Enhanced release of nitric oxide causes increased cytotoxicity of *S*-nitroso-*N*-acetyl-DL-penicillamine and sodium nitroprusside under hypoxic conditions

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S-Nitroso-*N*-acetyl-DL-penicillamine (SNAP) and sodium nitroprusside (SNP), both of which are known to release nitric oxide (•NO), exhibited cytotoxicity against cultivated endothelial cells. Under hypoxic conditions 5 mM SNAP and 20 mM SNP induced a loss in cell viability of about 90 % and 80 % respectively, after an 8 h incubation. Under normoxic conditions, cell death was only 45 % and 42 % respectively within the same time period. Concentrations of •NO liberated from SNAP and SNP were measured by the oxyhaemoglobin method and by two of the recently developed nitric oxide cheletropic traps (NOCTs). The •NO concentrations from SNAP and SNP increased from 74 μM and 28 μM to 136 μM and 66 μM respectively within 15 min of hypoxic incubation, and then decreased to 36 μM and 28 μM. In the respective normoxic incubations the •NO levels from SNAP and SNP remained in the region of about 30 μM and 20 μM respectively. In contrast, spermine/NO adduct (spermineNONOate) was shown to be more toxic under normoxic than under hypoxic conditions. Under either of these conditions, the concentration of •NO liberated from 2 mM spermineNONOate was about 20 μM. The results demonstrate that the cytotoxicity of SNAP and SNP, but not of spermineNONOate, is significantly enhanced under hypoxic compared with normoxic incubations. Studies on the •NO-releasing behaviour of these compounds indicate that the increased toxicity of SNAP and SNP under hypoxic conditions is related to the influence of O_2 on the chemical processes by which •NO is produced from the precursors, rather than to an increased sensitivity of the hypoxic cells towards •NO.

INTRODUCTION

Nitric oxide (•NO), which is secreted from various cell types, contributes to a variety of regulatory processes, such as neurotransmission, vasodilation and platelet aggregation [1]. In addition to these physiological effects, all of which are mediated via the activation of soluble guanylate cyclase, •NO is known to have bactericidal and tumoricidal potential [2]. Released in large amounts in response to cytokines and/or endotoxins, •NO participates in the cytotoxic activities of macrophages [3]. Diverse targets on the cell surface as well as within the cell, which include the thiol groups of proteins, have been suggested to account for the cytotoxicity of •NO. •NO is thought to inhibit key enzymes in mitochondrial respiration, DNA synthesis and iron metabolism [4].

Recent experiments suggest that the effectiveness of •NO as a cytotoxic molecule is modulated by reactive oxygen species such as the superoxide anion radical ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). The interaction of •NO with $O_2^{-\bullet}$ yielding the peroxynitrite anion ($ONOO^-$) increased the reactivity of the individual molecules towards many targets of cell metabolism [5–7]. It has also been demonstrated that the tumoricidal activity of •NO is enhanced by the co-operative action of •NO and H_2O_2 [8]. In synergy with endogenously produced reactive oxygen species, the damaging effects of •NO appear to be augmented [9]. Furthermore the formation of the highly reactive nitrogen dioxide ($^\bullet NO_2$), formed by reaction of •NO with O_2, has also been considered as a mediator of •NO toxicity [10]. From all of these observations it can be concluded that the toxic potential of •NO is likely to be increased in the presence of O_2, possibly by generating other toxic species.

To verify this hypothesis, in the present work we have studied the damaging effects of the •NO donors *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and sodium nitroprusside (SNP) against cultivated endothelial cells, taking into account that the formation of •NO from these compounds is generally considered to be independent of the presence of O_2. Surprisingly, we found that both of these •NO donors induced greater toxicity under hypoxic conditions compared with the respective normoxic incubations.

MATERIALS AND METHODS

Materials

Haemoglobin, SNP and spermine were from Sigma (Deisenhofen, Germany). Desferal and EDTA were purchased from Ciba–Geigy (Wehr, Germany) and Boehringer (Mannheim, Germany) respectively. Hanks balanced salt solution and RPMI 1640 medium were from Gibco (Eggenstein, Germany). 3-Morpholinosydnonimine-*N*-ethylcarbamide (SIN-1) was kindly provided by Cassella A. G. (Frankfurt, Germany). SNAP was prepared as described previously [11]. Spermine/NO adduct (SpermineNONOate) was from Molecular Probes (Leiden, The Netherlands). All other chemicals used were purchased from Merck (Darmstadt, Germany).

Culture conditions

Cultured liver sinusoidal endothelial cells from male Wistar rats were treated, with small modifications, as described elsewhere [12]. Briefly, the cells were seeded in RPMI 1640 medium supplemented with fetal calf serum (20 %, v/v; Biospa, Wedel, Germany), L-glutamine (2 mM; Gibco), dexamethasone (1 μM;

Abbreviations used: LDH, lactate dehydrogenase; •NO, nitric oxide; $O_2^{-\bullet}$, superoxide anion radical; $ONOO^-$, peroxynitrite anion; $^\bullet NO_2$, nitrogen dioxide; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside; SIN-1, 3-morpho-linosydnonimine-*N*-ethylcarbamide; spermineNONOate, spermine/NO adduct; 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-tetra-ene; NOCT-44K, 1,8-diphenyl-11,12-*trans*-dicarboxy-pentacyclo[6.6.1.1^{10,13}.0^{2,1}.0^{9,14}]hexadeca-2(7),3,5-triene-15-one; NOCT20, 7,8-dimethoxy-7,8-diphenyl-*o*-quinodimethane; NOCT-20K, 1,3-dimethoxy-1,3-diphenyl-2-indanone; THF, tetrahydrofuran.

