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Superoxide-Mediated Reduction of the Nitroxide Group Can Prevent Detection of Nitric Oxide by Nitronyl Nitroxides

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Nitronyl nitroxides (NN), a class of compounds which react with nitric oxide forming imino nitroxides, were applied in different systems for the detection of nitric oxide. Addition of a NN to planar monolayers of bovine aortic endothelial cells (BAEC) activated by Ca^{2+} ionophore A23187 immediately resulted in a strong decrease of the ozone-mediated $^{\bullet}NO$ chemiluminescence. Simultaneously, a rapid diminution of the electron spin resonance (ESR) signal intensity of the NN (without detectable formation of the corresponding imino nitroxide) was observed; superoxide dismutase partially inhibited this decrease in the NN concentration. Model experiments using hypoxanthine/xanthine oxidase in aqueous solution and KO_2 in dimethylsulfoxide as sources of $O_2^{\bullet-}$ revealed that there is a rapid reduction of nitronyl nitroxides by superoxide. The second order rate constant for the reaction of the water soluble NN with $O_2^{\bullet-}$ was determined to be $8.8 \cdot 10^5\ M^{-1}s^{-1}$, which is more than two orders of magnitude higher than the value reported previously for reaction with $^{\bullet}NO$ (Woldman *et al.*, BBRC 202, 195–203, 1994). Reduction of the nitronyl nitroxide was also observed in the presence of glutathione, ascorbic acid or rabbit liver microsomes. Incorporation of both nitronyl and imino nitroxides into liposomes strongly decreased reduction by superoxide and other reductants, however, in the presence of microsomes, there was no protective effect by liposomal encapsulation of NN. The results indicate that in biological systems (in addition to other reducing agents) the presence of superoxide can prevent the detection of nitric oxide using nitronyl nitroxides.

Keywords: Nitric oxide, superoxide, free radical, nitronyl nitroxide, hydroxylamine, electron spin resonance, chemiluminescence, endothelial cells, microsomes, liposomes

Abbreviations: Bovine aortic endothelial cells, BAEC; cholesterol, CH; 5,5-dimethyl-2,4-diphenyl-4-methoxy-2-imidazoline-3-oxide-1-oxyl, NN 2; 5,5-dimethyl-2,4-diphenyl-4-methoxy-2-imidazoline-1-oxyl, IN 2; 5,5-dimethylpyrroline-1-oxide, DMPO; dimethylsulfoxide, DMSO; 4,4,5,5-dimethyl-2-(4-trimethylammoniophenyl)-2-imidazoline-3-oxide-1-oxyl methyl sulfate, NN 1; 4,4,5,5-dimethyl-2-(4-trimethylammoniophenyl)-2-imidazoline-1-oxyl methyl sulfate, IN 1; ethylenediaminetetraacetic acid, EDTA; electron spin resonance, ESR; β-nicotinamide adenine dinucleotide phosphate (reduced form), NADPH; multilamellar vesicles, MLV; phosphate buffered saline, PBS; phosphatidylcholine, PC; reverse phase evaporation vesicles, REV; small unilamellar vesicles, SUV; superoxide dismutase, SOD; xanthine oxidase, XO

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INTRODUCTION

The role of nitric oxide in physiological and pathological processes has been extensively studied.[1,2,3] One of the crucial points in $^{\bullet}NO$ research is the specific and sensitive detection of this radical. Numerous methods have been developed for the quantification of nitric oxide including analytical methods such as $^{\bullet}NO$-mediated chemiluminescence[4] or electrochemical detection.[5] A highly sensitive technique using a porphyrin-based microsensor was described by Malinski *et al.*,[6] however, there are several unresolved problems in applying this method to biological systems.[7] $^{\bullet}NO$ detection by ESR spectroscopy has been performed using nitroso spin traps[8] or iron-nitrosyl complexes.[9] Promising techniques based on ESR spectroscopy of other reaction products of $^{\bullet}NO$ were proposed: spin trapping using cheletropic spin traps[10] and detection of imino nitroxides (IN) formed by a reaction of $^{\bullet}NO$ with nitronyl nitroxides (NN).[11] NN have also been found to exert physiological effects (inhibition of vasorelaxation in smooth muscle of rabbit aorta[12]). However, if these probes for nitric oxide are to be applied to biological systems, questions regarding their stability in these systems have to be considered. In general, nitroxides are intracellularly reduced to the corresponding hydroxylamines.[13] Recently, NN and, in particular, IN were found to be much more susceptible to thioglycerol-induced reduction than other nitroxides.[14] The oxidation of superoxide radicals by cyclic nitroxides yields hydroxylamines (ESR-silent) and molecular oxygen.[15] The presence of superoxide has been shown in different biological systems under physiological and, especially, under pathological conditions. Therefore, the simultaneous existence of $O_2^{\bullet -}$ and $^{\bullet}NO$ has to be considered. This study deals with reactions of nitronyl (and imino) nitroxides in cells, subcellular fractions and model systems with special emphasis on their interaction with superoxide radicals.

MATERIALS AND METHODS

Chemicals

Nitronyl nitroxides were synthesized as described by Woldman *et al.*[14] (NN 1) and Grigor'ev *et al.*[15] (NN 2). Figure 1 shows the structures of these nitroxides. 5,5-dimethylpyrroline-1-oxide (DMPO) was obtained from Aldrich and used as reported previously.[17] Xanthine oxidase (XO, EC 1.1.3.22) and superoxide dismutase (SOD, EC 1.15.1.1) were purchased from Boehringer Mannheim. Synthesis of peroxynitrite was accomplished according to Beckman *et al.*[18] All commercial chemicals were of the highest quality available.

