H_2O_2 in SIN-1 toxicity is suggested by the fact that 5 mM SIN-1 and 5 m-units/ml GOD produced similar amounts of H_2O_2, but, in contrast with 5 mM SIN-1, no significant effect on cell viability was observed in incubations with 5 m-units/ml GOD (Figure 5a). Co-operation between NO and H_2O_2, leading to an enhanced mortality of Fu5 cells, is also indicated when SNP or SNAP act in combination with GOD or XO (Figures 5a and 5b).

The experiments presented here provide no evidence for a significant role of $ONOO^-$ formation in NO cytotoxicity, but demonstrate an increase in cell injury when NO interacts with H_2O_2. However, the molecular mechanism of this interaction remains unknown. It has been suggested that a reaction of NO with H_2O_2 produces hydroxyl radicals in the gas phase as well as in solution (Kanner et al., 1991). Recently, the production of singlet oxygen, another highly reactive form of oxygen with cytotoxic potential, has been demonstrated (Noronha-Dutra et al., 1993). A non-chemical interplay between NO and H_2O_2, where the molecules act on different areas in the target cell, could also lead to stronger cytotoxicity. Here, cell death would be regarded as a summation of independent reactions.

Considering the surprising effect of H_2O_2 on NO toxicity, it is noteworthy that catalase, but not SOD, could protect against the toxicity of XO, a result which has also been observed in human fibroblasts (Simon et al., 1981), rat hepatocytes (Rubin and Farber, 1984) and mammalian epithelial cells (Link and Riley, 1988).

In organisms an interaction between NO and H_2O_2 may play a key role in inflammatory reactions and in immune defence. An intensification of the anti-microbial and the anti-tumoricidal activity of macrophages is expected to result from this interaction.

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