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Serva, Heidelberg, Germany), gentamycin (100 μg/ml; Sigma) and amphotericin B (5 μg/ml; Gibco) at 37 °C (in air/CO_2, 19:1). Subcultures were obtained after treatment with trypsin (0.25 % v/v trypsin in citrate saline) when cells had formed a confluent monolayer.

Incubation procedure

The cells were seeded in 25 cm² culture flasks (Falcon, Heidelberg, Germany) containing 5 ml of medium (2×10^5 cells/ml). After culturing for 24 h, the incubation medium was replaced with 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) equilibrated with 5 % CO_2. Hypoxic conditions were obtained by adding 5 ml of N_2-saturated (CO_2/N_2, 1:19) Krebs–Henseleit buffer. The flasks were flushed with CO_2/N_2 for 2 min at the beginning of the experiment and every 2 h during the course of the experiment, as described previously [13]. All experiments were performed at 37 °C and were started by exposing the cells to SNAP (2–10 mM), SNP (20 mM), spermineNONOate (2 mM) or SIN-1 (5 mM). Stock solutions of the compounds were prepared in Hanks balanced salt solution, except for SNAP, which was dissolved in DMSO. The reaction products (denitrosylated $^{\cdot}NO$ donors) from SNAP (5 mM), SNP (20 mM) and SIN-1 (5 mM) were prepared as described by Kröncke et al. [14]. Spermine was added to give a final concentration of 2 mM.

Cell viability

Lactate dehydrogenase (LDH) leakage was used to indicate loss of cell viability. At the indicated time points, supernatant samples were taken and the LDH activity was measured using a standard assay [15]. At the end of the incubation period, intracellular LDH activity was determined after lysis of the cells with Triton X-100 [1 % (v/v) in Hanks balanced salt solution]. Toxicity was defined as the LDH activity in the supernatant relative to the total LDH activity. In addition, the viability of cells was examined by counting the number of cells taking up Trypan Blue [16]. Both measures of cell viability gave the same results, therefore only the LDH leakage data will be shown.

Decomposition of SNAP

The decomposition of SNAP was determined spectrophotometrically. The experiments were performed at 37 °C in 5 ml of the air- or N_2-saturated Krebs–Henseleit buffer described above. The incubations were carried out in 25 cm² flasks both in cell-free systems and in the presence of cells. Briefly, the incubations were started by the addition of SNAP at a final concentration of 5 mM, and 200 μl samples were removed from the flasks at the indicated time points and were transferred to cuvettes containing 800 μl of Krebs–Henseleit buffer. The spectra were recorded in the visible region between 400 and 650 nm. The absorption coefficient, $\epsilon_{590} = 11\ M^{-1} \cdot cm^{-1}$, was used to quantify the concentration of SNAP [17]. The decomposition of SNAP was also studied in the presence of added Cu^{2+} ions ($CuSO_4$; 20 μM), desferal (200 μM) and/or EDTA (100 μM). To monitor SNAP decay continuously, measurements were also carried out with a stopped-flow system, which was equipped with a rapid scan unit for recording UV/visible spectra (λ = 350–700 nm) in 1 s time intervals. The incubations were carried out as described for the discontinuous experiments. After dissolution of SNAP in Krebs–Henseleit buffer, 5 ml was rapidly (within approx. 10 s) transferred to a syringe of the stopped-flow apparatus, the monitoring was started and spectra were recorded within a pre-determined time period.

Measurement of $^{\cdot}NO$ by the oxyhaemoglobin method

$^{\cdot}NO$ was determined spectrophotometrically by measuring the conversion of oxyhaemoglobin to methaemoglobin [18]. SNAP (2–10 mM), SNP (20 mM) and spermineNONOate (2 mM) were incubated under normoxic and hypoxic conditions as described above. At indicated times, 50 μl samples were added to 950 μl of Krebs–Henseleit buffer containing 7 μM oxyhaemoglobin. The formation of $^{\cdot}NO$ was quantified using the absorption coefficient, $\epsilon_{578} = 12.1\ mM^{-1} \cdot cm^{-1}$ [19].

ESR-spectroscopic measurement of $^{\cdot}NO$ with nitric oxide cheletropic traps (NOCTs)