Preparation of Liposomes

80 mg egg yolk phosphatidylcholine (PC, Lipoid) or PC and 19.2 mg cholesterol (CH, Merck) mixed at a molar ratio of 1:0.25 were dissolved in chloroform. The organic solvent was removed by rotary evaporation at 45°C. Multilamellar vesicles (MLV) were prepared by addition of 4 ml phosphate buffered saline (PBS, pH = 7.4) containing 1.6 mg nitroxide to the dry lipid film and shaking this mixture for 4 hours at room temperature. Unilamellar vesicles (SUV) were formed from MLV by sonication under nitrogen 4 × 4 min with a 50% pulsed input using a Branson Sonifier 260 (Branson Ultrasonic). For reverse phase evaporation vesicles (REV) preparation the lipid composition dissolved in freshly distilled diethyl ether was sonicated for 3 min directly with 4 ml nitroxide solution in PBS.[19] The organic solvent was removed under vacuum.

Preparation of Microsomes

Rabbit liver microsomes were prepared from phenobarbital-induced male rabbits by differential centrifugation[20] (protein content was (35 ± 5) mg/ml). The samples (in 0.1 M PBS, pH 7.4, 0.25 M sucrose) were frozen in liquid nitro-

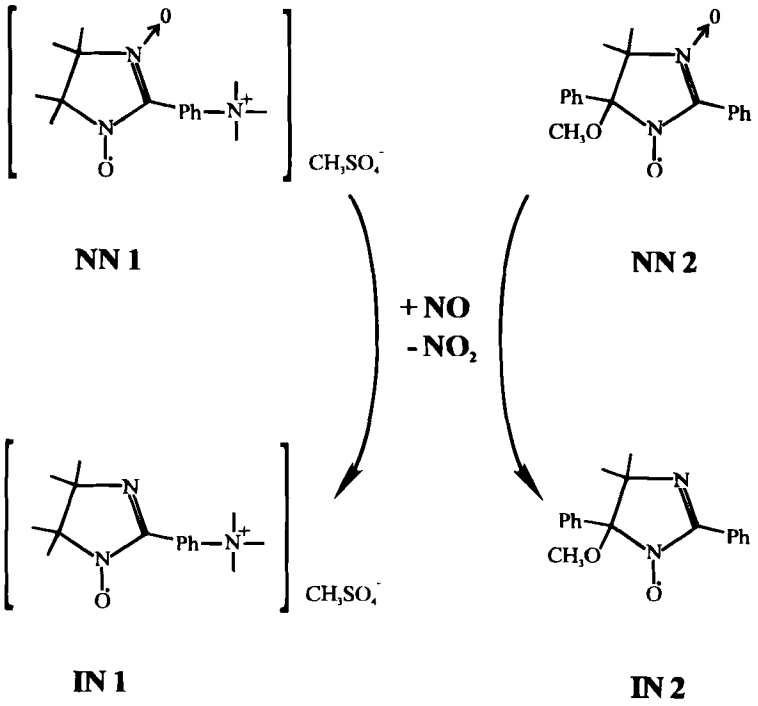

FIGURE 1 Structural formulae of the nitronyl nitroxides 4,4,5,5,-dimethyl-2-(4-trimethylammoniophenyl)-2-imidazoline-3-oxide-1-oxyl methyl sulfate (NN 1), 5,5-dimethyl-2,4-diphenyl-4-methoxy-2-imidazoline-3-oxide-1-oxyl (NN 2) and of the corresponding imino nitroxides 4,4,5,5-dimethyl-2-(4-trimethylammoniophenyl)-2-imidazoline-1-oxyl methyl sulfate (IN 1) and 5,5-dimethyl-2,4-diphenyl-4-methoxy-2-imidazoline-1-oxyl (IN 2), Ph indicates phenyl group

gen and stored at 253 K. Prior to the experiments, the frozen aliquots were thawed and diluted with PBS to a protein content of 0.3 mg/ml.

Endothelial Cell Culture

Bovine aortic endothelial cells (BAEC, passage 12 to 15)[21] were cultivated in Corning flasks (25 cm^2) in Minimum Essential Medium Eagle (Sigma) supplemented with 10% fetal calf serum (Sigma), 0.2 mM glutamine without antibiotics at 37°C in 5% CO_2 and 95% air. Subcultivation was made twice a week with trypsine/EDTA (each 0.25% v/v) in PBS. Quality of cultivation was verified by phase contrast microscopic cobblestone appearance at confluence, presence of factor VIII antigen, contents of alkaline phosphatase and angiotensin converting enzyme. The protein content of the cells was between 500 µg and 700 µg per flask. Experiments were performed in PBS supplemented with 1 mM Ca^{2+}, 0.5 mM Mg^{2+}, 10 mM glucose and 10 µM L-arginine after washing the cells three times with PBS.

