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Inhibitory and enhancing effects of NO on H_2O_2 toxicity: Dependence on the concentrations of NO and H_2O_2

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Abstract

Nitric oxide (NO) has been shown to both enhance hydrogen peroxide (H_2O_2) toxicity and protect cells against H_2O_2 toxicity. In order to resolve this apparent contradiction, we here studied the effects of NO on H_2O_2 toxicity in cultured liver endothelial cells over a wide range of NO and H_2O_2 concentrations. NO was generated by spermine NONOate (SpNO, 0.001–1 mM), H_2O_2 was generated continuously by glucose/glucose oxidase (GOD, 20–300 U/l), or added as a bolus (200 μM). SpNO concentrations between 0.01 and 0.1 mM provided protection against H_2O_2-induced cell death. SpNO concentrations >0.1 mM were injurious with low H_2O_2 concentrations, but protective at high H_2O_2 concentrations. Protection appeared to be mainly due to inhibition of lipid peroxidation, for which SpNO concentrations as low as 0.01 mM were sufficient. SpNO in high concentration (1 mM) consistently raised H_2O_2 steady-state levels in line with inhibition of H_2O_2 degradation. Thus, the overall effect of NO on H_2O_2 toxicity can be switched within the same cellular model, with protection being predominant at low NO and high H_2O_2 levels and enhancement being predominant with high NO and low H_2O_2 levels.

Keywords: *Nitrogen monoxide, nitric oxide, hydrogen peroxide, catalase, glutathione peroxidase, lipid peroxidation*

Abbreviations: *8-Br-cGMP, 8-bromoguanosine-3′,5′-cGMP; cGMP, cyclic guanosine monophosphate; GOD, glucose oxidase; H_2O_2, hydrogen peroxide; KH buffer, Krebs-Henseleit buffer; LDH, lactate dehydrogenase; NO, nitric oxide; NO_X, reactive nitrogen oxide species; PKG, cGMP-dependent protein kinase; SpNO, spermine NONOate; TBARS, thiobarbituric acid - reactive substances*

Introduction

It is well known that nitric oxide (NO) can elicit cell and tissue injury [1–5]. Beside NO itself, oxidation products of NO such as NO_2 or N_2O_3 and/or an interplay with reactive oxygen species (e.g. $O_2^{\cdot-}$, H_2O_2) contribute to injury [3,6,7]. As one of the interplays, co-operation of NO and hydrogen peroxide (H_2O_2) in their cytotoxicity against different cell types including rat liver endothelial cells, HepG2 cells, murine lymphoma cells, rabbit gastric mucosal cells, embryonic chick cardiomyocytes and a human epithelial ovarian cancer cell line has been described by several groups [7–13]. In recent experiments with isolated rat liver endothelial cells and hepatocytes, we have shown that NO inhibits H_2O_2 degradation by action on both H_2O_2-degrading pathways, catalase and the glutathione peroxidase pathway, and that this inhibition of H_2O_2 degradation can account for the co-operative action of NO and H_2O_2 (manuscript submitted for publication). Catalase was inhibited by NO itself, glutathione peroxidase by reactive nitrogen oxide species (NO_X), and the relative contribution of both pathways depended on both, the cell type and the H_2O_2 levels applied.

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Besides its injurious actions, NO has also been described to be cytoprotective in numerous *in vivo* and *in vitro* studies [2,3,14–19]. Along this line, protective effects of NO against H_2O_2 toxicity have also been reported in endothelial cells [20–23]. Although the differences in the effects observed—i.e. co-operative cytotoxic effects vs. cytoprotection—might be dependent on the cell type employed, they might also be dependent on experimental conditions such as concentrations of the reactive species applied etc. As comparative studies have not yet been done, we here set out to assess whether the occurrence of either enhancing or inhibitory effects of NO on H_2O_2 toxicity is dependent on NO and/or H_2O_2 concentrations.

Materials and methods

Materials

Horseradish peroxidase (grade I) and glucose oxidase (GOD, from *Aspergillus niger*) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Scopoletin (7-hydroxy-6-methoxy-2H-l-benzopyran-2-one), 8-bromoguanosine-3′,5′-cGMP (8-Br-cGMP), KT5823, α-tocopherol and hydrogen peroxide solution were obtained from Sigma-Aldrich (Taufkirchen, Germany). Spermine NONOate (SpNO) was from Situs (Düsseldorf, Germany), RPMI 1640 medium from Gibco (Karlsruhe, Germany) and C11-BODIPY$^{®581/591}$ (4,4-difluoro-5-(4-phenyl-l,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid) from Molecular Probes (Leiden, The Netherlands). All other chemicals used were purchased from Merck (Darmstadt, Germany).

Cell isolation and culture

A rat liver endothelial cell line, derived from the liver of a male Wistar rat, was used for the experiments. The cells had been isolated and characterized as described previously [24]. Cells were cultured in RPMI 1640 medium supplemented with fetal calf serum (20%), L-glutamine (2 mM), penicillin/streptomycin (50 U/ml and 50 μg/ml, respectively) and dexamethasone (1 μM). Subcultures were obtained by trypsinization (split ratio 1:3), and cells were used for experiments on day 6 or 7 after subcultivation.

Incubation procedure

At the beginning of the experiments, cells in 12.5 cm^2 culture flasks (BD Biosciences, Heidelberg, Germany) were washed three times with Hanks' balanced salt solution and then covered with 2.5 ml of modified Krebs–Henseleit buffer (KH: NaCl 115 mmol/l; $NaHCO_3$ 25 mmol/l; KCl 5.9 mmol/l; $MgCl_2$ 1.2 mmol/l; NaH_2PO_4 1.2 mmol/l; Na_2SO_4 1.2 mmol/l; $CaCl_2$ 2.5 mmol/l, Hepes 20 mmol/l; pH 7.4, supplemented with 10 mM glucose). All experiments were performed at 37°C. The NO donor SpNO was added to the incubation solution 15 min prior to addition of H_2O_2 or of GOD. Alternatively, cells were preincubated for 30 min with 8-Br-cGMP or KT5823; solvent controls were included. In other experiments, cells were preincubated with α-tocopherol as described by Martin et al. [25]. Briefly, cells were incubated for 20 h in cell culture medium containing α-tocopherol (added from a stock solution, 10 mg/ml in ethanol) in a final concentration of 60 μM; α-tocopherol was not added to the solutions during the experiments.