The oxyhaemoglobin assay is not specific for $^{\cdot}NO$, since in addition to other nitrogen–oxygen species, nitrite (NO_2^-), a major product of $^{\cdot}NO$ conversion in aqueous solutions [20], also reacts with oxyhaemoglobin. To support the results obtained with the oxyhaemoglobin method, we measured the concentration of $^{\cdot}NO$ released from SNAP and SNP under normoxic and hypoxic conditions in aqueous and non-aqueous solution using two of our recently developed NOCTs [21,22]. The advantages of using NOCTs is that $^{\cdot}NO$ and, with some derivatives, $^{\cdot}NO_2$ are specifically trapped, and monitoring and quantification of $^{\cdot}NO$ and/or $^{\cdot}NO_2$ production is easily achieved by ESR-spectroscopy of the resulting persistent nitroxide radicals. The water-soluble NOCT-44 (1,8-diphenyl-11,12-*trans*-dicarboxytetracyclo[8.4.1^{10,13}.0.0^{2,7}]pentadeca-1,3,5,7-tetraene) and the lipid-soluble NOCT-20 (7,8-dimethoxy-7,8-diphenyl-*o*-quinodimethane) were generated by UV-photolysis (Rayonet photoreactor; λ = 300 nm) of the corresponding ketones NOCT-44K {1,8-diphenyl-11,12-*trans*-dicarboxypentacyclo-[6.6.1.1^{10,13}.0^{2,1}.0^{9,14}]hexadeca-2(7),3,5-triene-15-one} and NOCT-20K (1,3-dimethoxy-1,3-diphenyl-2-indanone) in tetrahydrofuran (THF) and benzene solutions respectively, as described previously [21,23,24]. After 80 h photolysis at 10 °C, NOCT-44K was converted into NOCT-44 with a yield of 85–95 %, determined by ^{1}H NMR-spectroscopy (M. Bätz, H.-G. Korth and R. Sustmann, unpublished work). Photolysis of NOCT-20K gave 1,2-dimethoxy-1,2-diphenylbenzocyclobutene as the major product (> 90 %) and a stationary concentration of NOCT-20 of about 10^{-3} M. The photolysates were applied as follows. For NOCT-44, SNAP (5 mM) and SNP (20 mM) were incubated in 1 ml air- or Ar-saturated phosphate buffer (50 mM K_2HPO_4/50 mM KH_2PO_4, pH 7.8) at 37 °C. At different times, 50 μl samples were transferred by syringe to a septum-capped, Ar-flushed, dry quartz ESR tube (external diam. 3 mm). NOCT-44 (50 μl in THF) was added to the sample to give concentrations of NOCT-44 similar to those of the $^{\cdot}NO$ donor. The solutions were briefly mixed, and the tube was transferred to the ESR spectrometer. For NOCT-20, SNAP and SNP in 1 ml of air- or Ar-saturated THF were mixed with 1 ml of the photolysate of NOCT-20K, transferred to an ESR tube (external diam. 4 mm) and incubated at 37 °C. The concentrations of SNAP and the photolysate of NOCT-20K added were 5 mM. ESR spectra were recorded continuously over a period of 2 h.

The ESR spectra were recorded on a Bruker ER-420 spectrometer equipped with a double cavity and operating at 9.4 GHz. Microwave power levels of 2 mW and 1 G modulation amplitude were applied. The double cavity contained a calibrated spin-concentration standard. Spin concentrations were determined by double integration of the digitized spectra of the nitroxide radicals and comparison with the doubly integrated signal of the

spin standard, both spectra being recorded at the same instrument settings. ESR parameters were refined by computer simulation.

RESULTS

Toxicity of ·NO-donating compounds

When rat-liver sinusoidal endothelial cells were exposed to 5 mM SNAP, LDH leakage increased constantly from 15% after 2 h to 45% after 8 h of normoxic incubation (Figure 1a). Compared with the normoxic conditions, the damaging effect of 5 mM SNAP was significantly higher under hypoxic conditions. Cell death was approx. 50% after only 4 h and had increased to almost 90% after 8 h of incubation. SNAP (10 mM) induced toxicity of about 80% after 6 h under normoxic conditions (Table 1). The LDH leakage induced by 2 mM SNAP was only 15% after 6 h under hypoxic conditions. In experiments where 20 mM SNP was used results were similar to those obtained with 5 mM SNAP. SNP caused 42% cell death after 8 h of normoxic incubation, whereas nearly 80% of the cells were non-viable within the same time period under hypoxic conditions (Figure 1b). In contrast to these ·NO donors, 2 mM spermineNONOate induced slight cytotoxic effects of about 20% in the hypoxic incubations and 33% under normoxic conditions (Table 1). After 8 h of hypoxic incubation, SIN-1-induced LDH leakage was about 17%, and, under normoxic conditions, cell damage was almost complete at the end of the incubation period (Figure 1b). These results were expected, since O_2 is required for the decomposition of SIN-1 [25]. In normoxic and hypoxic controls, no loss of viability was observed (Figure 1a), and similarly, cell viability was not affected by the denitrosylated substances (results not shown), which is in agreement with data reported by Kröncke et al. [14].

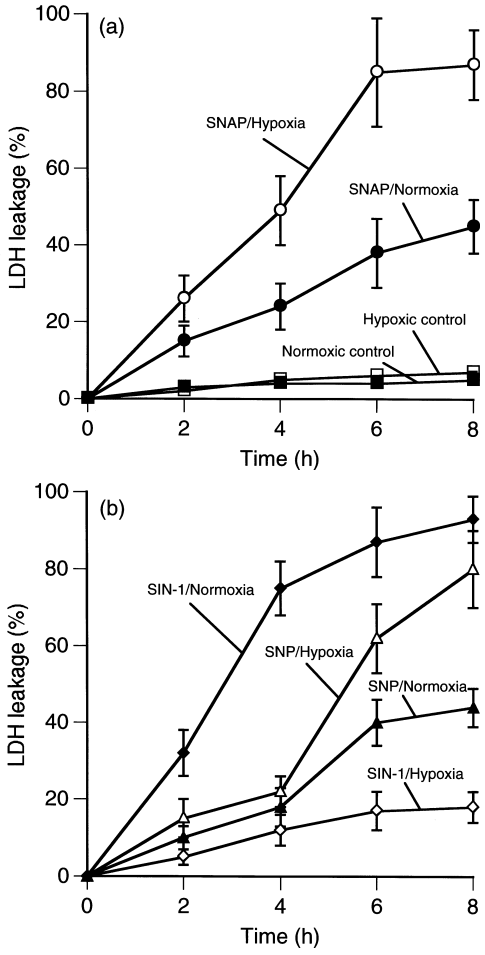

Figure 1 Toxicity of SNAP, SNP and SIN-1 to rat liver sinusoidal endothelial cells under normoxic and hypoxic conditions

The cells were exposed for 8 h to (**a**) 5 mM SNAP, (**b**) 20 mM SNP or 5 mM SIN-1 in 5 ml of Krebs–Henseleit buffer (pH 7.4) at 37 °C. Normoxic incubations were performed in air-saturated and hypoxic incubations in N_2-saturated Krebs–Henseleit buffer respectively. At the times indicated cell injury was estimated by LDH leakage. The values shown are the means ± S.D. of 7–8 experiments.

