Free Rad. Res., Vol. 21, No. 3, pp. 177–186
Reprints available directly from the publisher
Photocopying permitted by license only

SUPEROXIDE SCAVENGING ACTIVITY OF SPIN-LABELED NITROSOUREA AND TRIAZENE DERIVATIVES

VESSELINA GADZHEVA, KOHJI ICHIMORI*, HIROE NAKAZAWA* and ZAHARY RAIKOV

Department of Chemistry and Biochemistry, Medical University, 11 Armeiska Street, 6003 Stara Zagora, Bulgaria
**Department of Physiology 2, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa 259-11, Japan*

(Received October 22nd, 1993; in revised form April 8th, 1994)

Superoxide scavenging activities (SSA) of newly synthesized spin-labeled nitrosourea and triazene derivatives, and their precursor nitroxides were investigated by the ESR/spin-trapping method using the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and hypoxanthine/xanthine oxidase as the superoxide-generating system. The spin-labeled nitrosoureas, triazenes and their precursor nitroxides exhibited excellent SSA, whereas clinically used nitrosourea and triazene, which do not contain the nitroxide moiety, did not show any SSA. Furthermore, it was deduced that these nitroxides scavenge superoxide by redox cycling between nitroxide and corresponding hydroxylamine.

KEY WORDS: ESR, spin-trap, spin-labeled, nitroxide, nitrosourea, triazene.

INTRODUCTION

Nitrosoureas and triazenes are active alkylating chemotherapeutic agents used for the treatment of many clinical neoplasms.[1,2] In order to suppress cytotoxic side effects and to enhance accessibility of these drugs to tumor cells, we have synthesized a variety of spin-labeled nitrosoureas and triazenes in which the nitroxide structure is incorporated.[3,4,5] Some of these spin-labeled drugs exhibited less toxicity and higher antitumor activity than 1-cyclohexyl-3-chloroethyl-3-nitrosourea (CCNU) or 5-(3,3-dimethyltriazene-1-yl)-imidazole-4-carboxamide (DTIC), the clinically used analogues which do not contain the nitroxide moiety.[6,7] It was shown that the presence of nitroxide in the antitumor drugs increases the radiosensitizing properties and the rate of accumulation in target cells and decreases the toxicity, imparting a beneficial influence on the antineoplastic properties of a drug.[6,8] A recent study of Mitchell *et al.* showed that some nitroxides possess superoxide scavenging activity.[9] Thus, we hypothesized that the antioxidant action of the nitroxides may be responsible for the beneficial effects of spin-labeled compounds.

The present study was undertaken to determine the superoxide scavenging activities (SSA) of newly synthesized spin-labeled nitrosoureas, spin-labeled

Correspondence to: Kohji Ichimori, Department of Physiology 2, Tokai University, School of Medicine, Bohseidai, Isehara, Kanagawa 259-11, Japan. Tel: +81-463-93-1121 (ext. 2531) Fax: +81-463-93-6684.

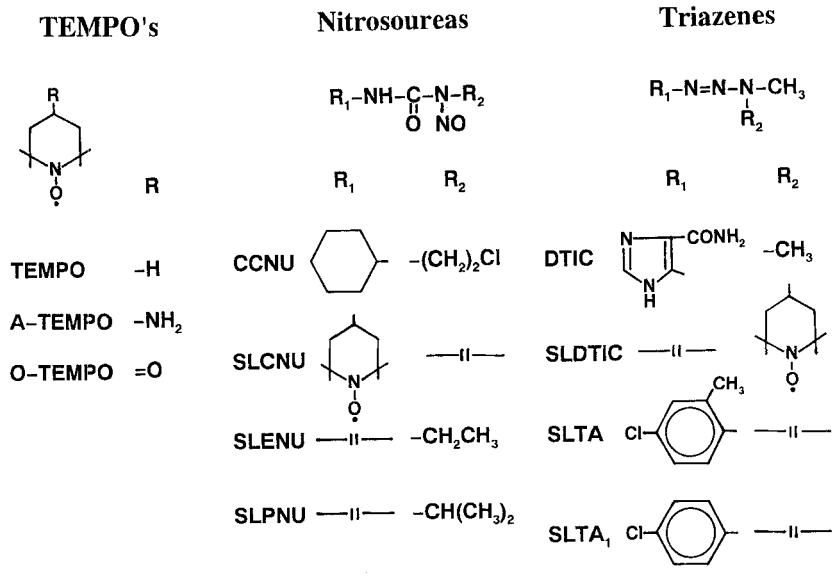

FIGURE 1 Schematic structures of TEMPO analogues, spin labeled nitrosoureas, spin labeled triazenes, CCNU and DTIC screened in the present study.