Assessment of cell injury

Lactate dehydrogenase (LDH) release was used to indicate occurrence of lethal cell injury [26].

Determination of H_2O_2 steady-state levels

H_2O_2 concentrations in the incubation medium were determined fluorimetrically by monitoring the horseradish peroxidase-dependent oxidation of scopoletin, using the assay conditions described previously (Ref. [27] for low H_2O_2 concentrations, Ref. [28] for higher H_2O_2 concentrations).

Determination of nitric oxide steady-state levels

NO concentrations in cell culture wells were determined with an electrochemical probe (ISO-NO electrochemical probe from World Precision Instruments, Berlin, Germany) as described in Ref. [29].

Determination of lipid peroxidation

Lipid peroxidation was assessed by the determination of thiobarbituric acid-reactive substances (TBARS) and by following the oxidation of the fluorescent reporter molecule C11-BODIPY$^{®581/591}$. TBARS were determined in the supernatant incubation solution after various incubation times as described previously [30]. In a second series, endothelial cells cultured on 12-well plates were loaded with the fluorescent, oxidation-sensitive fatty acid analogue C11-BODIPY$^{®581/591}$ (10 μM in KH buffer, 60 min loading at 37°C) [31,32], washed and then incubated at 37°C in dye-free, bicarbonate-free, Hepes-buffered modified KH buffer (supplemented with 10 mM D-glucose) in a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany). To part of the incubations, SpNO was added in a final concentration of 0.1 mM or 10 μM. Fluorescence was recorded at $\lambda_{exc} = 485$ nm, $\lambda_{em} = 520$ nm and $\lambda_{exc} = 560$ nm, $\lambda_{em} = 590$ nm in 60 s intervals using the top reading mode. After 15 min, GOD was added

in a final activity of 300 U/l, and fluorescence recordings were continued for 3 h. Values are given as a ratio of the intensity of the green fluorescence of the oxidized indicator ($\lambda_{exc} = 485$ nm, $\lambda_{em} = 520$ nm) to the intensity of the red fluorescence of the non-oxidized indicator ($\lambda_{exc} = 560$ nm, $\lambda_{em} = 590$ nm).

Statistics

All experiments were performed in duplicate and repeated 3–11 times. Data are expressed as means ± SD. Data obtained from two groups were compared by means of Student's *t*-test and comparisons among multiple groups were performed using an analysis of variance with Student-Newman-Keuls, Dunnett or Bonferroni post hoc comparisons, as appropriate. A p value of < 0.05 was considered significant.

Results

Cytotoxicity of hydrogen peroxide

When confluent cultures of liver endothelial cells were incubated in KH buffer with the hydrogen peroxide-generating system glucose (10 mM)/GOD, a GOD activity of 20 U/l did not cause any injury (Figure 1A), 50 U/l caused slight injury (Figure 1B), and higher GOD activities, i.e. 150 U/l (LDH release 43 ± 38% after 3 h) or 300 U/l (Figure 1C), led to a more pronounced and more rapid loss of viability.

When H_2O_2 was not generated continuously by the enzymatic system but authentic H_2O_2 was added as a bolus (200 μM), rapid and marked endothelial cell injury occurred (Figure 2).

Cytotoxicity of NO

Addition of 1 mM of the NO donor SpNO did not cause any cell injury over 3 h of incubation in the glucose-containing KH buffer (3 ± 1% LDH release after 3 h), but caused moderate injury thereafter (27 ± 21% after 5 h and 67 ± 19% after 7 h). SpNO at a concentration of 0.5 mM showed lower toxicity and SpNO at a concentration of 0.1 mM no toxicity at all (4 ± 1% after 7 h).

Enhancing and protective effects of NO on the toxicity of hydrogen peroxide

Although 20 U/l GOD were not toxic on their own, the additional administration of SpNO enhanced H_2O_2 toxicity (Figure 1A), in line with the co-operative effect of NO and H_2O_2 we described previously [7,8]. This enhancement of H_2O_2 toxicity by NO was dependent on the concentration of the NO donor: 0.1 mM SpNO only showed a tendency towards enhacement of H_2O_2 toxicity, 0.5 mM SpNO markedly increased H_2O_2 toxicity and 1 mM SpNO had an

