Low toxicity of nitric oxide against endothelial cells under physiological oxygen partial pressures

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Cultured rat liver endothelial cells were incubated with 1 and 2 mM spermineNONOate at different O_2 concentrations in the incubation atmosphere. (Z)-1-{N-[3-Aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino}diazene-1-ium-1,2-diolate (spermineNONOate), at 2 mM, was highly cytotoxic at 21 % and 95 % O_2 (as measured by lactate dehydrogenase release); more than 80 % of the cells were damaged after 6 h of incubation. Cytotoxicity induced by 2 mM spermineNONOate was significantly decreased at 10 %, 5 % and 0 % O_2 ; cell death was 54 %, 36 % and 25 % respectively after 6 h of incubation. In contrast, 1 mM spermineNONOate was almost non-toxic towards the cells. Only at 95 % O_2 was a slight damaging effect, of 25 %, observed. The nitric oxide ($\cdot NO$) concentrations released from 1 and 2 mM spermineNONOate were determined as varying between 5 and 12 μM and between 12 and 22 μM respectively as measured by the oxyhaemoglobin and the NO cheletropic spin-trapping methods. The decomposition rate of spermineNONOate

and the resulting $\cdot NO$ concentrations were independent of O_2 at all applied concentrations. Likewise, the steady-state concentrations of H_2O_2 remained at approx. 1 nM at all O_2 concentrations, as measured with the fluorescent dye scopoletin. L-Tyrosine and L-ascorbate, both of which are known to scavenge reactive nitrogen species, markedly diminished spermineNONOate-induced cytotoxicity at 95 % O_2 . The formation of 3-nitrotyrosine, indicating the reaction of L-tyrosine with nitrogen dioxide ($\cdot NO_2$) and/or peroxynitrite anions, was enhanced in incubations with spermineNONOate at 21 % and 95 % O_2 . The results demonstrate that at O_2 partial pressures typically found under physiological conditions and at $\cdot NO$ concentrations that can occur *in vivo*, $\cdot NO$ alone is almost non-toxic towards cultured rat liver endothelial cells. $\cdot NO$ at these concentrations *in vivo*, however, exerts toxic effects at supraphysiological O_2 partial pressures, owing to its oxidation to reactive nitrogen species such as $\cdot NO_2$.

INTRODUCTION

Nitric oxide ($\cdot NO$) is released from various cell types to contribute to a variety of physiological and pathophysiological processes [1]. As well as its function in the control of blood pressure, platelet aggregation and neurotransmission, $\cdot NO$ has been shown to convey the cytotoxic activity of macrophages [2]. The mechanisms by which $\cdot NO$ mediates its toxic effects are still poorly understood. Essential metabolic enzymes in the cytosol and in the nucleus as well as in the mitochondria have been reported to be inactivated by $\cdot NO$ [3–6]. As the half-life of $\cdot NO$ has been demonstrated to increase with decreasing oxygen partial pressures (p_{O_2}) [7], these effects of $\cdot NO$ should be especially pronounced at low p_{O_2} [8,9]. However, it has been suggested that the cytotoxicity of $\cdot NO$ is enhanced by interaction with reactive oxygen species such as the superoxide anion radical ($O_2^{\cdot -}$) and H_2O_2 [10,11]. Furthermore the highly reactive nitrogen dioxide ($\cdot NO_2$), formed by the autooxidation of $\cdot NO$, could possess a crucial role in $\cdot NO$ toxicity [12]. Hence the damaging potential of $\cdot NO$ should depend critically on the presence of O_2 and thus should be increased with increasing p_{O_2} .

To decide whether O_2 enhances or decreases the cytotoxic activity of $\cdot NO$ we studied the damaging effects of $\cdot NO$ against cultivated endothelial cells at different O_2 concentrations. As

$\cdot NO$ donor we selected (Z)-1-{N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino}diazene-1-ium-1,2-diolate (spermineNONOate) [13–15], which, as will be shown below, decomposes with the release of $\cdot NO$ independently of the presence of O_2 . We also determined the steady-state concentrations of H_2O_2 in the incubation medium at different O_2 concentrations to estimate the suspected contribution of H_2O_2 to $\cdot NO$ toxicity under such conditions. Furthermore, we studied the influence of L-tyrosine and L-ascorbate, both of which scavenge reactive nitrogen species, on $\cdot NO$ -induced toxicity, to evaluate whether the latter species are involved in cell damage, especially under hyperoxic conditions.