Table 1 Comparison of the toxicity of SNAP and spermineNONOate against endothelial cells and the ·NO concentrations released from these ·NO donors under normoxic and hypoxic conditions

Rat-liver sinusoidal endothelial cells were exposed for 6 h to various concentrations of ·NO donors in 5 ml of Krebs–Henseleit buffer (pH 7.4) at 37 °C. Normoxic incubations were performed in air-saturated and hypoxic incubations in N_2-saturated Krebs–Henseleit buffer. ·NO concentrations were determined by the oxyhaemoglobin method at 15 and 60 min. Cell injury was estimated by assay for LDH leakage at 6 h. The values represent the means ± S.D. of 3–4 determinations.

			·NO concentration (μM)	
O_2 content	Additions	LDH leakage (%)	15 min	60 min
Normoxia	5 mM SNAP	38 ± 9	31 ± 6	20 ± 3
Normoxia	10 mM SNAP	80 ± 7	44 ± 5	24 ± 3
Normoxia	2 mM SpermineNONOate	33 ± 5	18 ± 4	19 ± 6
Hypoxia	2 mM SNAP	15 ± 4	49 ± 6	40 ± 5
Hypoxia	5 mM SNAP	85 ± 12	136 ± 16	38 ± 6
Hypoxia	2 mM SpermineNONOate	20 ± 6	21 ± 6	18 ± 2

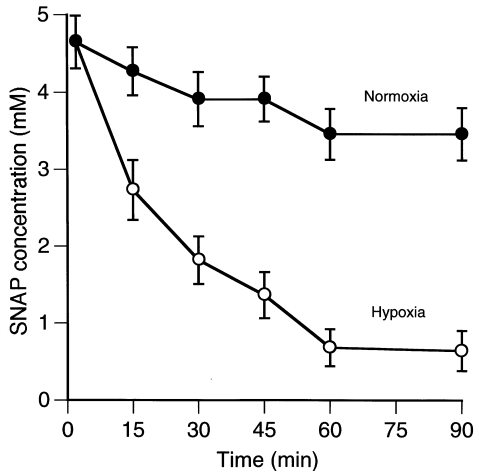

Figure 2 Decomposition of SNAP

SNAP (5 mM) was incubated in 5 ml of Krebs–Henseleit buffer (pH 7.4) at 37 °C for 90 min. Normoxic incubations were performed in air-saturated and hypoxic incubations in N_2-saturated Krebs–Henseleit buffer. At the times indicated SNAP concentrations were calculated from the absorption measured at 590 nm. Data represent means ± S.D. of seven experiments.

Decomposition of SNAP

In air-saturated Krebs–Henseleit buffer only a small decrease in the initial SNAP concentration (4.7 mM) was observed (Figure 2). After 60 min of incubation, the concentration was stationary

Table 2 The inhibitory effect of metal ion chelators on the decomposition of SNAP

SNAP (5 mM) was incubated in 5 ml of Krebs–Henseleit buffer (pH 7.4) at 37 °C. Desferal (200 μM) and EDTA (100 μM) were added to the flasks immediately before SNAP. Normoxic incubations were performed in air-saturated and hypoxic incubations in N_2-saturated Krebs–Henseleit buffer. SNAP concentrations were determined spectrophotometrically at 30 min. The values represent the means ± S.D. of 3–6 determinations. *Decreases as a percentage of the initial concentration of 4.7 mM.

O_2 content	Additions	SNAP (mM)	Decrease (%)*
Normoxia	None (control)	3.73 ± 0.24	20
Normoxia	200 μM Desferal	4.25 ± 0.20	9
Normoxia	100 μM EDTA	4.25 ± 0.21	9
Hypoxia	None (control)	1.82 ± 0.11	60
Hypoxia	200 μM Desferal	3.85 ± 0.18	17
Hypoxia	100 μM EDTA	4.00 ± 0.19	14

at 3.5 mM. In N_2-saturated Krebs–Henseleit buffer the decay of SNAP gave a much lower final concentration (0.7 mM) within the same time period. Results similar to those of the cell-free system were obtained in the respective incubations of SNAP in the presence of cells and were confirmed by continuous monitoring of the SNAP absorption with the stopped-flow apparatus (results not shown).

The decomposition of SNAP was strongly inhibited in the presence of the metal-ion chelators desferal and EDTA. Under normoxic conditions, the SNAP concentration decreased by only 9%, but in the control a loss of 20% was observed after 30 min (Table 2). Under hypoxic conditions, desferal or EDTA reduced the decay of SNAP to 17% and 14% respectively, whereas in the control the SNAP concentration decreased by 60%. In incubations with Cu(II) ions, the decay of SNAP was total within 8 min. This effect was again abolished by EDTA (results not shown).

