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ENDOTHELIAL DAMAGE INDUCED BY NITRIC OXIDE: SYNERGISM WITH REACTIVE OXYGEN SPECIES

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Interactions of reactive oxygen and nitrogen species to mediate endothelial cell damage were studied in vitro. S-Nitroso-N-acetyl-DL-penicillamine (SNAP), 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1) and sodiumnitroprusside (SNP) were used as NO·-donating agents. The toxicity of SIN-1 (5 mM), which produces both O_2^{-}· and NO·, was reduced when catalase was added to remove H_2O_2 whereas superoxide dismutase had a marginal protective influence. Low doses of H_2O_2 producing enzymes added to low doses of SNAP (1 mM) or SNP (5 mM) substantially increased toxicity. Such damage was absent when catalase was present, but was still seen in the presence of superoxide dismutase. Non toxic doses of KCN (1 mM), antimycin A (1 µM), and rotenone (0.5 µM) in order to increase endogeneously produced reactive oxygen species increased toxic effects by 20 - 30 % ($p<0.05$). In our experiments we provide evidence that extracellularly produced H_2O_2 rather than O_2^{-}· enhances toxicity of NO· against endothelial cells. Likewise, endogeneous production of reactive oxygen species may increase toxicity of NO·.

Nitric oxide (NO·) is known to play an important role in biological systems. This short lived radical gas has the lowest molecular weight of any known bioactive secretory product of mammalian cells. It possesses critical functions in the control of blood pressure, neurotransmission and cytotoxicity (1). The latter effects of secreted NO· is believed to determine the efficacy of macrophages against invading organisms and tumor cells. Large quantities of NO· can be produced by these cells for long periods of time whenever they are activated by endotoxins, exotoxins, lipoteichoic acid or cytokines like γ-Interferon, tumor necrosis factor α, or interleukin 1, all of which have been shown to be important mediators in critically ill patients (2-4). Cellular targets include key enzymes in respiration, glycolysis,

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Abbreviations: CAT: catalase, GOD: glucose oxidase, ROS: reactive oxygen species, SIN-1: 3-morpholinosydno-nimine-N-ethylcarbamide, SNAP: S-Nitroso-N-acetyl-DL-penicillamine, SNP: sodium nitroprusside, SOD: superoxide dismutase, XOD: xanthine oxidase.

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regulation of iron metabolism, and DNA-repair (5-8). In addition, reactive oxygen species (ROS) have been shown to participate in cellular damage in sepsis or reperfusion injury after organ transplantation or infarction (9, 4). With its radical nature NO· can rapidly react with oxygen and its derivatives to form other potentially toxic radicals. For example, peroxynitrite (ONOO·-) may rapidly be formed in aqueous solutions by an interaction between O_2·- and NO·. Peroxynitrite decays, once protonated, to the very reactive hydroxyl radical OH· and nitrogen dioxide radical NO_2· (1). Whether toxic effects of NO· are dependent on the presence of reactive oxygen species at all is not known. Respiring cells endogenously produce oxygen radicals such as O_2·- and H_2O_2 that can react with NO· inside the cell. The sources of these intrinsic radicals are dominated by leakage from mitochondrial electron transport chains which can make up to 4% of the total oxygen consumption (10). To clarify the role of extracellularly and intracellularly produced reactive oxygen species on toxicity induced by NO·, we exposed endothelial cells to these radicals together with enzymes to remove superoxide or hydrogen peroxide.

METHODS

Cell culture

Sinusoidal rat liver endothelial cells were isolated and treated as described previously (11): cells were cultured in RPMI medium (Boehringer) at 37°C (21% O_2 / 5% CO_2), supplemented with fetal calf serum (20%, Sigma), glutamine (2 mM, Gibco, Eggenstein, Germany), gentamicin (100 µg/ml, Sigma), dexamethasone (1 µmol/L, Merck), amphotericin B (5 µg/ml), and grown on fibronectin (Sigma) coated culture flasks (Falcon, Heidelberg, Germany).

Cytotoxicity assay

During the experimental incubation cells in confluency were used. On the day of the experiment, cells were washed and kept in Krebs Henseleit buffer (pH 7.4, supplemented with 10 mM glucose and 20 mM HEPES). Experiments were started by exposing cells to S-nitroso-N-acetyl-DL-penicillamine (SNAP; kindly provided by Dr. H.G. Korth, Institute of organic chemistry, University of Essen, Germany), sodium nitroprusside (SNP; Merck, Darmstadt, Germany), 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1; Casella AG, Frankfurt, Germany), KCN (Sigma, Deisenhofen, Germany), rotenone (Sigma), and antimycin A (Sigma) as indicated. When xanthine oxidase (XOD, Boehringer, Mannheim, Germany) or glucose oxidase (GOD, grade II, Boehringer) were used, hypoxanthine (1 mM) and glucose (10 mM) were added to saturate enzymatic fomation of reactive oxygen species, respectively. Superoxide dismutase (SOD; 5000 U/mg, Boehringer) and catalase (CAT; from bovine liver; 65.000 U/mg, Boehringer) were used to enzymatically remove O_2·- and H_2O_2, respectively. Samples of 20 µl were aspirated from the incubation medium at predefined time points. Measurements of lactate dehydrogenase activity (LDH, kit tox-7, Sigma) released from endothelial cells and trypan blue exclusion were applied to indicate viability (11).