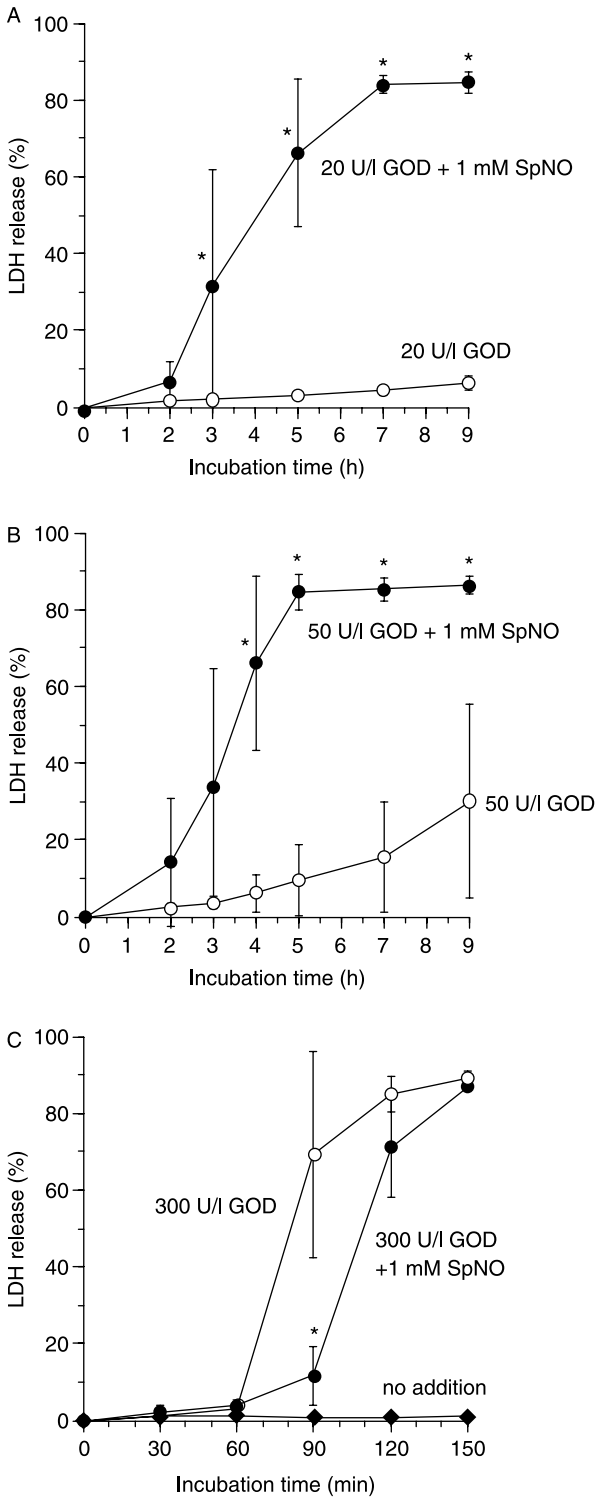


Figure 1. Enhancing and inhibitory effects of the NO donor SpNO on hydrogen peroxide toxicity to liver endothelial cells exposed to a hydrogen peroxide-generating system. Cultured rat liver endothelial cells were incubated with the hydrogen peroxide-generating system glucose/GOD and/or the NO donor SpNO in KH buffer. To this end, cells were preincubated or not with 1 mM SpNO for 15 min (in KH buffer containing 10 mM D-glucose), then (time "0"), GOD was added to this medium in a final activity of 20 U/l (A), 50 U/l (B) or 300 U/l (C). Cell injury was assessed by the release of LDH. Data are means ± SD of 3–5 experiments. *Significantly different to the respective incubation without SpNO, $p < 0.05$.

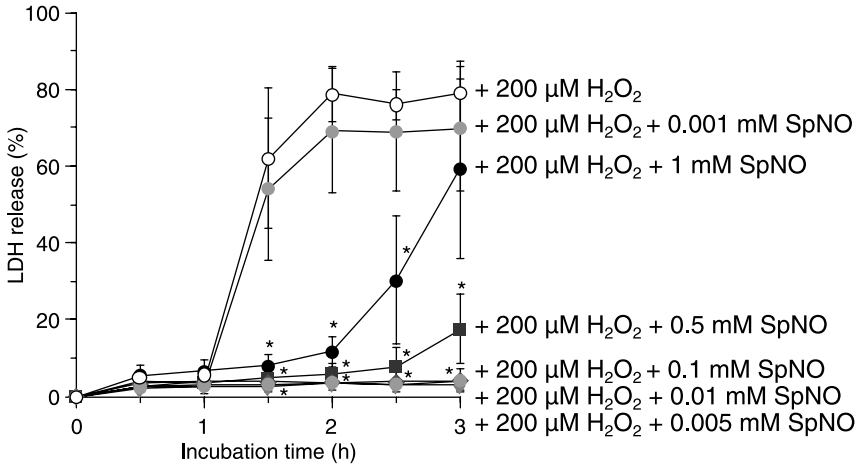


Figure 2. Effects of different SpNO concentrations on the endothelial cell injury elicited by a bolus of hydrogen peroxide. Cultured liver endothelial cells were preincubated or not with different concentrations of the NO donor SpNO in KH buffer containing 10 mM D-glucose for 15 min. Then (time "0"), a bolus of authentic H_2O_2 (final concentration 200 μM) was added. Cell injury was assessed by the release of LDH. Data are means ± S.D. of 4 experiments. * Significantly different to the respective incubation without SpNO, $p < 0.05$.

even more pronounced effect (Figure 3A). Similar enhancement of H_2O_2 toxicity by 1 mM SpNO was observed when 50 U/l GOD was used (Figure 1B). Again, the enhancement was less pronounced with 0.5 mM SpNO and only marginal with 0.1 mM SpNO (data not shown).

At an intermediate GOD activity, i.e. 150 U/l GOD, however, 1 mM SpNO only increased H_2O_2 toxicity in 5 out of 11 experiments, and was protective in six experiments. SpNO in a concentration of 0.1 mM, however, consistently provided protection against the injury induced by 150 U/l GOD, even in those experiments where 1 mM SpNO enhanced cell injury (data not shown). When cells were exposed to 300 U/l GOD, 1 mM SpNO did not cause any aggravation of injury but consistently provided protection (Figure 1C). Similarly, 0.1 mM SpNO or even 10 μM SpNO provided protection (Figure 3B).

As the time course and extent of endothelial cell injury induced by the "intermediate" GOD activity of 150 U/l showed a relative high variability, we grouped these experiments according to the time course/extent of the injury (LDH release after 3 h $<40\%$ or $>40\%$), and surprisingly, in all experiments with a slow time course/smaller extent of the injury ($n = 5$) 1 mM SpNO strongly enhanced H_2O_2-induced cell injury, while in all experiments with a more rapid H_2O_2-induced cell injury 1 mM SpNO was protective ($n = 6$).

When H_2O_2 was applied as a bolus, 1 mM SpNO was also consistently protective, although the protection was only partial (Figure 2). The protection by SpNO was enhanced at lower SpNO concentrations, i.e. 0.5 mM SpNO provided almost complete and 0.1 mM SpNO provided complete protection. Even concentrations as low as 5 and 10 μM SpNO were fully protective and only at 1 μM SpNO, the protective effect was lost.

Thus, it proved to be dependent on both, SpNO concentrations and intensity of H_2O_2-induced cell injury whether SpNO aggravated H_2O_2-induced cell injury or protected against H_2O_2-induced cell injury, with aggravation being predominant at high SpNO concentrations (>0.1 mM) and low/lower H_2O_2-induced cell injury (20–50 U/l GOD, less sensitive cell passages with 150 U/l GOD) and protection being predominant at low SpNO concentrations (<0.1 mM) and high/rapid H_2O_2-induced cell injury (150 U/l GOD in the more sensitive cultures, 300 U/l GOD, H_2O_2 bolus).

Steady-state levels of NO

For the experimental setting used in the current study, the steady-state levels of NO (in the incubation medium) are shown in Figure 4 for the borderline SpNO concentration of 0.1 mM, i.e. the SpNO concentration on the verge between protection and enhancement of H_2O_2 toxicity. 0.1 mM SpNO gave peak NO concentrations of 1–2 μM NO.