·NO concentrations

Oxyhaemoglobin method

The concentration of ·NO released from 5 mM SNAP under normoxic conditions remained at about 30 μM for 45 min and then decreased to 10 μM after 90 min of incubation (Figure 3a; Table 1). In hypoxic incubations, the ·NO concentration, determined 2 min after the start of the incubation, was already at 74 μM. ·NO peaked at 136 μM after 15 min and then decreased constantly to 36 μM after 90 min of incubation. The concentration of ·NO released from 10 mM SNAP peaked at 44 μM after 15 min and decreased to 24 μM after 60 min of normoxic incubation (Table 1). In the hypoxic incubations, the ·NO released from 2 mM SNAP was 49 μM after 15 min and fell slightly to 40 μM after 60 min. The results of experiments with SNAP in the presence of cells were similar to those obtained in the cell-free system (results not shown).

The concentration of ·NO recorded during the decay of 20 mM SNP remained between 15 μM and 25 μM during 90 min of normoxic incubation (Figure 3b). In the hypoxic incubations, significantly higher ·NO concentrations were again found. The ·NO concentration was 28 μM after 2 min, peaked at 66 μM after 15 min of incubation and decreased to 28 μM after 60 min of incubation. The time courses of the ·NO concentrations in the presence of cells were similar to those without cells (results not shown).

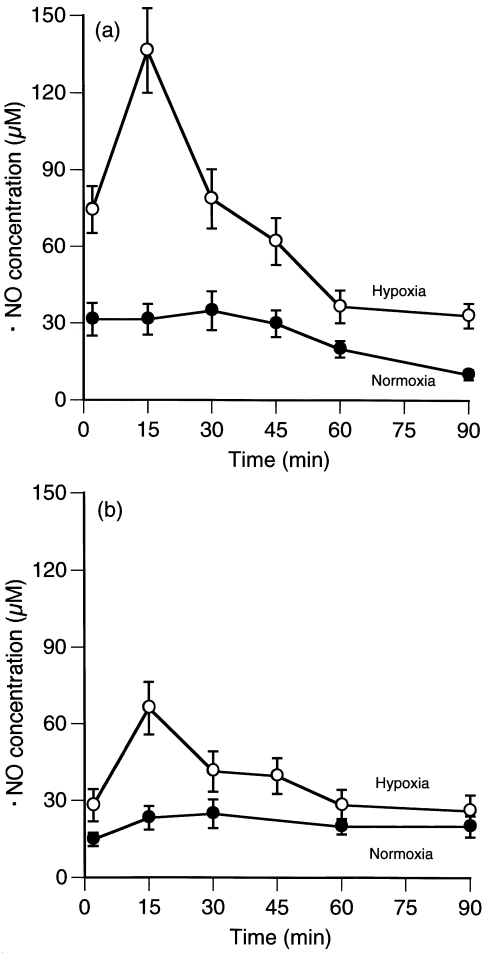

Figure 3 ·NO concentrations

(**a**) 5 mM SNAP and (**b**) 20 mM SNP were incubated in 5 ml of Krebs–Henseleit buffer (pH 7.4) at 37 °C for 90 min. Normoxic incubations were performed in air-saturated and hypoxic incubations in N_2-saturated Krebs–Henseleit buffer. At the times indicated, samples were taken and the ·NO concentrations were determined spectrophotometrically by the oxyhaemoglobin method. The values are the means ± S.D. of 3–4 experiments.

The concentration of ·NO released from 2 mM spermine-NONOate remained at around 20 μM during 60 min of both normoxic and hypoxic incubations (Table 1).

Trapping of ·NO by NOCTs

NOCT-44 and NOCT-20 form persistent nitroxide radicals in the reaction with ·NO (Figure 4). The ESR hyperfine splittings a and g factors of the ·NO adducts of the NOCTs [NOCT-44NO: $a(^{14}N) = 2.23$ mT, $g = 2.0063$; NOCT-20NO: $a(^{14}N) = 1.15$ mT, $g = 2.0063$] corresponded to data of similar nitroxides previously reported [21,26]. From 5 mM SNAP, the concentration of ·NO trapped with NOCT-44 remained in the region of 16 μM during 60 min of normoxic incubation (Figure 5). Again, markedly increased ·NO concentrations were observed under hypoxic conditions, with a peak value of 113 μM after 20 min of incubation. The ·NO concentration then decreased to about 33 μM after 40 min. These observations were paralleled by the release of ·NO from SNAP in THF solution, continuously monitored by trapping with NOCT-20 (results not shown). Using this approach, we found that ·NO production under