Statistical analysis

To compare the measured results in different groups descriptive statistical parameters (mean and SD) were calculated and differences were tested for significance using an ANOVA. The Student-Newman-Keuls test was used to compare individual results with the control. P values < 0.01 were considered to be significanct.

RESULTS

Endothelial toxicity of NO·-donating substances

SNP, SNAP, and SIN-1 exhibited a concentration dependent cytotoxicity against endothelial cells (Fig. 1). A detectable increase in LDH-activity in the supernatant was not apparent until 2 hrs of incubation. While a significant toxicity of SNP was seen at a concentration of 10 —mmol/L, SIN-1 and SNAP showed a similar cytotoxic potential at much lower concentrations (2 mmol/L and 1mmol/L, respectively). Ethanol as well as denitrosylated NO· donors (12 hrs in solution containing 1 mM cystein) did not cause any cytotoxicity.

Both, SOD (50 U/ml) and CAT (50 U/ml) had no influence on the toxicity of 20 mmol/L SNP and 5 mmol/L SNAP. After 6 hrs of incubation, cellular damage caused by SIN-1 (5 mmol/l) was reduced from 79 ± 7 % to 26 ± 5 % ($p<0.0001$), when CAT (50 U/ml) was added. In contrast, SOD (50 U/ml) only marginally influenced the damaging effect of SIN-1 (72 ± 5 %; Fig. 2).

Influence of enzymatically produced ROS on endothelial viability

O_2-· was produced enzymatically by the hypoxanthine/xanthine oxidase reaction. XOD caused a concentration dependent endothelial cytotoxicity which was apparent at XOD-activities above 5 mU/ml (18 ± 6 %; 6 h of incubation). At the highest concentrations used (20 mU/ml), toxicity increased to 69 ± 8 % after 6 h of incubation. O_2-· spontaneously dismutates to H_2O_2, which was accelerated by SOD. At XOD activities of 20 mU/ml, SOD had no

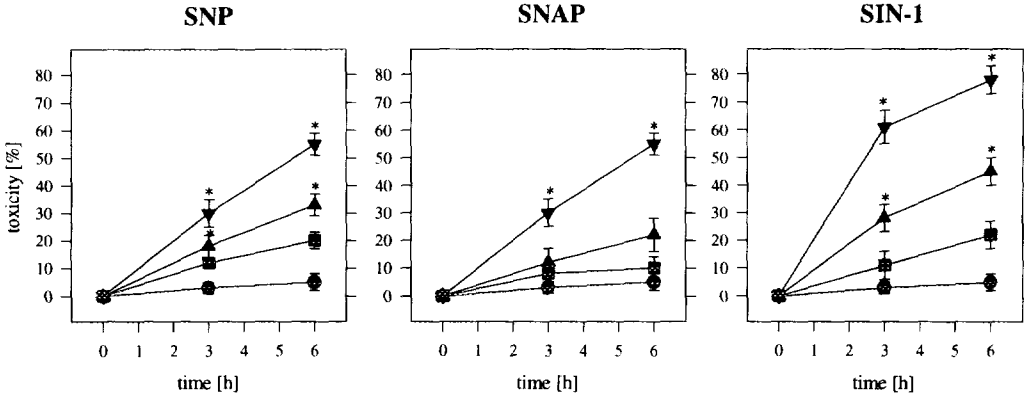

Fig. 1.
Toxicity against endothelial monolayers caused by SNP, SNAP and SIN-1: SNP was used in concentrations of 5 mM (■), 10 mM (▲), and 20 mM (▼). SNAP and SIN-1 were added in concentrations of 1 mM (■), 2 mM (▲) and 5 mM (▼). Symbols represent means and SEM of experiments performed in duplicate in 3 independent series. Controls incubated in buffer (●). (*) significant changes in comparison to the controls.