Hydrogen peroxide steady-state levels

In liver endothelial cell cultures incubated in KH buffer (supplemented with 10 mM glucose) in the absence of GOD, H_2O_2 steady-state levels were below 0.3 μM. The addition of 20 U/l GOD led to an increase in H_2O_2 steady-state levels that reached a maximum at 1–2 h of incubation (Figure 5A), levels at 1 h being 3.2 ± 1.5 μM. When 50 U/l GOD were applied, H_2O_2 steady-state levels rose higher, reaching 13.9 ± 6.2 μM after 1 h of incubation, and remained at this level for at

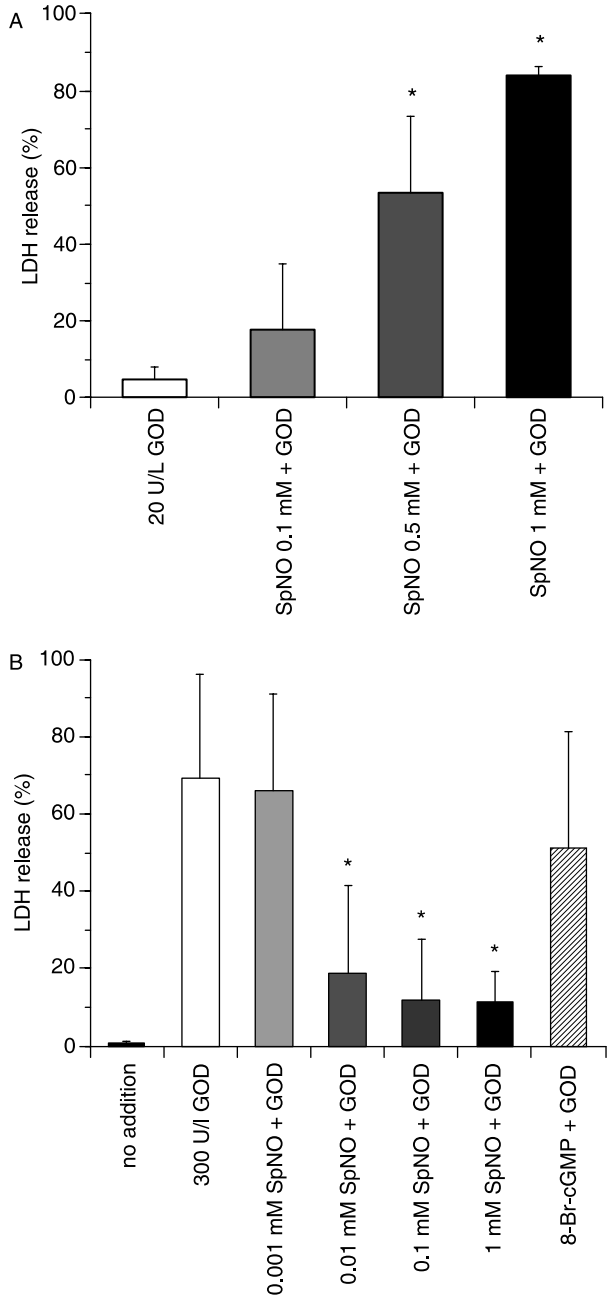


Figure 3. Effects of different SpNO concentrations on hydrogen peroxide toxicity to liver endothelial cells exposed to a hydrogen peroxide-generating system. Cultured liver endothelial cells were preincubated or not with different concentrations of the NO donor SpNO in KH buffer containing 10 mM D-glucose for 15 min, then (time "0"), GOD was added to this medium in a final activity of 20 U/l (A) or 300 U/l (B). In order to assess whether guanylate cyclase might be involved in cytoprotection by NO, additional cultures were preincubated with 8-Br-cGMP (50 μM, 30 min preincubation) instead of the NO donor (B). Cell injury was assessed after 7 h (A) or after 90 min (B) by the release of LDH. Data are means ± S.D. of 3 experiments. * Significantly different to the respective incubation with GOD alone (without SpNO/8-Br-cGMP), $p < 0.05$.

least 4 h (values after 5 h of incubation: 12.4 ± 9.6 μM). In the presence of 150 U/l GOD, H_2O_2 steady-state levels rose to 40.3 ± 9.8 μM after 2 h of incubation, and with 300 U/l GOD, H_2O_2 steady-state levels after 2 h

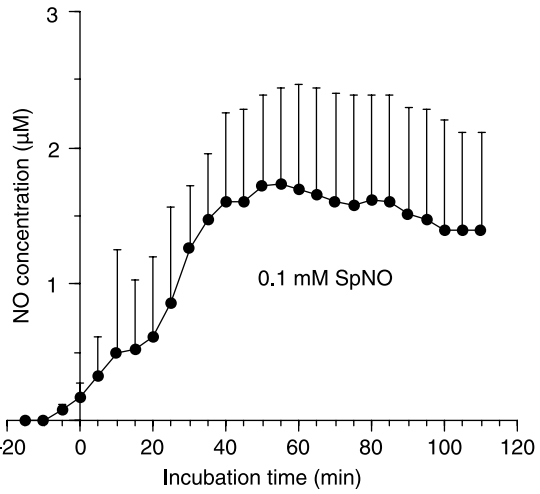


Figure 4. NO steady-state levels in liver endothelial cell cultures exposed to 0.1 mM of the NO donor SpNO. NO steady-state levels in liver endothelial cell cultures incubated with 0.1 mM SpNO in KH buffer (supplemented with 10 mM D-glucose) at 21% O_2 (as in the experiments shown in Figures 1–3, 5–9) were monitored continuously with an NO-sensitive electrode. To facilitate comparison with the other figures, the usual preincubation period is shown in the negative range of the *x*-axis (i.e. time "0" equals the time point when GOD or H_2O_2 was added in the experiments shown in Figures 1–3 and 5). Data are means ± S.D. of 4 experiments.

were 89.4 ± 2.9 μM. A *bolus* of 200 μM H_2O_2 has previously been shown to be degraded by the same cell type in the same experimental system to concentrations below 100 μM within 10 min and below 20 μM after about 36 min (manuscript submitted for publication).