MATERIALS AND METHODS

Materials

Oxygen gas mixtures were purchased from Messer Griesheim (Oberhausen, Germany). Horseradish peroxidase (grade I; 2500 units) was from Boehringer (Mannheim, Germany). 2,2'-Dipyridyl (2,2'-DPD), diethylenetriaminepenta-acetic acid (DTPA), haemoglobin, scopoletin (7-hydroxy-6-methoxy-2H-1-benzopyran-2-one) and spermine were obtained from Sigma

Abbreviations used: 2,2'-DPD, 2,2'-dipyridyl; DTPA, diethylenetriamine-pentaacetic acid; LDH, lactate dehydrogenase; NOCT, nitric oxide cheletropic trap; NOCT-44, 1,8-diphenyl-11,12-trans-dicarboxytetracyclo[8.4.1^{10,13}0.0^{2,7}]pentadeca-1,3,5,7-tetraene; $O_2^{\cdot -}$, superoxide anion radical; ONOO⁻, peroxynitrite anion; p_{O_2} , oxygen partial pressure; spermineNONOate, (Z)-1-{N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino}diazene-1-ium-1,2-diolate.

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(Deisenhofen, Germany). The $\cdot\text{NO}$ donor spermineNONOate was purchased from Molecular Probes (Leiden, The Netherlands). Hanks balanced salt solution and RPMI 1640 medium were from Gibco (Eggenstein, Germany). All other chemicals used were obtained from Merck (Darmstadt, Germany).

Culture conditions

Cultured sinusoidal liver endothelial cells from male Wistar rats were treated as described elsewhere [16], with small modifications. Briefly, the cells were seeded in RPMI 1640 medium supplemented with 20 % (v/v) fetal calf serum (Biospa, Wedel, Germany), 2 mM L-glutamine (Gibco), 1 μM dexamethasone (Serva, Heidelberg, Germany), 100 $\mu\text{g}/\text{ml}$ gentamicin (Sigma) and 5 $\mu\text{g}/\text{ml}$ amphotericin B (Gibco) at 37 °C in air/ CO_2 (19:1).

Incubation procedure

The cells were seeded in 20 cm^2 culture dishes with a gas-permeable bottom (Heraeus, Osterode, Germany) containing 5 ml of medium (2×10^5 cells/ml). After 24 h of culturing, the incubation medium was replaced by 5 ml of Krebs–Henseleit buffer [115 mM NaCl/25 mM NaHCO_3 /6 mM KCl/1.2 mM MgCl_2 /1.2 mM NaH_2PO_4 /1.2 mM Na_2SO_4 /2.5 mM CaCl_2 (pH 7.4)], supplemented with 10 mM glucose and 20 mM Hepes. The Krebs–Henseleit buffer had been equilibrated for 45 min with gas mixtures of the following compositions: CO_2/N_2 (1:19, 0 % O_2), $\text{O}_2/\text{CO}_2/\text{N}_2$ (1:1:18, 5 % O_2), $\text{O}_2/\text{CO}_2/\text{N}_2$ (2:1:17, 10 % O_2), $\text{O}_2/\text{CO}_2/\text{N}_2$ (21:5:74, 21 % O_2) and O_2/CO_2 (19:1, 95 % O_2). The dishes were placed in air-tight vessels with a gas inlet and a gas outlet (Dinkelberg, Neu-Ulm, Germany) and were subsequently regassed for a further 10 min. All experiments were performed at 37 °C and were started by exposing the cells to spermineNONOate (1 or 2 mM). L-Tyrosine (2 mM), L-ascorbate (100 μM), 2,2'-DPD (100 μM) or DTPA (100 μM) were added before spermineNONOate. Lactate dehydrogenase (LDH) leakage and Trypan Blue exclusion were used to indicate loss of cell viability [16].