ESR Experiments

ESR experiments were carried out at room temperature (21°C) on a Bruker ECS 106 X-band spectrometer (equipped with a high sensitivity rectangular-mode cavity ER 4102 ST). The studies on the decay of nitroxides in the presence of reducing agents or cells were performed in air-saturated solutions. At predesignated times, aliquots were taken from the supernatant (PBS, pH 7.4) and placed into a flat quartz cell. The following standard conditions were used for ESR spectroscopy: modulation frequency,

100 kHz; modulation amplitude, 0.08 mT; field set, 347.5 mT; scan range, 5 mT; microwave power, 10 mW. Nitroxide concentrations were determined by double integration after baseline correction (relative deviation of data, ≤ 10% of absolute values). The rate constant for the reaction of the nitroxide with $O_2^{\bullet -}$ was determined by a competition kinetics approach according to Samuni *et al.*[22]

Chemiluminescence Measurements

The release of nitric oxide from the cells was monitored at 37°C by ozone-mediated chemiluminescence (without a reducing system) using a Sievers 270B analyzer (Sievers Research Inc., Boulder, CO). For continuous measurements, the headspace gas was forced by vacuum directly to the reaction chamber. The instrument was calibrated using diluted •NO solutions prior to the measurement.

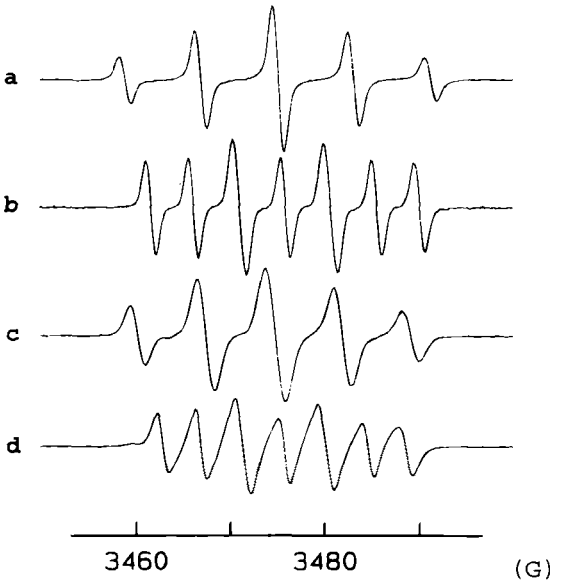

FIGURE 2 ESR spectra of 10 μM NN 1 (a), IN 1 (b) in PBS and of NN 2 (c), IN 2 (d) in dimethylsulfoxide (imino nitroxides b and d were obtained by injection of •NO gas into solutions a and c). Hyperfine splitting parameters of the nitroxides: NN 1, $a_N^{(1)} = a_N^{(3)} = 0.805$ mT; IN 1, $a_N^{(1)} = 0.97$ mT, $a_N^{(3)} = 0.45$ mT; NN 2, $a_N^{(1)} = a_N^{(3)} = 0.72$ mT; IN 2, $a_N^{(1)} \approx 0.88$ mT, $a_N^{(3)} = 0.40$ mT.

RESULTS

The reaction of nitric oxide with nitronyl nitroxides was investigated in both protic (aqueous solution) and aprotic medium (dimethylsulfoxide, DMSO). Figure 2 shows the ESR spectra of NN 1 and NN 2 in PBS and DMSO, respectively, and of the corresponding imino nitroxides IN 1 and IN 2 obtained after injection of •NO gas into NN solutions. The spectra of IN 1 and IN 2 were also observed after addition of NN 1 and NN 2 to a •NO-liberating compound (0.5 mM *S*-Nitroso-*N*-acetylpenicillamine).

In order to investigate the cellular-induced decay of NN 1 and IN 1, the compounds were added to the supernatant (PBS) of BAEC monolayers, and the nitroxide radical decay over time of incubation was studied by EPR spectroscopy (Fig. 3). The nitronyl and the imino nitroxide displayed sufficient stability in the presence of the intact monolayer. It is well known[13] that nitroxides are metabolized by subcellular fractions (cytosol, mitochondria, microsomes). Consistent with these literature data, addition of NN 1 to rabbit liver microsomes in the presence of NADPH resulted in a rapid decrease of the ESR signal intensity. The effect of NADPH-supplemented microsomes and superoxide dismutase on the NN stability was investigated in more detail (Fig. 4). In the absence of NADPH, no effect on the nitroxide concentration was found. Addition of a 200-fold excess of NADPH to NN 1 in the absence of microsomes resulted in a slow decrease of the nitroxide concentration (half-life, approx. 8 min). In the presence of both microsomes and NADPH, the nitroxide concentration declined rapidly to the limit of detection; addition of SOD to this reaction mixture resulted only in a moderate effect. Recently, incorporation of NN into liposomes was shown to be effective in the protection of the nitroxides from reductive attack.[14] In the presence of microsomes how-

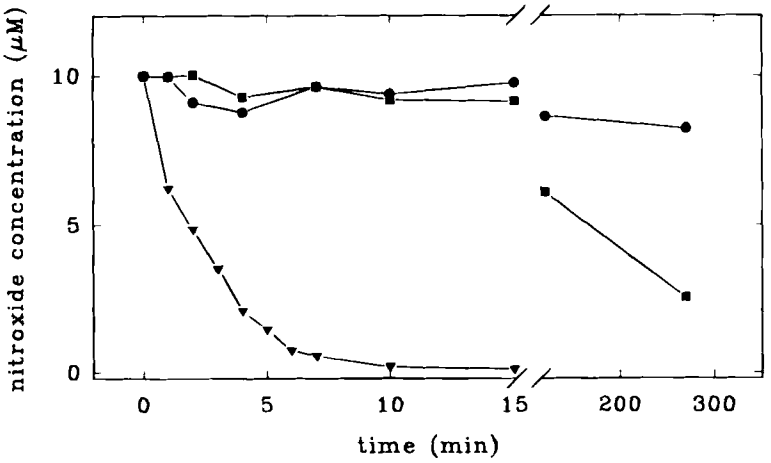

FIGURE 3 Time dependence of the concentration of 10 μM NN 1 (●) and IN 1 (■) added to the medium (PBS) of bovine aortic endothelial cell monolayers (samples taken from the supernatant) and of 10 μM NN 1 in the presence of rabbit liver microsomes (▼)

ever, this protective effect of liposome encapsulation was not observed.