Effects of NO/NO_x on hydrogen peroxide steady-state levels

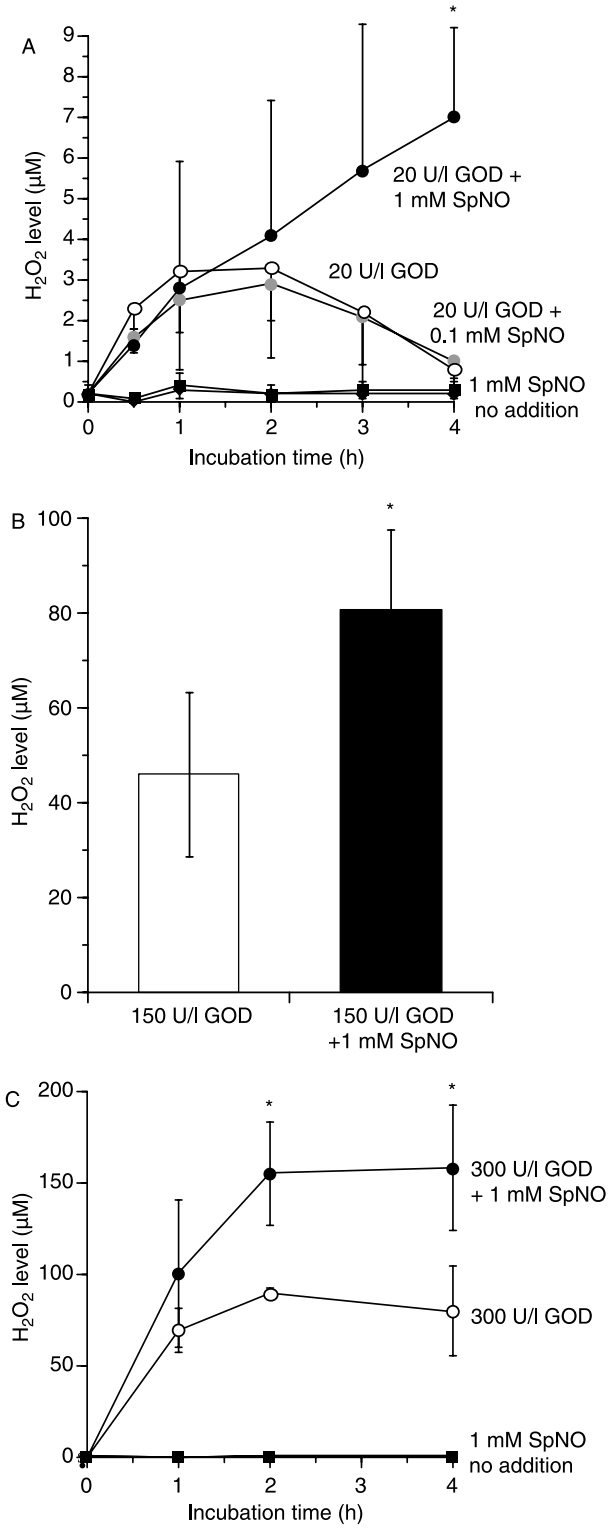
As described previously, SpNO (1 mM) added in addition to 20 U/l GOD did not affect H_2O_2 levels up to 1 h, but increased H_2O_2 levels thereafter (Figure 5A). In contrast to 1 mM SpNO—and in line with its only marginal effect on viability (Figure 3A)—0.1 mM SpNO did not alter H_2O_2 steady-state levels significantly (Figure 5A).

Also at higher GOD activities, i.e. in the range where protective effects by SpNO were observed, the addition of 1 mM SpNO strongly raised H_2O_2 steady-state levels (Figure 5B,C). That SpNO (1 mM) also inhibits the degradation of a 200 μM H_2O_2 bolus by liver endothelial cells has already been shown in the same system (manuscript submitted for publication).

No evidence for an involvement of a cGMP-dependent pathway in the protection by NO against H_20_2

A major action of NO is the activation of guanylate cyclase with subsequent formation of cyclic guanosine monophosphate (cGMP). Protective effects of cGMP have been reported in several models of cell injury

[14,18,19], and have, among others, been attributed to activation of cGMP-dependent protein kinase (PKG) [14,19]. Therefore, we tested whether the protective effect of SpNO can be mimicked by 8-Br-cGMP, a soluble and membrane-permeable cGMP analogue. Preincubating the cells with 8-Br-cGMP (50 μM) for 30 min prior to the addition of GOD or of a bolus of H_2O_2, a protocol that provided protection in other models of cell injury [19], did neither protect liver endothelial cells against the injury elicited by glucose/GOD (300 U/l; Figure 3B) nor against the injury elicited by a bolus of authentic H_2O_2 (200 μM; Figure 6). Along the same line, an inhibitor of PKG, KT5823 (5 μM), did not prevent NO-mediated protection of endothelial cells against H_2O_2-induced injury (Figure 6). Thus, protection by NO against H_2O_2-induced injury appears to be independent of cGMP and PKG.



Effect of NO on lipid peroxidation

NO has also been described to inhibit lipid peroxidation [15,16]; therefore, we tested this possibility next. H_2O_2 application, whether by the continuously H_2O_2-generating system glucose/GOD (data not shown) or by bolus addition (Figure 7), initiated lipid peroxidation as detected by the formation of TBARS.

The H_2O_2-induced formation of TBARS was inhibited by SpNO—strongly with SpNO concentrations of 1 mM but already (and even slightly better) with SpNO concentrations of 0.1 mM or 10 μM. This was confirmed using a fluorescent reporter molecule for lipid peroxidation, the fluorescent fatty acid analogue C11-BODIPY$^{®581/591}$, the fluorescence of which shifts from red to green upon oxidation of its conjugated double bond [31,32]. After addition of 300 U/l GOD, the green/red fluorescence ratio of C11-BODIPY$^{®581/591}$ increased, indicating oxidation of this indicator (Figure 8), and 0.1 mM SpNO (Figure 8) or 10 μM SpNO (data not shown) completely inhibited this oxidation (in the presence of the NO donor, the fluorescence ratio remained even below the values of control cells not exposed to exogenous H_2O_2).