Decomposition of spermineNONOate

SpermineNONOate decomposition was determined spectrophotometrically. The experiments were performed at 37 °C in 25 cm^2 culture flasks (Falcon, Heidelberg, Germany) containing 5 ml of Krebs–Henseleit buffer. The flasks were sealed with rubber stoppers equipped with two steel cannulas for sampling and gas inlet as described previously [17]. A value of molar absorption coefficient (ϵ_{252}) of 8.5 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ was used to quantify the concentration of spermineNONOate [13].

Measurement of $\cdot\text{NO}$ released from spermineNONOate

$\cdot\text{NO}$ was determined spectrophotometrically by measuring the conversion of oxyhaemoglobin into methaemoglobin [18]. SpermineNONOate (1 or 2 mM) was incubated in culture flasks at 37 °C as described above. The formation of $\cdot\text{NO}$ was quantified by using a molar absorption coefficient (ϵ_{578}) value of 12.1 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ [19]. To support the results obtained with the oxyhaemoglobin method (this assay is not specific for $\cdot\text{NO}$) we also measured the concentration of $\cdot\text{NO}$ by application of one of our recently developed nitric oxide cheletropic traps (NOCTs), the water-soluble 1,8-diphenyl-11,12-*trans*-dicarboxytetracyclo-[8.4.1^{10.13}.0.0^{2.7}]pentadeca-1,3,5,7-tetraene (NOCT-44). The advantage of NOCTs is the specific trapping of $\cdot\text{NO}$ and its easy monitoring and quantification by ESR spectroscopic detection of the resulting persistent nitroxide radicals. The ESR spec-

troscopic data for the $\cdot\text{NO}$ adduct of NOCT-44 (NOCT-44NO: $a(^{14}\text{N}) = 2.23$ mT, $g = 2.0063$) corresponded to data for similar nitroxides reported previously [20]. SpermineNONOate (2 mM) was incubated in 1 ml of phosphate buffer [50 mM K_2HPO_4 /50 mM KH_2PO_4 (pH 7.4)] at 37 °C equilibrated with 21 % O_2 or Ar (0 % O_2). ESR spectra of the resulting nitroxide radicals were recorded at 20 °C on a Bruker ER-420 spectrometer as described previously [20].

Measurement of the steady-state concentrations of H_2O_2

Steady-state concentrations of H_2O_2 in the incubation medium were detected by the H_2O_2 -sensitive fluorescent dye scopoletin [21,22]. Taking into account the fact that H_2O_2 diffuses freely through cell membranes, we used this method as an indicator for the endogenous production of H_2O_2 from endothelial cells. The cells were seeded in 3.8 cm^2 culture plates (Becton Dickinson, Heidelberg, Germany) with 1 ml of RPMI 1640 medium. After 24 h at 37 °C in air/ CO_2 (19:1) the cells were washed twice with Hanks balanced salt solution, pH 7.4, and 500 μl of Krebs–Henseleit buffer was added. During the incubation the culture plates were kept in air-tight vessels at 37 °C. At indicated times, cells were gassed with 21 % O_2 or 95 % O_2 for 5 min and subsequently for another 3 min with the respective gas mixture after the samples had been taken. Samples of the incubation medium were diluted 1:4 with Hanks balanced salt solution, pH 7.4, containing 0.1 mM DTPA, 2.5 units/ml horseradish peroxidase and 0.01 μM scopoletin as hydrogen donor. The fluorescence of reduced scopoletin was determined after excitation at 350 nm. The loss of fluorescence with oxidation catalysed by horseradish peroxidase/ H_2O_2 was detected at 460 nm on a spectrophotofluorimeter (Aminco-Bowman, Silver Spring, MD, U.S.A.). The assay was standardized with known concentrations of H_2O_2 prepared by diluting 30 % (w/v) H_2O_2 .