Figure 5 demonstrates the reduction of NN 1 by other biologically relevant reductants. The addition of 1 mM glutathione resulted in a slow decrease of the ESR signal intensity of 10 μM NN1 In contrast, the addition of 400 μM ascorbic acid to 10 μM NN 1 lead to a rapid and full decay of the nitroxide and the appearance of an ESR signal with a splitting of 0.17 mT at g = 2.005, clearly indicating formation of the ascorbyl radical. The addition of SOD did not exert a discernable effect, whereas encapsulation of the nitroxide into liposomes (MLV) resulted in complete inhibition of this reduction process.

When NN 1 was added to A23187-stimulated endothelial cells a strong inhibition of the ozone-mediated $^{\bullet}NO$ chemiluminescence was observed (Fig. 6) which was accompanied by a much faster decrease of the nitroxide concentration (<10% of

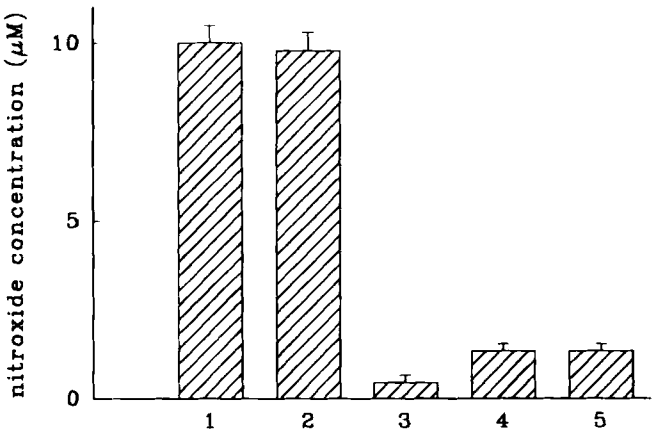

FIGURE 4 Concentration of NN 1 (determined by ESR spectroscopy) in the presence of microsomes (mean + S.D.; incubation time, 5 min; incubation medium, PBS): (1) 10 μM NN 1; (2) 10 μM NN 1, microsomes without NADPH; (3) 10 μM NN 1, microsomes, 1.5 mM NADPH; (4) 10 μM NN 1, microsomes, 1.5 mM NADPH, 100 U/ml SOD; (5) 10 μM NN 1 in liposomes, microsomes, 1.5 mM NADPH.

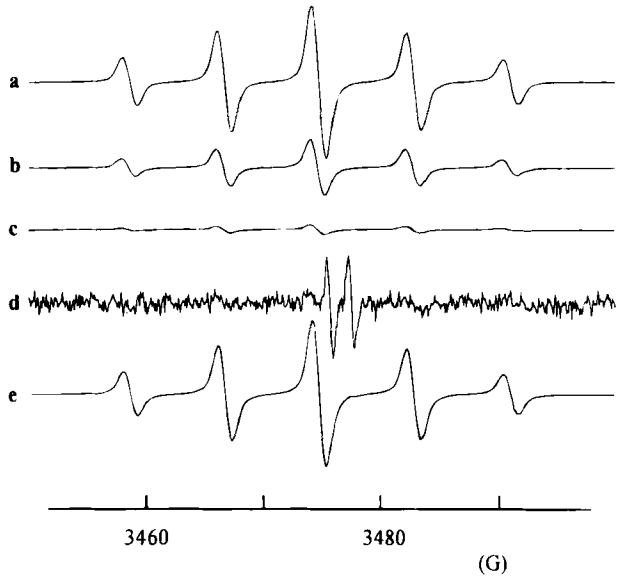

FIGURE 5 Reduction of ESR signal intensity of 10 μM NN 1 dissolved in PBS (a, reference spectrum) in the presence of 1 mM glutathione (b, 10 min; c, 30 min) and 0.4 mM ascorbic acid (d, 1 min) and effect of NN 1 encapsulation into multilamellar vesicles (e, conditions identical to d)

control intensity) compared to resting cells without any indication for the formation of IN 1 (inset Fig. 6). In the presence of SOD, the decay of the nitroxide was inhibited (approx. 70% of control intensity).