This inhibitory effect on lipid peroxidation was thus not only present, but also occurred at similar concentrations as the protective effect of NO—and thus appears to be a likely explanation for the NO-induced protection observed. This was reinforced by the findings that preincubating the cells with the

Figure 5. Increase in hydrogen peroxide steady-state levels in endothelial cell cultures in the presence of higher concentrations of the NO donor SpNO. Cultured liver endothelial cells were preincubated or not with different concentrations of the NO donor SpNO in KH buffer containing 10 mM D-glucose for 15 min. Then (time "0"), GOD was added to this medium in a final activity of 20 U/l (A), 150 U/l (B) or 300 U/l (C). Hydrogen peroxide steady-state levels were assessed in samples taken at different time points (B: 150 min) using the horseradish peroxidase-catalyzed oxidation of scopoletin. Calibration was performed using authentic hydrogen peroxide. Data are means ± S.D. of 4–5 experiments. ⋆ Significantly different to the respective incubation without SpNO, $p < 0.05$.

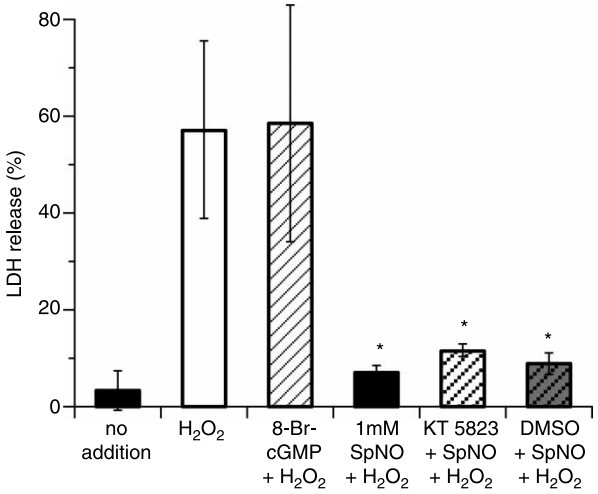


Figure 6. No evidence for an involvement of a cGMP-dependent pathway in the protective effect of NO against hydrogen peroxide toxicity. Cultured liver endothelial cells were preincubated with the NO donor SpNO (1 mM), the cGMP analogue 8-Br-cGMP (50 μM) and/or the PKG inhibitor KT5823 (5 μM), or the solvent of the latter (DMSO), in KH buffer (supplemented by 10 mM D-glucose) for 15 min (SpNO) or 30 min (8-Br-cGMP, KT5823, solvent). Then, a bolus of authentic H_2O_2 (final concentration 200 μM) was added, and cells were incubated for 90 min. Cell injury was assessed by the release of LDH. Data are means ± S.D. of 3 experiments. ⋆ Significantly different to the respective incubation with H_2O_2 alone, $p < 0.05$.

potent inhibitor of lipid peroxidation, α-tocopherol, mimicked the protective effect of SpNO (Figure 9), while in α-tocopherol-pretreated cells SpNO did not provide any further protection.

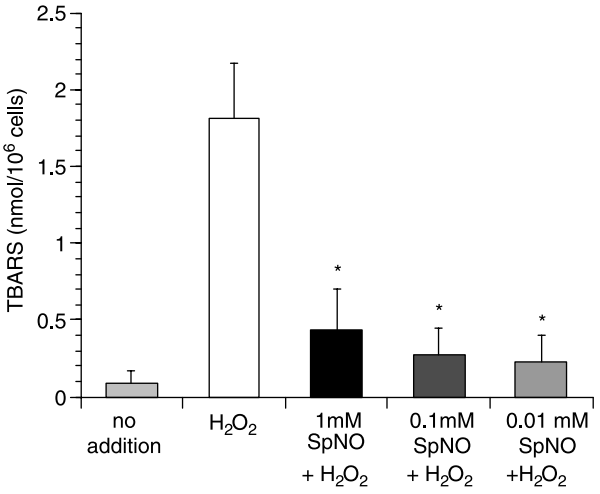


Figure 7. Inhibition of the formation of TBARS by the NO donor SpNO and its dependence on the SpNO concentration. Cultured liver endothelial cells were preincubated or not with different concentrations of the NO donor SpNO in KH buffer containing 10 mM D-glucose for 15 min. Then, a bolus of authentic H_2O_2 (final concentration 200 μM) was added. As markers of lipid peroxidation, TBARS were determined after 3 h of incubation. Data are means ± S.D. of 4 experiments. ⋆ Significantly different to the respective incubation without SpNO, $p < 0.05$.

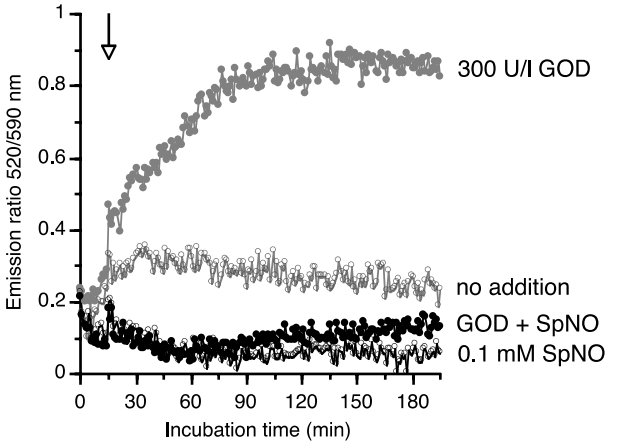


Figure 8. Inhibition of lipid peroxidation by the NO donor SpNO. Liver endothelial cells cultured on 12-well plates were loaded with the fluorescent, oxidation-sensitive fatty acid analogue C11-BODIPY®581/591 (10 μM, 60 min), and then incubated at 37°C in modified KH buffer (supplemented with 10 mM D-glucose) in a fluorescence microplate reader. To part of the incubations, SpNO was added in a final concentration of 0.1 mM at time zero. Fluorescence was recorded at $\lambda_{exc} = 485$ nm, $\lambda_{em} = 520$ nm and $\lambda_{exc} = 560$ nm, $\lambda_{em} = 590$ nm. After 15 min, GOD was added to part of the wells in a final activity of 300 U/l (arrow), and fluorescence recordings were continued for 3 h. Values are given as a ratio of the intensity of the green fluorescence of the oxidized indicator ($\lambda_{exc} = 485$ nm, $\lambda_{em} = 520$ nm) to the intensity of the red fluorescence of the non-oxidized indicator ($\lambda_{exc} = 560$ nm, $\lambda_{em} = 590$ nm). Data shown represent a representative recording from one of five experiments performed in duplicates.