Measurement of 3-nitrotyrosine formation

To estimate the generation of reactive nitrogen species [e.g. $\cdot\text{NO}_2$ and/or peroxynitrite anion (ONOO^-)] in the incubations with spermineNONOate, the nitration of L-tyrosine to yield 3-nitrotyrosine was followed [23,24]. 3-Nitrotyrosine was assayed spectrophotometrically at 428 nm [24]. Briefly, spermineNONOate (2 mM) was dissolved in Krebs–Henseleit buffer equilibrated with 0 %, 5 %, 10 %, 21 % or 95 % O_2 at 37 °C as described above. L-Tyrosine (2 mM) was added before the incubations. After 6 h of incubation, samples were taken and the absorbance was measured. 3-Nitrotyrosine production was quantified by calibration with known concentrations of 3-nitrotyrosine.

Statistics

Data are given as means $\pm$ S.D. Statistical analysis was performed with a one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered significant.

RESULTS

Toxicity of spermineNONOate

At 0 % and 5 % O_2 , the toxicity induced by 2 mM spermineNONOate against endothelial cells remained below 20 % within 4 h of incubation and was only slightly increased, to 25 % and 36 % respectively, after 6 h (Figure 1; Table 1). The damaging effect of 2 mM spermineNONOate was, however, significantly higher at 10 %, 21 % and 95 % O_2 . At 10 % O_2 , LDH leakage increased to 46 % after 4 h of incubation, whereas at 21 % O_2 it

Figure 1 Toxicity of spermineNONOate at different O_2 concentrations

Sinusoidal rat liver endothelial cells were exposed to 2 mM spermineNONOate at 37 °C in Krebs–Henseleit buffer equilibrated with 0%, 5%, 10%, 21% and 95% O_2 . At the times indicated, samples were taken and cell injury was estimated by LDH leakage. Data are means $\pm$ S.D. for five to seven experiments. *Significant difference from values at 0% O_2 by one-way ANOVA ($P < 0.05$).

Table 1 O_2 dependence of the $\cdot NO$ cytotoxicity

Sinusoidal rat liver endothelial cells were exposed for 4 h to spermineNONOate at 37 °C in Krebs–Henseleit buffer equilibrated with 0%, 5%, 10%, 21% and 95% O_2 . After 4 h, samples were taken and cell injury was estimated by LDH leakage. Results are means $\pm$ S.D. for four to seven experiments. *Significant difference from values at 0% O_2 by one-way ANOVA ($P < 0.05$).

O_2 concentration (%)	[SpermineNONOate] (mM) ...	LDH leakage (%)		
		0 (control)	1	2
0		4 $\pm$ 2	6 $\pm$ 2	12 $\pm$ 3
5		6 $\pm$ 2	8 $\pm$ 3	20 $\pm$ 9
10		5 $\pm$ 2	11 $\pm$ 4	46 $\pm$ 6*
21		6 $\pm$ 3	15 $\pm$ 4*	73 $\pm$ 10*
95		6 $\pm$ 2	25 $\pm$ 6*	95 $\pm$ 5*

reached 73 % within the same period. At 95 % O_2 , cell death was approx. 89 % after 2 h and 95 % after 4 h of incubation. SpermineNONOate, at 1 mM, induced only weak toxicity against the cells (Table 1). After 4 h of incubation, cell death remained below 15 % in the incubations up to 21 % O_2 and was somewhat increased to only 25 % in the incubations at 95 % O_2 . In the controls, i.e. in the absence of spermineNONOate, LDH leakage did not exceed 6 % over the whole duration of the experiment for all O_2 concentrations (Table 1). Spermine (2 mM), the by-product of spermineNONOate decomposition, did not cause cytotoxicity above the control level (results not shown).

$\cdot NO$ release from spermineNONOate

No effect of oxygen on the decay rate of spermineNONOate was observed. The initial concentration of spermineNONOate (1.5 mM) decreased at 0 %, 21 % and 95 % O_2 , with almost identical first-order rate constants of 1.1×10^{-4} , 1.3×10^{-4} and $1.1 \times 10^{-4} s^{-1}$ respectively, to give residual concentrations of 0.32, 0.40 and 0.38 mM respectively after 4 h of incubation (Figure 2). Results similar to those in the cell-free system were obtained in incubations of spermineNONOate in the presence of cells (results not shown).