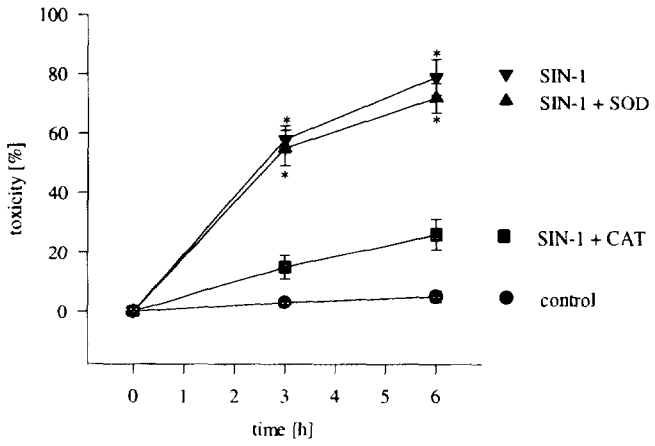

Fig. 2.
Protection from SIN-1 toxicity by Catalase: SIN-1 (5 mM) was added to endothelial cells for 6 h together with SOD (50 U/ml) or CAT (50 U/ml). Shown are mean and SEM values of 3 independent experiments.
(*) significant differences compared to controls.

influence on LDH defined viability, whereas CAT reduced the damage to 14 ± 6 % ($p<0.0001$). The activities of GOD in glucose containing buffer at which cellular damage was seen were similar (5 mU/ml; 16 ± 5 %) compared to XOD and increased to 72 ± 8 % at 6 h of incubation when 20 mU/ml were used ($p<0.0001$). CAT (50 U/ml) almost completely abrogated the cytotoxic effects of GOD (20 mU/ml).

Combined action of reactive oxygen and nitrogen species generated outside endothelial cells

In Fig. 3 results of experiments carried out with low concentrations of both SNAP (1 mM, 22 ± 6 %) and XOD (5 mU/ml, 18 ± 5 %) are shown. The cytotoxicity produced by SNAP plus XOD increased compared to their action when used alone (65 ± 5 %, $p<0.0001$). Inhibition of these damaging combinations was marginal when SOD was added to reduce O_2-·. In contrast to these findings, CAT showed a significant reduction in cytotoxicity of 5 mU/ml XOD plus 1 mM SNAP (23 ± 3; $p<0,0001$), again indicating that H_2O_2 rather than O_2- potentiates the damaging effect of NO·. Using GOD (5 mU/ml; 16 ± 5% toxicity of sole action) and SNAP (1 mM) in combination similar results were found: endothelial viability decreased to 34 ± 8 % ($p<0.001$) after 6 h. In this series, a protective effect of CAT could also be observed (50 U/ml; 21 ± 4 %).

Influence of antimycin A, KCN and rotenone on toxicity induced by SNAP and SNP

Antimycin A (1 μM), KCN (1mM), and rotenone (0.5 μM) were used to inhibit the respiratory chain at different levels. In these concentrations no cellular damage had been

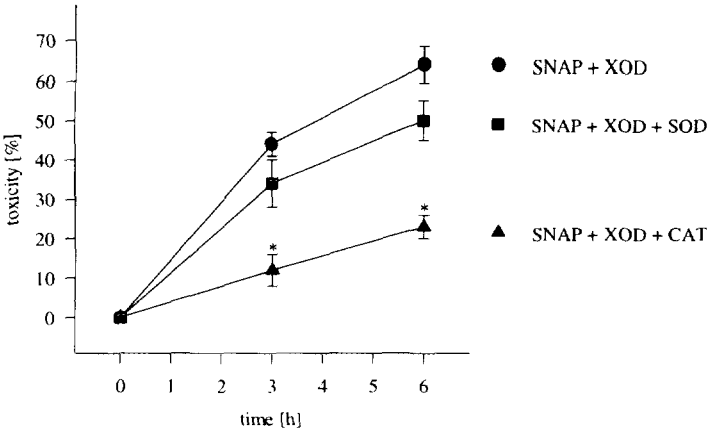

Fig. 3.
Additive actions of hydrogen peroxide with SNAP in the killing of endothelial cells: Over a period of 6 h SNAP (1 mM) was coincubated with XOD (5 U/ml). SOD (50 U/ml) or CAT (50 U/ml) were added as indicated. The graph represents measured values (mean and S.E.M.) of 3 independent experiments.
(*) see Fig. 1.

observed in controls during the incubation period of 6 hrs. Regardless of the NO·-donor used, antimycin A, rotenone and KCN had no protective effect on NO· induced cell death (Fig. 4), but accelerated the toxic effect mediated by both SNAP and SNP, respectively.