These data prompted an investigation into the interaction of nitronyl nitroxides with $O_2^{\bullet-}$ in aqueous and aprotic solutions (Fig. 7). Using XO/HX it was found that the generation of superoxide radicals in the presence of nitronyl nitroxides led to a rapid (SOD-sensitive) diminution of the nitroxide ESR signal intensity. Under these conditions neither xanthine oxidase nor hypoxanthine alone exerted any effect on the concentration of NN 1. Analogous results were obtained by the addition of superoxide to NN 2

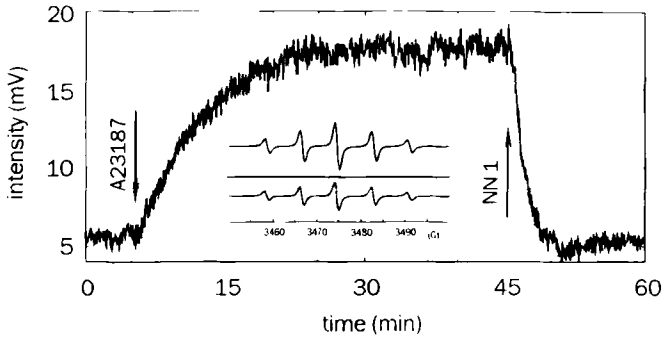

FIGURE 6 Continuous chemiluminescence measurement of A23187 (10 μM)-induced •NO liberation from monolayers of bovine aortic endothelial cells and effect of the addition of 10 μM NN 1. **Inset:** ESR spectra of NN 1; upper spectrum, reference spectrum of 10 μM NN 1 in PBS (without cells); middle spectrum obtained after addition of 10 μM NN 1 to the cell monolayer (cell medium, PBS; incubation time, 1 min); lower spectrum, conditions identical to middle spectrum except for presence of 100 U/ml SOD

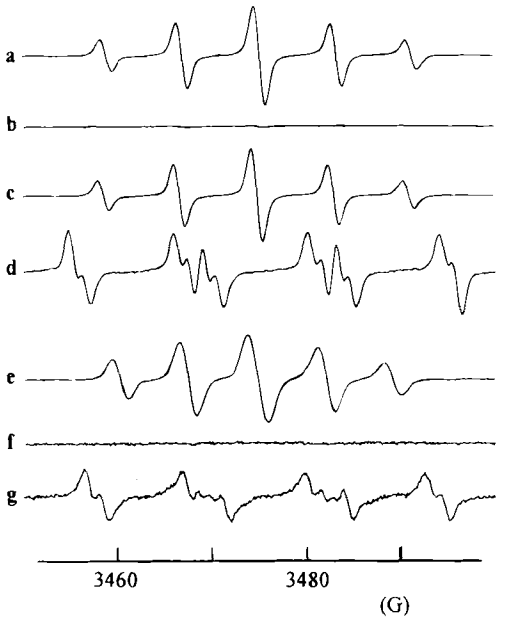

FIGURE 7 Interaction of nitronyl nitroxides with superoxide radicals in PBS (a–d) and dimethylsulfoxide (e–g); ESR spectra obtained from: (a) 10 μM NN 1, 0.1 U/ml xanthine oxidase; (b) 10 μM NN 1, 0.1 U/ml xanthine oxidase, 50 μM hypoxanthine; (c) 10 μM NN 1, 0.1 U/ml xanthine oxidase, 50 μM hypoxanthine, 100 U/ml superoxide dismutase; (d) DMPO/OOH, generated by addition of 0.1 M DMPO to 0.1 U/ml xanthine oxidase, 50 μM hypoxanthine; (e) 10 μM NN 2; (f) 10 μM NN 2, 1 mM KO_2 solution; (g) DMPO/•OOH, generated by addition of 0.1 M DMPO to 1 mM KO_2 solution.

Hyperfine splitting constants of DMPO adducts: a_N = 1.17 mT, a_H^β = 1.43 mT, a_H^γ = 0.13 mT (c) a_N = 1.05 mT, a_H^β = 1.29 mT, a_H^γ = 0.11 mT (f).

dissolved in DMSO as well as by the reaction of IN with $O_2^{\bullet-}$ (data not shown in detail).

When superoxide radicals were generated by XO/HX in the presence of liposome encapsulated NN 1, only a slow decrease of the ESR signal (approx. 20% within 3 hours) was found (Fig. 8). Addition of 10 μl •NO gas led to the formation of IN 1 with a yield of about 90%. However, the imino nitroxide was found to be considerably more susceptible to superoxide-mediated reduction compared to the corresponding nitronyl nitroxide. Incorporation of a preformed imino nitroxide into liposomes resulted in a slightly higher stability of the nitroxide during the first minutes of incubation.

Figure 9 underlines the higher reduction rate of IN compared to NN. Relatively low amounts of XO/HX were added to an equimolar mixture of both nitroxides. After 1 min, an almost complete reduction of the imino nitroxide was observed whereas the decrease in the concentration of the nitronyl nitroxide was low (<5%).

DISCUSSION

The introduction of the spin trapping technique for the detection of low concentrations of short-lived radicals[23] established a basis for new and fascinating insights into the role of free radicals in biological systems. The application of nitronyl nitroxides for the detection of nitric oxide is similar in its approach. The stability of the nitroxides is an important aspect, for "conventional" spin trapping as well as for •NO detection by NN. There are several reactants in biological systems known to reduce nitroxides to the respective hydroxylamine.[24] This study illustrates the susceptibility of nitronyl- and of imino nitroxides to reductive attack not only by reducing agents such as GSH or ascorbic acid but by $O_2^{\bullet-}$. An increase in the intracellular calcium concentration by A23187 results in $O_2^{\bullet-}$ formation in endothelial cells,[25] *e.g.*, by activation of the arachidonic acid metabolism.[26] Even the amount of superoxide released from an A23187-stimulated monolayer of endothelial cells is sufficient for a fast (SOD-sensitive) decrease of the nitronyl nitroxide concentration. This decrease presumably prevents the ESR spectroscopic detection of imino nitroxides although •NO formation was proven *in situ* unambiguously by ozone-mediated chemiluminescence (which, in turn, was completely suppressed by addition of 50 μM N^G-nitro-*L*-arginine methyl ester and increased by addition of SOD[27]). Consequently, the imino nitroxide should be formed by the reaction of NN 1 with •NO. The lack of detection of IN1 by ESR spectroscopy is explained by (i) the lower sensitivity compared to the chemiluminescence method (•NO concentrations detected in