Figure 2 Decomposition of spermineNONOate

SpermineNONOate (1.5 mM) was incubated for 4 h at 37 °C in Krebs–Henseleit buffer equilibrated with 0% ($\blacktriangle$), 21% ($\bullet$), and 95% ($\blacklozenge$) O_2 . At the times indicated, samples were taken and the spermineNONOate concentrations were calculated from the absorption measured at 252 nm. The continuous curves are the least-squares fits to a first-order rate law. The correlation coefficient was at least 0.993. Data are means $\pm$ S.D. for five experiments.

Figure 3 Concentrations of $\cdot NO$

SpermineNONOate (filled symbols, 2 mM; open symbols, 1 mM) was incubated for 2 h at 37 °C in Krebs–Henseleit buffer equilibrated with 0% ($\blacktriangle$, $\triangle$), 21% ($\bullet$, $\circ$), and 95% ($\blacklozenge$, $\lozenge$) O_2 . At the times indicated, samples were added to a 7 μM oxyhaemoglobin solution in Krebs–Henseleit buffer. The $\cdot NO$ concentrations were determined by the oxyhaemoglobin method. The empirical fitting of the data are a guide for the eye. Data are means $\pm$ S.D. for four experiments.

At 0 % O_2 , the initial $\cdot NO$ concentration, i.e. 2 min after the beginning of the incubation, produced from 2 mM spermineNONOate, was 14 μM , as determined by the oxyhaemoglobin method (Figure 3). The $\cdot NO$ level then increased slightly to 22 μM within 40 min, after which it slowly decreased to 15 μM within 2 h. At 21 % and 95 % O_2 , similar time courses were found. Comparable $\cdot NO$ concentrations between 12 and 26 μM were determined by trapping with NOCT-44 for the first 2 h of incubation at 0 % and 21 % O_2 respectively (results not shown). Solutions containing 1 mM spermineNONOate liberated $\cdot NO$ in the concentration range 5–12 μM within 1 h of incubation at 0 %, 21 % and 95 % O_2 (Figure 3). In the presence of cells very similar results were obtained (results not shown).

Figure 4 Steady-state concentrations of H_2O_2

Krebs–Henseleit buffer (37 °C) equilibrated with 21% O_2 was added to sinusoidal rat liver endothelial cells grown in 3.8 cm^2 culture plates. During the incubation time the culture plates were kept in air-tight anaerobic vessels at 37 °C. The cells were gassed with 21% and 95% O_2 for 5 min (arrows) and subsequently for another 3 min with the respective gas mixture after samples were taken. At indicated time points the H_2O_2 concentrations were calculated from the loss of scopoletin fluorescence detected in the presence of horseradish peroxidase (2.5 units/ml). The decrease in scopoletin fluorescence was measured at 460 nm after excitation at 350 nm. Data are means $\pm$ S.D. for six experiments.

Table 2 Formation of 3-nitrotyrosine

Sinusoidal rat liver endothelial cells were exposed to 2 mM spermineNONOate at 37 °C in Krebs–Henseleit buffer equilibrated with 0%, 5%, 10%, 21% and 95% O_2 . L-Tyrosine (2 mM) was added before spermineNONOate. After 6 h, samples were taken and 3-nitrotyrosine was assayed spectrophotometrically at 428 nm. Data are means $\pm$ S.D. for three experiments. Abbreviation: n.d., not detectable.

O_2 concentration (%)	3-Nitrotyrosine (μM)
0	n.d.
5	2.0 ± 0.4
10	2.9 ± 0.6
21	6.5 ± 1.2
95	10.3 ± 1.5

Table 3 Influence of L-tyrosine, L-ascorbate and iron chelators on $\cdot\text{NO}$ -mediated cytotoxicity at 95% O_2

Sinusoidal rat liver endothelial cells were exposed to 2 mM spermineNONOate at 37 °C in Krebs–Henseleit buffer equilibrated with 95% O_2 . L-Tyrosine (2 mM), L-ascorbate (100 μM) or one of the iron-chelating substances 2,2'-DPD or DTPA (100 μM each) were added before the incubations. After 6 h, samples were taken and cell injury was estimated by lactate dehydrogenase (LDH) leakage. Data are means $\pm$ S.D. for four to six experiments. *Significant difference from values at 2 mM spermineNONOate without any additions by one-way ANOVA ($P < 0.05$).