Discussion

We have previously shown that NO and H_2O_2 act co-operatively in their cytotoxic action on liver endothelial cells, and that this co-operativity is due to inhibition of H_2O_2 degradation by NO (manuscript submitted for publication). However, protective effects of NO on H_2O_2 toxicity have also been described, and here we could show a switch between the enhancing and a protective effect of NO on H_2O_2 toxicity in the same cellular system—solely dependent on NO and H_2O_2 levels (Figure 10). In general, the enhancing effect of SpNO occurred when toxicity by H_2O_2 alone was relatively low and NO concentration high (NO concentration $>1-2$ μM), and the protective effect predominated when toxicity by H_2O_2 alone was high and/or the concentration of NO was low (i.e. below $1-2$ μM).

The enhancement of H_2O_2 toxicity with high NO concentrations (the SpNO concentration of 0.5 mM has previously given an NO steady-state concentration around 3 μM in the same cell culture model) [29] is in line with previous observations showing that inhibition of glutathione peroxidase required SpNO concentrations in excess of 0.1 mM, with marked inhibition being observed at 1 mM SpNO (around 10 μM NO; manuscript submitted for publication). Inhibition of glutathione peroxidase appeared to be caused by

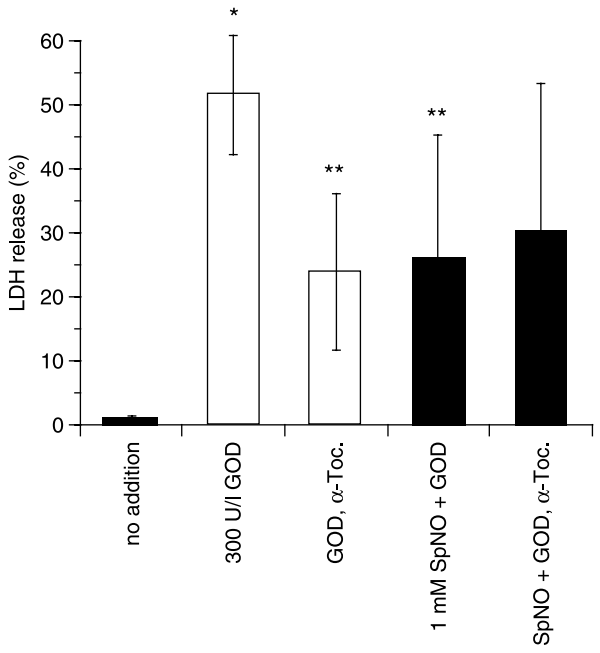


Figure 9. α-Tocopherol preincubation mimics the protective effect of SpNO on hydrogen peroxide toxicity to liver endothelial cells. Cultured liver endothelial cells were preincubated or not with α-tocopherol (α-Toc.; 60 μM in cell culture medium, 20 h) and then treated or not with the NO donor SpNO (1 mM) in KH buffer containing 10 mM D-glucose for 15 min. Thereafter (time "0"), GOD was added in a final activity of 300 U/l. Cell injury was assessed after 120 min by the release of LDH. Data are means ± SD of four experiments. *Significantly different to the respective incubation without GOD, $p < 0.05$. **Significantly different to the respective incubation with GOD alone (without SpNO/α-tocopherol), $p < 0.05$.

oxidation products of NO, reactive nitrogen oxide species (NO_X), not by NO itself, and was therefore a delayed effect occurring after about 2 h of NO exposure (Figure 10). Inhibition of catalase also occurs in the presence of NO. In contrast to inhibition of glutathione peroxidase, inhibition of catalase is caused by NO itself [10,33], and is therefore an immediate—and reversible—effect. It required similar NO concentrations as inhibition of glutathione peroxidase, however, it appeared to contribute to the decreased H_2O_2 degradation of liver endothelial cells confronted with NO only at high H_2O_2 levels (manuscript submitted for publication), a finding that is in line with the predominant role of glutathione peroxidase in H_2O_2 degradation of endothelial cells in the lower H_2O_2 concentration range [34–36]. In the present study, these inhibitory effects of high concentrations of NO on H_2O_2 degradation—whether by inhibition of glutathione peroxidase or of catalase—appeared to occur throughout, irrespective of the concentration of H_2O_2 applied, even under conditions where high levels of NO proved to be protective (Figure 5A–C).

The most likely mechanism for the protective effect of NO is an inhibition of lipid peroxidation by NO, as has been described previously [13,15,16]. H_2O_2-induced cell injury is mediated, besides by a calcium-dependent pathway, by an iron-dependent pathway that results in hydroxyl radical formation and subsequent lipid peroxidation (Figure 10) [28,37,38]. In the presence of glucose [37], and, in fact, in liver endothelial cells under most conditions (unpublished results), the iron-dependent pathway is the predominant one, and lipid peroxidation occurs

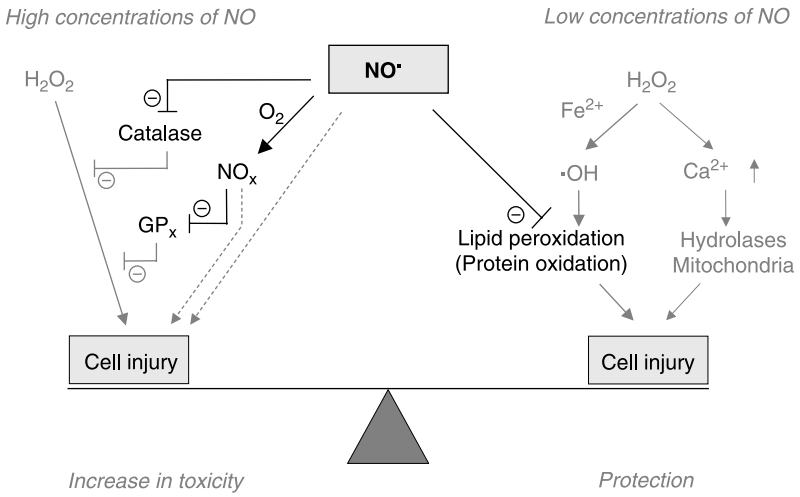


Figure 10. Enhancing and protective effects of NO on H_2O_2 toxicity. NO inhibits the H_2O_2-degrading enzyme catalase, and oxidation products of NO, NO_X, inhibit the H_2O_2-degrading enzyme glutathione peroxidase (GP_X). By these ways, H_2O_2 steady-state levels are increased, leading to enhancement of cell injury. On the other hand, NO inhibits lipid peroxidation and thus exerts protective effects. While for the latter effects low (submicromolar) NO concentrations are sufficient, the inhibition of H_2O_2 degradation requires higher NO levels (>1–2 μM). Thus, high NO concentrations cause enhancement of toxicity (unless H_2O_2-induced injury is severe and occurs rapidly, when protection by inhibition of lipid peroxidation can prevail), while low NO concentrations exert protection (mainly via inhibition of lipid peroxidation). For further details, refer to text.