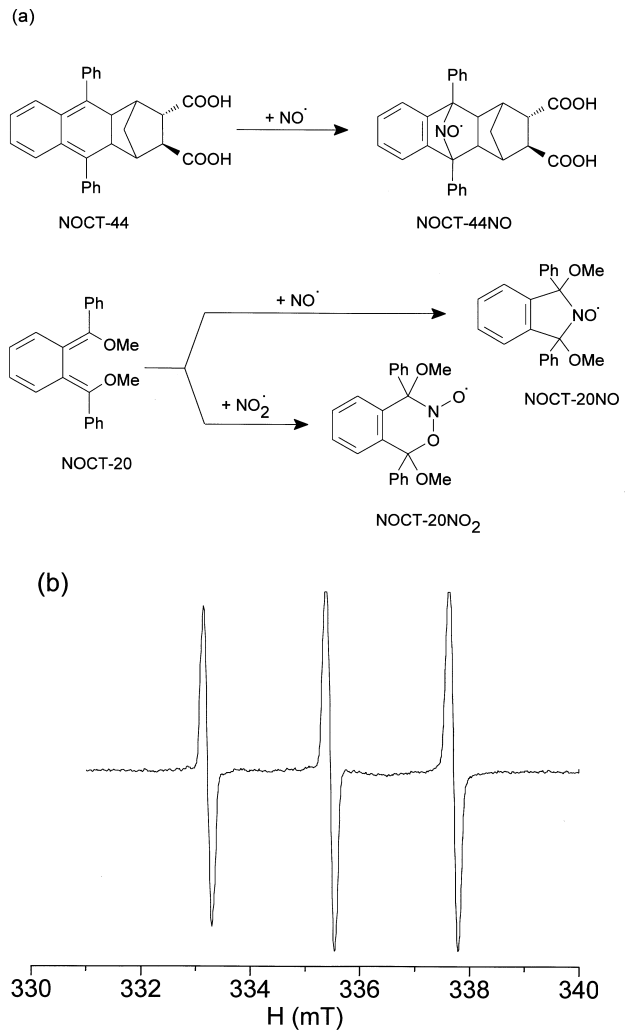

Figure 4 Reaction of ·NO with NOCT-44 and of ·NO and $\cdot NO_2$ with NOCT-20, and the ESR spectrum of the nitroxide radical NOCT-44NO

(**a**) Scheme of the reactions of ·NO with NOCT-44 and of ·NO and $\cdot NO_2$ with NOCT-20 yielding the respective nitroxide radicals NOCT-44NO, NOCT-20NO and NOCT-20NO_2. (**b**) ESR spectrum of NOCT-44NO in THF/H_2O–phosphate buffer (pH 7.8) at 25 °C [$a(^{14}N) = 2.23$ mT, $g = 2.0063$].

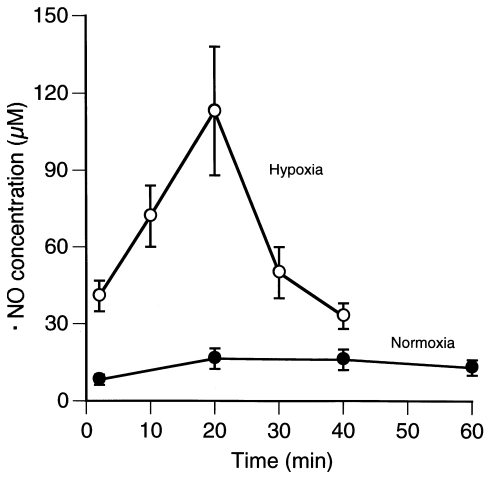

Figure 5 ·NO concentrations from SNAP, trapped by NOCT-44

SNAP (5 mM) was incubated in 1 ml of phosphate buffer (pH 7.8) at 37 °C for 60 min. Normoxic incubations were performed in air-saturated and hypoxic incubations in N_2-saturated phosphate buffer. At the times indicated the ·NO concentrations were determined by the ·NO-induced conversion of NOCT-44 into the nitroxide radical NOCT-44NO. The spin concentrations were measured by ESR spectroscopy. The values are the means ± S.D. of four experiments.

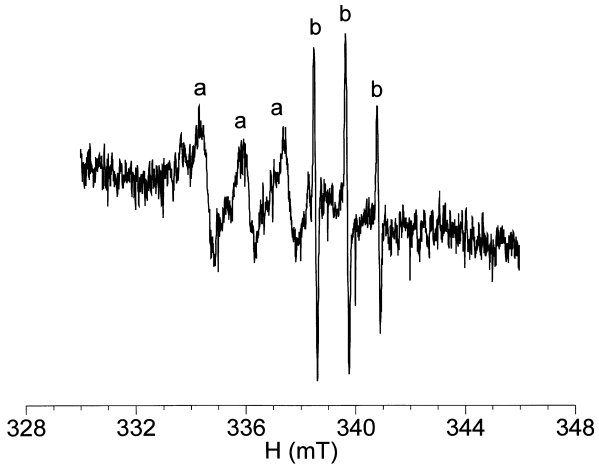

Figure 6 ESR signals of $[Fe(CN)_4NO]^{2-}$ and NOCT-20NO

A mixture of SNP (0.5 M) and ascorbic acid (0.3 M) in phosphate buffer (pH 7.8) and NOCT-20 in benzene was incubated at 25 °C for 10 min in the cavity of the ESR spectrometer. The ESR signals of $[Fe(CN)_4NO]^{2-}$ are indicated by peaks marked a and those of NOCT-20NO by peaks marked b. $[Fe(CN)_4NO]^{2-}$: $a(^{14}N) = 1.48$ mT, $g = 2.0279$; NOCT-20NO: $a(^{14}N) = 1.15$ mT, $g = 2.0063$.

normoxic conditions was not significant, whereas, in the hypoxic incubations, the build-up of the NOCT-20NO ESR signal followed roughly ($k = 2.5 \times 10^{-4}\ s^{-1}$) the same time course as that measured for the decay of SNAP in aqueous solution by UV/visible spectroscopy (see Discussion). A final concentration of 61 μM NOCT-20NO was attained after 90 min of incubation.