SpermineNONOate concentration (mM)	Addition	LDH leakage (%)
0	None (control)	5 ± 2
2	None	91 ± 6
2	L-Tyrosine (2 mM)	$57 \pm 9^*$
2	L-Ascorbate (100 μM)	$25 \pm 3^*$
2	2,2'-DPD (100 μM)	92 ± 5
2	DTPA (100 μM)	94 ± 5

Steady-state concentrations of H_2O_2

H_2O_2 concentrations were estimated from horseradish peroxidase-catalysed oxidation of the fluorescent dye scopoletin to the non-fluorescent oxidized form of the compound. Low levels of H_2O_2 (in nanomolar ranges) were found to be already present in the incubation medium (Figure 4). These amounts decomposed rapidly in the presence of endothelial cells. No increase in H_2O_2 concentration was observed, either at 21% O_2 or after subsequent regassing with 95% O_2 (Figure 4).

Formation of 3-nitrotyrosine

The decomposition of spermineNONOate was also found to result in the formation of 3-nitrotyrosine, indicating the nitration of L-tyrosine by reaction with $\cdot\text{NO}_2$ and/or ONOO^- . The concentration of 3-nitrotyrosine increased with increasing O_2 concentrations (Table 2). At 0% O_2 , formation of 3-nitrotyrosine could not be detected within 6 h of incubation. At 5% and 10% O_2 , 3-nitrotyrosine concentrations of 2.0 and 2.9 μM respectively were formed, whereas at 21% O_2 6.5 μM 3-nitrotyrosine was measured after 6 h. The highest concentration of 3-nitrotyrosine (10.3 μM) was determined at 95% O_2 . Similar results were obtained in the incubations without cells (results not shown).

Influence of L-tyrosine, L-ascorbate and iron chelators on the $\cdot\text{NO}$ -mediated toxicity

The cytotoxicity induced by 2 mM spermineNONOate in the incubations at 95% O_2 was diminished from 91% to 57% after 6 h in the presence of 2 mM L-tyrosine (Table 3). With 100 μM L-ascorbate, the protection against spermineNONOate-mediated LDH leakage at 95% O_2 was much greater than that afforded by L-tyrosine. Only 25% of the cells were damaged in the incubations with spermineNONOate in the presence of L-ascorbate after 6 h (Table 3).

In contrast with the results obtained with L-tyrosine and L-ascorbate, the iron-chelating compounds 2,2'-DPD and DTPA did not exert measurable protective effects on the spermineNONOate-induced cytotoxicity (Table 3). In the control incubations without spermineNONOate, none of the above substances had any influence on the cell viability (results not shown).

DISCUSSION

The cytotoxicity of the $\cdot\text{NO}$ donor spermineNONOate against endothelial cells was found to be strongly dependent on the concentration of O_2 in the incubation atmosphere (Figure 1; Table 1). The pronounced O_2 dependence of cytotoxicity was shown not to be due to an enhanced decay of spermineNONOate, i.e. due to an increased $\cdot\text{NO}$ level, as the rate of decomposition of spermineNONOate was found to be almost independent of O_2 (Figure 2). Recently we demonstrated that the decay of *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and sodium nitroprusside (SNP), two frequently used $\cdot\text{NO}$ donors, was significantly diminished in the presence of O_2 [25]. This behaviour was followed by severe biological consequences, which were attributed to the higher $\cdot\text{NO}$ levels produced under hypoxic conditions. In contrast with SNAP and SNP, similar concentrations of spermineNONOate were shown to liberate more or less the same amount of $\cdot\text{NO}$ in each of the incubations (Figure 3), proving that the $\cdot\text{NO}$ -releasing process from this precursor is not affected by O_2 . It should be considered, however, that a large portion of $\cdot\text{NO}$ might be lost by diffusion into the gas phase rather than undergoing reaction with O_2 . Considering the independence of the spermineNONOate decomposition of O_2 , thereby yielding similar $\cdot\text{NO}$ concentrations in all of the incu-

bations, one has to conclude that O_2 must be directly involved in the mechanism by which $\cdot NO$ mediates its toxic effects.