(Figures 7 and 8). NO, itself a radical and being present in the lipid phase of the cellular membranes in far higher concentrations than in the aqueous phase of the cell culture system, is likely to combine with radical intermediates of lipid peroxidation, e.g. peroxyl radicals, and thus to interrupt this chain reaction [15,16,39,40]. This effect apparently only requires fairly low NO concentrations (in the aqueous phase); the 10 μM SpNO that were already fully protective in the current study yielded maximal NO steady-state levels of about 300 nM in the same cell culture model as used here [29]. This fits well with a recent report by Hummel et al. [40], showing that ferrous iron + dioxygen-induced lipid peroxidation in HL-60 and U937 cells was inhibited by NO steady-state levels >75 nM. These NO concentrations are far lower than those that are required for the inhibition of H_2O_2 degradation. Thus, it is explainable that at low NO concentrations protection by NO was predominent (compare results with 150 U/l GOD). At high NO concentrations, both, inhibition of H_2O_2 degradation (Figure 5) and inhibition of lipid peroxidation (Figure 7) occurred. In general, with high NO concentrations, enhancement of H_2O_2-induced cell injury was observed with lower H_2O_2 levels and/or lower cell injury induced by H_2O_2 alone. However, protection prevailed when high H_2O_2 amounts were applied and H_2O_2-induced cell injury was rapid (and lipid peroxidation prominent). With "intermediate" H_2O_2 levels as demonstrated by the results with 150 U/l GOD, protective and enhancing effects appeared to be in a delicate balance and the resulting effect was thus sometimes switched by minor alterations such as cell passage. In line with the finding that inhibition of lipid peroxidation by NO might account for a major part of the protective effect of NO (although we cannot rule out additional inhibitory effects of NO on protein oxidation), preincubation of the cells with α-tocopherol yielded similar protection as NO, and in α-tocopherol-pretreated cells NO did no longer exert (additional) cytoprotection (Figure 9).

In the model used here, i.e. acute exposure of endothelial cells to H_2O_2 in serum-free (i.e. transferrin-free) KH buffer, the mechanism proposed by Kotamraju et al. [22], namely inhibition of transferrin receptor-mediated iron uptake by NO, can not contribute to the protection provided by NO. Inhibition of mitochondrial respiration by NO, as suggested to contribute to protection against peroxide-mediated delayed cell death [23], is also unlikely, as in the model employed here and under the oxygen partial pressures (i.e. ambient oxygen) used here, 10 μM SpNO did not inhibit the mitochondrial respiratory chain to any appreciable extent [29]. Mediation of protective effects of NO by cGMP and PKG has also been reported in several models of cell injury, e.g. in models of ischemia/reperfusion injury [14,19]. However, the protection of NO against H_2O_2 toxicity in our experiments was neither mimicked by 8-Br-cGMP nor inhibited by the PKG inhibitor KT5823. Therefore, this pathway does not appear to be involved in the protection by NO against H_2O_2 toxicity in our experiments.

The cytotoxicity high NO concentrations elicited in the absence of H_2O_2 is due to NO_X as we have shown previously (Figure 10) [29]; the well-known inhibition of cytochrome oxidase by NO, which occurs in competition to oxygen, does not contribute significantly to the endothelial cell injury in our model as experiments were performed at 21% O_2 and as the experiments with the highly glycolytic liver endothelial cells [41] were performed in the presence of glucose [29]. We cannot exclude that NO, which exerts multiple effects in biological systems [2,3,42], might contribute to cytoprotection or to enhancement of H_2O_2-induced cell injury by other ways; however, inhibition of lipid peroxidation and inhibition of H_2O_2 degradation can fully account for the protection against and the enhancement of H_2O_2 cytotoxicity, respectively, in the model studied here, and can sufficiently explain the at first view contradictory results on the effects of NO on H_2O_2 toxicity.

Taking into consideration the NO concentrations which are assumed to occur *in vivo* under physiological (well below 1 μM NO) and pathophysiological conditions (presumably below 10 μM NO) [3,43,44], one should expect that under most conditions, the protective effects of NO prevail. On the other hand, physiological *in vivo* concentrations of H_2O_2 have been reported to be in the range of 1–100 nM [45], so that under pathological conditions in which elevated amounts of both NO and reactive oxygen species are formed, e.g. during inflammatory reactions or during reperfusion of tissues, inhibition of catalase and/or glutathione peroxidase might also contribute significantly to the injurious process. The balance of these processes is likely to vary considerably depending on the conditions, cell types involved, oxygen partial pressure, etc. so that overall a very delicate balance between enhancing and protective effects of NO is to be expected under these conditions. Furthermore, on the tissue level the vasodilatory actions of NO are, besides direct cellular effects as discussed in this article, likely to contribute to tissue protection [2,46]. In addition, H_2O_2 has been described to enhance eNOS activity [47], the NO thus released might contribute to the protection against (or the enhancement of) the oxidative stress generated by the H_2O_2 that stimulates its release.

In conclusion, we have shown here that the inhibition of H_2O_2 degradation requires relatively high NO concentrations while inhibition of lipid peroxidation only requires low NO concentrations. Thus, at low NO concentrations protection against H_2O_2 toxicity can be observed. At high NO concentrations, both inhibition of H_2O_2 degradation

and inhibition of lipid peroxidation can be observed and it appears to be dependent on the relative importance of both processes whether NO enhances or inhibits H_2O_2-induced cell injury.

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