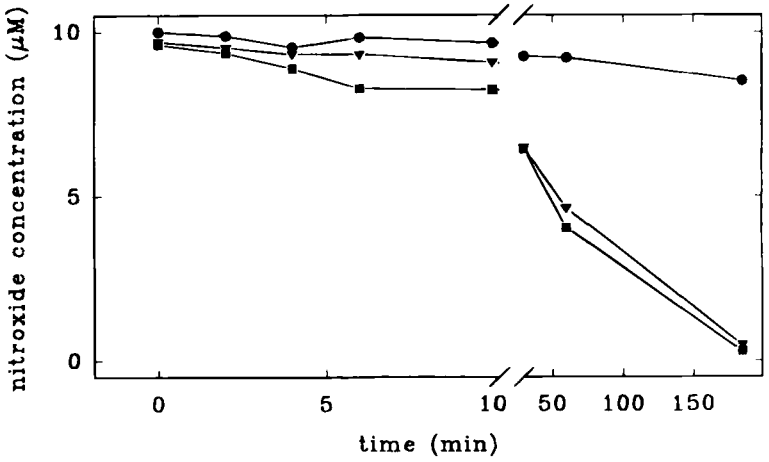

FIGURE 8 Time dependence of the concentration of 10 μM NN 1 (●, incorporated into large unilamellar liposomes), approx. 9 μM IN 1 (■, formed by injection of 10 μl •NO gas into vesicles containing NN 1) and 10 μM preformed IN 1 (▼, incorporated into large unilamellar liposomes) in PBS during generation of superoxide radicals by 0.1 U/ml xanthine oxidase and 50 μM hypoxanthine

these experiments are in the range of 10 nmol/l) and (ii) the observation that there is a much faster reduction of IN (>2 orders of magnitude as seen by thioglycerol-induced reduction[14]) compared to NN. In additional experiments (data not shown in detail), cell damage (*e.g.*, measured by release of lactate dehydrogenase) has been observed after treatment of the BAEC with 10 μM A23187. Thus, it cannot be excluded that a liberation of other reducing agents from the cells may be involved in the nitroxide reduction although, from the data presented (inset Fig. 6), superoxide is considered to play a major role. An interaction of NN 1 with peroxynitrite formed by the reaction of $O_2^{\bullet-}$ with •NO does not contribute to the nitroxide reduction: the decrease of the nitroxide concentration was less than 10% within 30 minutes in the presence of 20-fold excess of peroxynitrite. The reaction of NN with $O_2^{\bullet-}$ was also observed in model systems (Fig. 7). The presence of superoxide was demonstrated by ESR spectra which are clearly assignable to DMPO adducts of superoxide in both aqueous solution and DMSO.[28] The second order rate constant for the reaction of NN 1 with $O_2^{\bullet-}$ was determined to be $8.8 \pm 0.9 \cdot 10^5$ $M^{-1}s^{-1}$. This value is slightly higher but in the same range as the rate constant obtained for another nitroxide (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, TEMPO).[22] As the rate constant for the reaction of NN 1 with •NO is $k = 6 \cdot 10^3$ $M^{-1}s^{-1}$ [14] nitric oxide must be present in large excess compared to superoxide in order to obtain detectable amounts of the corresponding imino nitroxide.

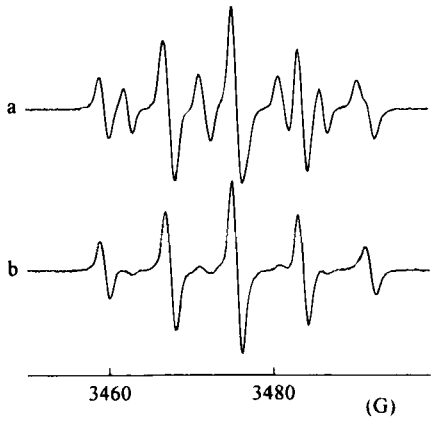

FIGURE 9 ESR spectra obtained from an equimolar (10 μM) mixture of NN and IN in PBS in the absence (a) and presence of 20 mU/ml xanthine oxidase and 10 μM hypoxanthine (b; incubation time, 1 min)

In agreement with literature data,[13] superoxide-independent reactions (cytochrome P-450 reductase activity) were observed in microsomes

leading to the reduction of the nitroxides. It was shown by Woldman *et al.*[14] that incorporation of nitronyl nitroxides into liposomes decreases the reduction of the radical considerably. This protection is of special importance regarding the attack of superoxide (Fig. 7). There are contradictory results regarding the permeation of $O_2^{\bullet-}$ through lipid bilayers.[29,30] The data presented here supports a slower permeation of superoxide into the inner compartment of the liposome compared to its free diffusion in the pure aqueous phase. The above-mentioned fast reduction of the imino nitroxide was also observed using $O_2^{\bullet-}$ as a reductant: additional experiments (Fig. 9) revealed that the rate constant for this reduction is nearly two orders of magnitude greater than that of nitronyl nitroxide. Thus, Fig. 8 allows to conclude that even the decreased flow of superoxide is sufficient for a much stronger reduction of the imino nitroxide. Similar reduction kinetics were observed for preformed IN encapsulated into liposomes and IN obtained from NN reduced consecutively by $^{\bullet}NO$ and $O_2^{\bullet-}$ (Fig. 8 b/c) suggesting that liposome damage attributable to the presence of both superoxide and nitric oxide may not be significant. On the other hand, no protection is afforded in the presence of microsomes. This effect may be due to a fusion between microsomal lipid residues and liposomes.[31]