DISCUSSION

Our results show that apart from their regulatory functions both oxygen and nitrogen derived radical species can cause cytotoxicity (12). Biological activity of SNAP, SNP, and

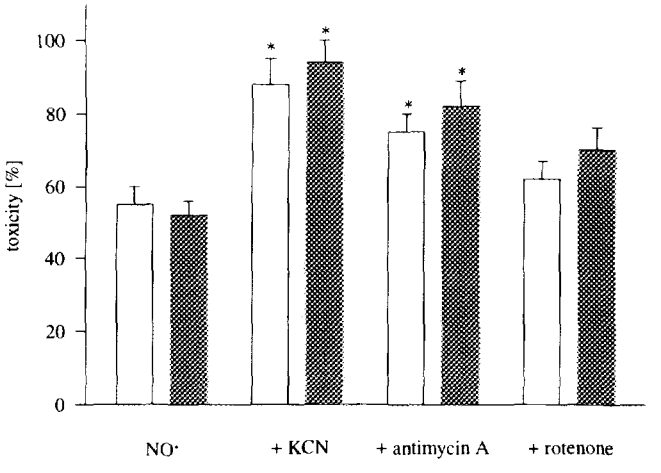

Fig. 4.
Effect of SNAP and SNP on viability of KCN (1 mM), antimycin A (1 μM) or rotenone (0.5 μM) treated endothelial cells after 6 h. White bars represent mean ± S.E.M. of SNAP (5 mM) and hatched bars represent incubations with SNP (20 mM) in 4 independent experiments.
(*) significant difference compared to NO· -treated group.

SIN-1 is known to be mediated by their release of nitric oxide (13). In our experiments all NO·-donating agents caused endothelial damage (Fig. 1). These results are in agreement with reports about the cytotoxic potential of NO· against endothelial cells (14, 15).

It is well established, that reactive oxygen species have damaging effects against cellular proteins, DNA and lipids eventually leading to cell death (16-18). Confirmation of measurable toxicity was found by the experiments using xanthine oxidase and glucose oxidase with saturated substrate concentrations. Using SOD to dismutate O_2-· which is primarily produced by XOD we could demonstrate that H_2O_2 rather than O_2-· damages endothelial cells which is in agreement with data reported for other cell types (19-22).

Apart from the direct action of each species against different cellular targets, the effects of combined radical actions are ill defined. For example, peroxynitrite has been proposed to be generated by an interaction of nitric oxide with superoxide leading to even more cellular damage (23). Recently, hydrogen peroxide rather than superoxide has been shown to augment the cytotoxic potential of NO· against malignant hepatoma cells (19). The molecular mechanism, however, of this synergism remains speculative. It has been suggested that highly reactive hydroxyl radicals or singlet oxygen is generated by an interaction between H_2O_2 and NO· (24, 25). Alternatively, peroxynitrite may have been formed provided that nitrosonium cation (NO^+) is present to react with H_2O_2 (26). In contrast to an increase in cytotoxicity, nitric oxide has also been suggested to protect against cellular damage of hydrogen peroxide in rodent lung fibroblasts and mesencephalic dopaminergic cells (27). Whether these data reflect cell specific changes in susceptibility, higher doses of XOD (40 mU/ml), or a different source of NO·-donating substances ("NONOates") is still open to question.

Toxicity of SIN-1, which produces both O_2-· and NO·, was not reduced by superoxide dismutase but by catalase, suggesting that O_2-· is a minor contributor to NO· induced damage. An increase in NO· toxicity was only apparent when hydrogen peroxide, dismutated from O_2-·, was present. Under these conditions SOD again was not protective. The data reported here support a cooperative action of nitric oxide with hydrogen peroxide. This interpretation leads us to hypothesize, that the amount of coproduced reactive oxygen and nitrogen species in activated leukocytes may regulate their cytotoxic potentials, whereas separated production of each reactive species may serve regulatory functions.

Reactive oxygen species like superoxide or hydrogen peroxide are produced inside respiring cells. Toxicity of NO· may therefore be modulated by an intracellular interaction with O_2-· or H_2O_2. Inhibitors of the respiratory chain are known to increase O_2-· production as molecular oxygen is not reduced in the appropriate way (28, 29). All blocking agents applied increased toxicity of NO·-donors. This additional damage could have been caused by interactions that were seen in extracellular combinations of hydrogen peroxide with nitric

oxide. Alternatively, the substances used could have caused sublethal damage in part due to loss of ATP. Endothelial cells may need energy from the respiratory chain to detoxify the ubiquitous reaction products of nitric oxide and therefore, the measured toxicity may represent an additive effect occuring independently of ATP depletion.

In summary, we believe that the toxicitiy of NO· is primarily determined in the presence of hydrogen peroxide rather than superoxide. This synergism may have important implications for the understanding of the pathophysiology of sepsis, reperfusion injury and shock. Locally dysregulated production of either species (e.g. after the exposure of cytokines) may be fatal when produced together, but regulate normal cellular functions when acting solely. A second possible explanation may arise from a regulated coproduction when cytotoxicity is required. To separate these functions further studies are needed.

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