Unfortunately, the determination of ·NO production from 20 mM SNP in aqueous buffer solution by application of NOCT-44 was not possible. No ESR signals of NOCT-44NO were detected either in the presence or absence of O_2. It is well established that SNP does not release ·NO spontaneously but requires an initial reduction step [27]. We now found that the ·NO adduct of NOCT-44, the nitroxide NOCT-44NO, is rapidly degraded under the reductive conditions that are necessary for SNP to generate ·NO. However, trapping of ·NO by NOCT-44 was observed by UV-photolysis of SNP (results not shown). Complementary experiments revealed that the ESR signal of NOCT-44NO (approx. 10^{-4} M), independently generated from ·NO gas, is destroyed within seconds by the addition of equimolar amounts of reducing agents such as $FeCl_2$, glucose or ascorbic acid. On the other hand, the addition of these compounds to SNP solutions resulted in the formation of the well-known ESR spectrum of the $[Fe(CN)_4NO]^{2-}$ complex [27,28], the intermediate from which ·NO is believed to be released. The formation of ·NO under such conditions was confirmed by experiments in which benzene solutions of NOCT-20 were injected into ESR tubes containing aqueous solutions of SNP (0.5 M) and one of the reductants (0.3 M) mentioned above. Measuring both liquid phases simultaneously, the ESR signals of both $[Fe(CN)_4NO]^{2-}$ and NOCT-20NO were detected (Figure 6). The signals of the

latter radical increased with time. Repeating the foregoing experiments with air-saturated solutions gave similar ESR spectra, although the release of ·NO was somewhat slower. Interestingly, in addition to the spectra of $[Fe(CN)_4NO]^{2-}$ and NOCT-20NO we also observed the ESR signal of the $\cdot NO_2$ adduct of NOCT-20, NOCT-20NO_2 [$a(^{14}N) = 2.65$ mT, $g = 2.0058$], confirming the expected oxidation of ·NO to $\cdot NO_2$ under these conditions.

DISCUSSION

SNAP and SNP damaged the rat-liver sinusoidal endothelial cells used in the present experiments (Figure 1), in agreement with results of experiments with other cultivated rat cells such as mesangial [29], hepatoma [8] and pancreatic islet cells [14]. Surprisingly, the toxic effects of both ·NO donors were significantly greater under hypoxic conditions compared with the normoxic incubations (Figure 1). These results were unexpected, since the presence of O_2 has been suggested to be a basic requirement for ·NO toxicity [5,8,10]. In our experiments we found that equivalent concentrations of SNAP as well as SNP released much higher concentrations of ·NO under hypoxic than under normoxic conditions (Figure 3). For SNAP, the decomposition, and thereby the amount of ·NO liberated, were shown to be increased by approx. 500% in the absence of O_2 (Figure 2). Least-squares fitting of the data to an empirical rate law $c_t = c_o \times e^{-kO_2 t} + c_\infty$ yields apparent first-order rate constants of $k^{O_2} = 4.1 \times 10^{-4} \cdot s^{-1}$ and $k^{N_2} = 7.2 \times 10^{-4} \cdot s^{-1}$. Thus the rate constants are only slightly affected by O_2; however, since the initial rate of decay is increased approx. 5-fold, and the decomposition occurs to a greater extent (final concentration of SNAP of 0.7 mM versus 3.5 mM), a greater amount of ·NO is produced under hypoxia. Accordingly the enhanced toxicity of SNAP and SNP in the hypoxic incubations could be attributed to the more pronounced release of ·NO from these compounds under hypoxic conditions producing higher ·NO concentrations (Table 1, Figure 3). Thus the increased cytotoxicity is specifically related to the chemistry of the individual ·NO-releasing compound.

The biological effects of *S*-nitrosothiols, including SNAP, have been well characterized. The decomposition of these compounds has been shown to be accompanied by the formation of the corresponding disulphides and by the release of ·NO [30,31]. These processes depend on parameters such as temperature, light and pH [32], but little is known about the role of O_2 in this scheme. Though ·NO is liberated from SNAP in an apparent homolytic cleavage of the S–N bond [30,33], it is now established that SNAP does not release ·NO spontaneously but that this reaction is catalysed by trace amounts of transition metal ions, most likely copper and iron [34]. Recent investigations by Askew et al. [35] revealed a linear relationship between the rate of decay of *S*-nitrosothiols, including SNAP, and the concentration of Cu^{2+} ions. Thorough complexation of the transition metal ions resulted in an almost complete suppression of decay. This is also confirmed in our experiments by the inhibitory effects of desferal and EDTA (Table 2). Therefore, in our system, the decay of SNAP and the consequent generation of ·NO must also be related to trace contamination with transition metals. Similar time profiles of ·NO concentration were observed in experiments with and without cells, and we believe that contamination of the buffer solutions initiated the decomposition of SNAP. Since a similar rate of decay of SNAP was also observed in THF solution, one must conclude that the level of contamination is approximately the same in both solvents. Within this context, the protective effect of O_2, depicted by the time profiles in Figure 2, is a consequence of the interaction of O_2 with catalytically active species involved in the catalytic cycle of decomposition. The reactions may be rather complex, taking into account the possible redox interconversions of metal ions. In the case of copper, Askew et al. [35] discussed the likely involvement of Cu^{2+}-nitrosothiol complexes as well as Cu^{2+}/Cu^{+} redox cycles. We hypothesize that O_2 is able to react with the transition metal of the catalytic cycle by transforming it into (an) inactive species. The direct function of O_2 concentration in the decay of *S*-nitrosothiols has been demonstrated recently by Dicks et al. [36]. The authors showed that the decomposition of *S*-nitrosothiols is catalysed by Cu^{+} but not by Cu^{2+}. The decreased decomposition of the *S*-nitrosothiols in the presence of O_2 is attributed to the oxidation of Cu^{+} to Cu^{2+}, thus removing the active catalyst from the system.