Experiments were performed using A23187-stimulated BAEC monolayers to compare the sensitivity of the SOD-inhibitable nitroxide reduction to "conventional" spin trapping of superoxide. Due to the low concentration of $O_2^{\bullet-}$, formation of this species could not be detected clearly by ESR spectroscopy using 100 mM DMPO as a spin trapping agent. The high sensitivity of the nitronyl and imino nitroxides to superoxide-mediated reduction may offer another conceivable application for these substances: superoxide detection by determination of the SOD-inhibitable reduction of the nitroxide. A more exact quantification may be possible by reoxidation of the hydroxylamines as described for spin adducts of α-phenyl-N-*tert*-butylnitrone.[32] In preliminary experiments using different oxidants (MnO_2, $K_3Fe(CN)_6$)[33] it was found that a reoxidation of the hydroxylamines of both nitronyl and imino nitroxides is possible. Control experiments also revealed the possibility of a $^{\bullet}NO$-independent formation of the imino nitroxide during the reoxidation of NN.

Superoxide reacts efficiently with nitric oxide,[34] peroxynitrite formed by this reaction is thought to be a highly cytotoxic intermediate.[35] Recently, nitroxides without nitrone group (*e.g.*, TEMPO) were found to exert protective effects in quinone-mediated cytotoxicity.[36] Thus, from a pharmacological point of view, scavenging of both superoxide and nitric oxide may be an additional and promising feature of nitronyl nitroxides.

ESR spectroscopy of nitronyl- and imino nitroxides is a reliable tool to study reactions of nitric oxide in the absence of reducing agents. However, the results indicate limitations concerning the applicability of nitronyl nitroxides for $^{\bullet}NO$ detection in biological systems: the sensitivity of this method is lower compared to other techniques (such as ozone-mediated chemiluminescence), partly due to rapid reduction of the nitroxide. Superoxide, if present, can contribute to this reduction to a great extent. Encapsulation of nitronyl nitroxides into liposomes decreases its reduction by membrane-impermeable compounds, however, there are situations in biological environment and model systems where the stability of liposomes is questionable. On the other hand, scavenging of both superoxide and nitric oxide may have beneficial pharmacological implications which warrant further investigations.

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References

[1] Palmer, R. M. J., Ferrige, A. G. and Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

[2] Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1991) Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacological Reviews*, **43**, 109–142.

[3] Moncada, S. and Higgs, A. (1993) Mechanisms of Disease—The L-Arginine Nitric Oxide Pathway. *New England Journal of Medicine*, **329**, 2002–2012.

[4] Archer, S. (1993) Measurement of nitric oxide in biological models. *FASEB-Journal*, **7**, 349–360.

[5] Kiechle, F. L. and Malinski, T. (1993) Nitric Oxide—Biochemistry, Pathophysiology, and Detection. *American Journal of Clinical Pathology*, **100**, 567–575.

[6] Malinski, T. and Taha, Z. (1992) Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. *Nature*, **358**, 676–678.

[7] Malinski, T., Taha, Z., Grunfeld, S., Burewicz, A., Tomboulian, P. and Kiechle, F. (1993) Measurements of Nitric Oxide in Biological Materials Using a Porphyrinic Microsensor. *Analytica Chimica Acta*, **279**, 135–140.

[8] Arroyo, C. M. and Kohno, M. (1990) Difficulties encountered in the detection of nitric oxide (NO) by spin trapping techniques. A cautionary note. *Free Radical Research Communicatons*, **14**, 145–155.

[9] Lancaster, Jr, J. R. and Hibbs, Jr, J. B. (1990) EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proceedings of the National Academy of Sciences of the United States of America*, **37**, 1223–1227.

[10] Korth, H.-G., Sustmann, R., Lommes, P., Paul, T., Ernst, A., de Groot, H., Hughes, L. and Ingold, K. U. (1994) Nitric Oxide Cheletropic Traps (NOCTs) with Improved Thermal Stability and Water Solubility. *Journal of the American Chemical Society*, **116**, 2767–2777.

[11] Joseph, J., Kalyanaraman, B. and Hyde, J. S. (1993) Trapping of nitric oxide by nitronyl nitroxides: an electron spin resonance investigation. *Biochemical and Biophysical Research Communications*, **192**, 926–934.

[12] Akaike, T., Yoshida, M., Miyamoto, Y., Sato, K., Kohno, M., Sasamoto, K., Miyazaki, K., Ueda, S. and Maeda, H. (1992) Antagonistic action of Imidazolineoxyl N-oxides against endothelium-derived relaxing factor/NO· through a radical reaction. *Biochemistry*, **32**, 827–832.

[13] Iannone, A., Bini, A., Swartz, H. M., Tomasi, A. and Vannini, V. (1989) Metabolism in rat liver microsomes of the nitroxide spin probe TEMPOL. *Biochemical Pharmacology*, **38**, 2581–2586.