At 0% and 5% O_2 , toxicity of $\cdot NO$ released from 2 mM spermineNONOate did not lead to more than 20% cell death after 4 h of incubation (Figure 1; Table 1). Under these conditions the concentrations of $\cdot NO$ (approx. 20 μM ; Figure 3) might not be sufficient to induce significant toxic effects. In similar experiments where under entirely hypoxic conditions endothelial cells were exposed to much higher $\cdot NO$ concentrations (at least 70 μM), cell death was much more pronounced [25]. Hence $\cdot NO$ itself seems to be toxic at higher concentrations. In contrast with the observations described above, the toxic effects of $\cdot NO$ released from 2 mM spermineNONOate were significantly increased at 10% and 21% O_2 and were strongly pronounced at 95% O_2 (Figure 1). Consequently O_2 enhances the damaging potential of $\cdot NO$. This effect is reasonably explained by the oxidation of $\cdot NO$, yielding $\cdot NO_2$ and other reactive nitrogen species derived from it [26,27]. Part of the effect might also result from the interaction of $\cdot NO$ with the reactive oxygen species $O_2^{\cdot -}$ and/or H_2O_2 . In recent experiments the cytotoxic activity of $\cdot NO$ was found to be enhanced by co-operative action with H_2O_2 , although a direct interaction of H_2O_2 and $\cdot NO$ seems unlikely [28], as well as by endogenously produced reactive oxygen species [11,29]. Increased reactivity also has been suggested to be due to an interplay of $\cdot NO$ with $O_2^{\cdot -}$ via the ONOO $^-$ pathway [30–32].

Enhanced oxygen levels might up-regulate the endogenous formation of reactive oxygen species from cells, thereby probably increasing the cytotoxic potential of $\cdot NO$. We therefore determined the steady-state concentrations of H_2O_2 (which diffuses freely through cell membranes [33]) to gain information about the H_2O_2 production from endothelial cells. In the present study, increasing O_2 concentrations were shown not to alter the steady-state concentrations of H_2O_2 , remaining at approx. 1 nM, which is at the detection limit of the assay used in this study (Figure 4). One can suggest either that endothelial cells produce only very small amounts of H_2O_2 even in situations where the cells are exposed to high O_2 concentrations, or, although formed at increased rates at high O_2 concentrations, H_2O_2 is still effectively degraded by catalase and glutathione peroxidase within the cells, resulting in no significant increase in the steady-state level. According to previous studies [11,29], several times higher concentrations of H_2O_2 are required to explain a co-operative action of $\cdot NO$ with H_2O_2 in cytotoxicity. In addition to these results, H_2O_2 was found to be present in nanomolar concentrations in the incubation medium (Krebs–Henseleit buffer with supplements). These small amounts were decomposed rapidly by the cells (Figure 4) and, in agreement with the above observations, should be without influence on their viability. Thus we conclude that at least for this type of cell the increased toxicity of $\cdot NO$ at 95% O_2 is not due to an enhanced intracellular production of H_2O_2 .

The fact that we were unable to detect a significant increase in the H_2O_2 concentration renders it rather unlikely that with increasing O_2 concentration the formation of $O_2^{\cdot -}$ by the endothelial cells was markedly increased. Hence the formation of toxic levels of ONOO $^-$ is also unlikely under these conditions.