The decomposition of pure solutions of SNP, and thereby the release of ·NO, has been shown to be mainly due to photochemical reactions [37,38]. Under physiological conditions, ·NO release from SNP also occurred [38,39]. However, as mentioned previously, the formation of ·NO from SNP is dependent on the effectiveness of various reducing metabolites found in biological systems [40,41]. In the present study, apparent concentrations of ·NO were measured in the incubations with SNP in Krebs–Henseleit buffer (Figure 3b). The ·NO concentrations were generally lower than those obtained with SNAP, indicating either a lower rate of decomposition and/or subsequent reactions of the released ·NO with the constituents. Because similar results were obtained in the presence and absence of cells, the actual reducing agent responsible for the decomposition of SNP must be a constituent of our buffer solution. A likely candidate is glucose, which was present at a concentration of 10 mM. The observation of the characteristic ESR spectrum of the $[Fe(CN)_4NO]^{2-}$ complex in the reaction of SNP with the reducing agents mentioned, as well as the trapping of ·NO with NOCT-20 in the corresponding two-phase experiment, supports this view. Similar to the experiments with SNAP, the concentrations of ·NO were significantly higher under hypoxic conditions compared with normoxic incubations (Figure 3b). Because of the difficulties encountered with the application of the water-soluble ·NO-trap, in this case NOCT-44, the results obtained with the oxyhaemoglobin method could not be independently supported by the NOCT method. One might argue that the oxyhaemoglobin/methaemoglobin conversion is the result of a direct reaction of SNP with oxyhaemoglobin, since it is known that iron–nitrosyl complexes can react directly with oxyhaemoglobin via nitrosyl–ligand transfer [42], i.e. without the necessity to release ·NO into the solution. Reduced haemoglobin has been found to cause ·NO release from SNP [27]. However, the artificial production of ·NO by a direct SNP–oxyhaemoglobin interaction would not explain the strikingly parallel behaviour found in the cell viability experiments.

The effect of O_2 on the ·NO level produced by SNP is yet more difficult to explain than in the case of SNAP. There is some evidence that the effects are basically similar, i.e. a suppression of the decomposition of the ·NO donor by interaction of O_2 with reactive intermediate(s). Though the mechanism of ·NO liberation from SNP under reductive conditions is still not completely known, the following sequence is generally accepted [28]:

$$[Fe(CN)_5NO]^{2-} \xrightarrow{+e^-} [Fe(CN)_5NO]^{3-}$$

$$[Fe(CN)_5NO]^{3-} \xrightarrow{fast} [Fe(CN)_4NO]^{2-} + CN^-$$

$$[Fe(CN)_4NO]^{2-} \rightarrow \rightarrow [Fe(CN)_6]^{4-} + \cdot NO$$

There are reports in the literature that the first reduction step is reversible in the presence of O_2 [40], which indeed would diminish the rate of formation of the reactive species and hence the final concentration of ·NO. However, our ESR experiments provided no clear indication for such a situation because the signal intensity of the intermediate $[Fe(CN)_4NO]^{2-}$ complex was approximately the same in air-and N_2-saturated SNP–reductant mixtures. Clearly, further work is necessary to elucidate the effect of O_2 on the production of ·NO from SNP.

In addition to the faster and more pronounced release of ·NO, the enhanced toxicity of SNAP and SNP under hypoxic conditions could also result from an increased sensitivity of the hypoxic cells towards ·NO [43]. However, this is highly unlikely since an increased toxicity of ·NO under hypoxic conditions would be expected when equal concentrations of ·NO are compared under normoxic and hypoxic conditions. Just the opposite was true. The cytotoxicity of SNAP, when equal ·NO concentrations were released under normoxic and hypoxic conditions, was significantly reduced rather than increased in the hypoxic incubations (Table 1). These observations were confirmed by using spermineNONOate as ·NO-donating compound. SpermineNONOate, which liberated ·NO independently of the O_2 content, was less toxic to endothelial cells under hypoxic than normoxic conditions (Table 1). The similar ·NO concentrations under normoxic and hypoxic conditions in the experiments where different SNAP concentrations were used, and especially in those experiments where ·NO was liberated by spermine-NONOate (Table 1), also exclude the possibility that a decreased breakdown of ·NO accounts for the enhanced toxicity of SNAP and SNP in the hypoxic cells. Hence, the more pronounced release of ·NO from SNAP and SNP is a sufficient explanation for the enhanced cytotoxicity of both compounds under hypoxic conditions. The increased toxicity of ·NO under normoxic conditions when matched concentrations of ·NO were used supports our assumption that the toxic potential of ·NO should be increased in the presence of O_2.

The biological properties of SNAP and SNP have been well documented as both of these substances are of pharmacological and clinical interest. The Fe–NO group is suggested to be responsible for the vasodilatory activity of SNP [27], while the *S*-nitroso group determines the biological activity of SNAP [44]. However, the toxic effects of both compounds have been attributed to the reactivity of ·NO. Accordingly, denitrosylated products were without effect on cell viability. We have shown here that ·NO release from SNAP and SNP is significantly enhanced under hypoxic conditions leading to pronounced damage to endothelial cells. The role of O_2 in these reactions might be profound when the role of ·NO as a messenger and cytotoxic molecule is considered. The dependence on the O_2 content offers new aspects for the biological effectiveness of SNAP and SNP.

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