[14] Woldman, Y. Yu., Khramtsov, V. V., Grigor'ev, I. A., Kirilyuk, I. A. and Utepbergenov, D. I. (1994) Spin trapping of nitric oxide by nitronylnitroxides: measurement of the activity of NO synthase from rat cerebellum. *Biochemical and Biophysical Research Communications*, **202**, 195–203.

[15] Samuni, A., Krishna, C. M., Mitchell, J. B., Collins, C. R. and Russo, A. (1990) Superoxide reaction with nitroxides. *Free Radical Research Communications*, **9**, 241–249.

[16] Grigor'ev, I. A., Volodarsky, L. B., Starichenko, V. F., Shchukin, G. I., Kirilyuk, I. A. (1985) Route to stable nitroxides with alkoxy groups at α-carbon—the derivatives of 2- and 3-imidazolines. *Tetrahedron Letters*, **26**, 5085–5088.

[17] Haseloff, R. F., Blasig, I. E., Meffert, H. and Ebert, B. (1990) Hydroxyl radical scavenging and antipsoriatic activity of benzoic acid derivatives. *Free Radical Biology and Medicine*, **9**, 111–115.

[18] J. S. Beckman, T. W. Beckman, J. Chen, P. A. Marshall and B. A. Freeman (1990) Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 1620–1624.

[19] Szoka, F. and Papahadjopulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proceedings of the National Academy of Sciences of the United States of America*, **75**, 4194–4198.

[20] Van der Hoeven, T. A. and Coon, M. J. (1974) Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenindinucleotide phosphate—cytochrome P-450 reductase from rabbit liver microsomes. *Journal of Biological Chemistry*, **249**, 6302–6310.

[21] Halle, W., Siems, W. E., Jentsch, K. D., Teuscher, E. und Göres, E. (1984) Die in vitro kultivierte Aorten-Endothelzelle in der Wirkstofforschung—Zellphysiologische Charakterisierung und Einsatzmöglichkeiten der Zellinie BKEz-7. *Pharmazie*, **39**, 77–81.

[22] Samuni, A., Mitchell, J. B., DeGraff, W., Krishna, C. M., Samuni, U. and Russo, A. (1991) Nitroxide SOD-mimics: Modes of action. *Free Radical Research Communications*, **12–13**, 187–194.

[23] Janzen, E. G. and Blackburn, B. J. (1968) Detection and identification of short-lived free radicals by an electron spin resonance trapping technique. *Journal of the American Chemical Society*, **90**, 5909–5910.

[24] Rosen, G. M., Cohen, M. S., Britigan, B. E. and Pou, S. (1990) Application of spin traps to biological systems. *Free Radical Research Communications*, **9**, 187–95.

[25] Kooy, N. W. and Royall, J. A. (1994) Agonist-induced peroxynitrite production from endothelial cells. *Archives of Biochemistry and Biophysics*, **310**, 352–359.

[26] Kuehl, F. A. and Ekgan, R. A. (1980) Prostaglandins, arachidonic acid and inflammation. *Science*, **210**, 978–984.

[27] Zöllner, S., Haseloff, R. F., Mertsch, K. and Blasig, I. E. (1994) Ca^{2+}-induzierte NO-Freisetzung durch Monolayer von Aortenendothelzellen. In *Endothelfunktion und Arteriosklerose* (ed. H. Heinle, H. Schulte and H. K. Breddin), Deutsche Gesellschaft für Arteriosklerose-forschung, Tübingen, pp. 41–45.

[28] Buettner, G. R. (1987) Spin trapping: ESR parameters of spin adducts. *Free Radical Biology and Medicine*, **3**, 259–303.

[29] Mao, G. D. and Poznansky, M. J. (1992) Electron spin resonance study on the permeability of superoxide radicals in lipid bilayers and biological membranes. *FEBS-Letters*, **305**, 233–236.

[30] Rumyantseva, G. V., Weiner, L. M., Molin, Yu. N. and Budger, V. G. (1979) Permeation of liposome membrane by superoxide radical. *FEBS-Letters*, **108**, 477–480.

[31] Pistolesi, R., Corazzi, L. and Arienti, G. (1990) Microsomal protein mediates a pH-dependent fusion of liposomes to rat brain microsomes. *Membrane Biochemistry*, **9**, 253–261.

[32] Sentjurc, M. and Mason, R. P. (1992) Inhibition of radical adduct reduction and reoxidation of the corresponding hydroxylamines in in vivo spin trapping of carbon tetrachloride-derived radicals. *Free Radical Biology and Medicine*, **13**, 151–160.

[33] Hudlický, M. (1990) Oxidations in Organic Chemistry (ACS Monograph 186). American Chemical Society, Washington, D.C.

[34] Rubanyi, G. M., Ho, E. H., Cantor, E. H., Lumma, W. C. and Botelho, L. C. (1991) Cytoprotective function of nitric oxide: inactivation of superoxide radicals produced by human leukocytes. *Biochemical and Biophysical Research Communications*, **181**, 1392–1397.

[35] Radi, R., Beckman, J. S., Bush, K. M. and Freeman, B. A. (1991) Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Archives of Biochemistry and Biophysics*, **288**, 481–487.

[36] Zhang, R., Hirsch, O., Mohsen, M. and Samuni, A. (1994) Effects of Nitroxide Stable Radicals on Juglone Cytotoxicity. *Archives of Biochemistry and Biophysics*, **312**, 385–391.