With regard to the present results, the oxidation of $\cdot NO$ by O_2 might have greater implications for cell-damaging processes than hitherto assumed. The reaction of $\cdot NO$ with O_2 [$k = (2\text{--}6) \times 10^6 \text{ M}^{-2} \cdot \text{s}^{-1}$] has widely been shown to yield first of all the highly reactive $\cdot NO_2$ [34,35]. N_2O_3 was discussed as being formed in a secondary, diffusion-controlled ($k = 1.1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$) reaction of $\cdot NO$ with $\cdot NO_2$ in aqueous solution. The formation of $\cdot NO_2$ and thereby its contribution to cell damage in the respective experiments is in agreement with the observation that L-tyrosine

and L-ascorbate had strong protective effects against the toxicity of spermineNONOate at 95% O_2 (Table 3). $\cdot NO_2$ is well known to oxidize L-tyrosine, generating phenoxyl radicals, which can react further with $\cdot NO_2$ to form 3-nitrotyrosine [23,24]. By measuring the formation of 3-nitrotyrosine, additional evidence for the contribution of $\cdot NO_2$ was provided (Table 2). The amount of 3-nitrotyrosine (in the micromolar range) compared with the initial concentrations of spermineNONOate and L-tyrosine (2 mM each) led us to suggest that (1) the reaction of $\cdot NO$ with O_2 to form $\cdot NO_2$ was not complete and/or (2) other intermediates/products were formed, e.g. N_2O_3 , not being scavenged by L-tyrosine [36]. The participation of other such reactive nitrogen species (N_2O_3 , N_2O_4 , NO^+) is also suggested by the pronounced protection exerted by L-ascorbate. L-Ascorbate is discussed as being an effective scavenger of such species [37–39]. Iron-dependent processes, such as hydroxyl radical formation, are not assumed to account for the enhanced toxicity at increased O_2 concentration. Both DTPA and the penetrable iron-chelating compound 2,2'-DPD (the former acts to chelate only extracellular iron) did not influence spermineNONOate-induced cell damage (Table 3). We therefore suggest that the increased toxicity of $\cdot NO$ with increasing O_2 is due to the formation of reactive nitrogen species, such as $\cdot NO_2$, resulting from the autoxidation of $\cdot NO$.

Gas-phase O_2 concentrations of 0–15% correspond to the mean p_{O_2} commonly present under physiological conditions (e.g. 5% ≈ 35 mmHg). Maximum p_{O_2} values of approx. 100 mmHg are typically found in the lung. In most other tissues values between 1 and 60 mmHg have been determined; e.g. the mean p_{O_2} in the liver amounts to approx. 20 mmHg [40]. According to our results at such p_{O_2} values, $\cdot NO$ would not be assumed to exert crucial toxic effects against endothelial cells (Table 1). This fact is of great importance for the viability of endothelial cells, because they produce $\cdot NO$ to mediate physiological functions. $\cdot NO$ concentrations commonly produced endogenously from cultured endothelial cells ranged from 100 to 500 nM [41] and thus were much lower than those released in our experimental system. However, higher and therefore cytotoxic levels of $\cdot NO$ might be reached locally when $\cdot NO$ is liberated from cells over long periods in response to stimulants [26]. Various cell types, including those localized in the endothelium, have been shown to produce $\cdot NO$ concentrations up to 5 μM after stimulation [42], values comparable to those released from 1 mM spermineNONOate (Figure 4). As demonstrated here, such concentrations do not induce toxic effects as long as the O_2 concentration is kept below 21% (Table 1). Thus a direct action of $\cdot NO$, e.g. by inhibitory binding of cytochrome *c* oxidase [5,6,43] and/or depression of the enzymic activity of glyceraldehyde-3-phosphate dehydrogenase [44], is unlikely to be responsible for cell death observed under these experimental conditions. It should be considered that endothelial cells as used in the present study have been suggested to be relatively tolerant to the inhibition of both mitochondrial and glycolytic ATP production. By up-regulation of glyceraldehyde-3-phosphate dehydrogenase, endothelial cells rather than other cells maintain sufficient ATP levels via the glycolytic pathway [44,45]. Thus it remains to be shown whether the present results also apply to other cell types, especially to those that essentially depend on mitochondrial ATP production.

This work was supported by the Deutsche Forschungsgemeinschaft, Gr 815/7-1.

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