triazenes and their precursor nitroxides, using the electron paramagnetic resonance (ESR)/spin-trapping method. The underlying mechanism for the reaction between these compounds and superoxide was also investigated in detail.

MATERIALS AND METHODS

Chemicals

The structures of compounds used for SSA measurements are shown in Figure 1. CCNU and DTIC were obtained from the Bristol-Myers Squibb Co. (Connecticut, U.S.A.). 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Shonan Analytic Center (Tokyo). TEMPO and A-TEMPO were purchased from Aldrich (Milwaukee, U.S.A.). O-TEMPO was synthesized by the procedure of Rosancev and Zhdanov.[10] SLCNU, SLENU, SLPNU, SLDTIC, SLTA, and $SLTA_1$ were synthesized by the procedure of Raikov *et al.*[3,4] and Gadzheva and Raikov.[5] The test compounds were dissolved in ethanol/sodium phosphate buffer (PBS) (pH 7.4) (v/v;1/10) and SLDTIC, SLTA and $SLTA_1$ in dimethylformamide/PBS (v/v;1/10). Cow milk xanthine oxidase and trolox were obtained from Boehringer (Germany) and Aldrich, respectively. Recombinant human superoxide dismutase was donated by Nihon Kayaku (Tokyo). Other chemicals were purchased from Wako Pure Chemical Co. (Tokyo). These compounds were used without further purification.

Superoxide Scavenging Activity Assay

Superoxide scavenging activity (SSA) of these compounds was determined by the ESR/spin-trapping method. Briefly, DMPO traps superoxide generated from the hypoxanthine/xanthine oxidase (HX/XO) system and turns into DMPO-OOH which is an ESR-detectable spin adduct. If a superoxide scavenger is present, DMPO and the scavenger react with O_2^- competitively as in the following reactions and the ESR signal of DMPO-OOH is diminished:

$$\text{DMPO} + O_2^- + H^+ \xrightarrow{k_d} \text{DMPO-OOH} \quad (1)$$

$$\text{Scavenger} + O_2^- \xrightarrow{k_s} \text{Product} \quad (2)$$

where k_d and k_s are second-order rate constants of DMPO and scavengers of superoxide, respectively. If the concentrations of DMPO and scavenger are much higher than that of O_2^-, the following equation can be easily derived from Eqs. (1) and (2):[11]

$$I_o/I = 1 + k_s[\text{S}]/k_d[\text{D}] \quad (3)$$

where I and I_o are the ESR signal intensities of DMPO-OOH with and without the scavenger, respectively, and [S], and [D] are the initial concentrations of scavenger and DMPO, respectively. Since k_d has been reported to be $30\,M^{-1}\,sec^{-1}$ at pH 7.4[12], k_s can be estimated from the slope of the regression line of $I_o/I - 1$ vs [S]/[D]. IC_{50}, which is the drug concentration at 50% inhibition of the DMPO-OOH ESR signal, was also calculated as another index of SSA. Thus, SSA can be expressed as k_s and IC_{50}. The relationship between k_s and IC_{50} is derived as follows:

$$k_s = k_d[\text{D}]/IC_{50} \quad (4)$$

For the actual SSA measurements, hypoxanthine (HX, 0.5 mM), diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid (DETAPAC, 0.85 mM), DMPO (69 mM or 690 mM) and various concentrations of the test compounds were mixed in 0.1 M PBS (pH 7.4). Superoxide generation was initiated by the addition of XOD (0.1 Unit/ml) and an aliquot of reaction mixture was then transferred into a quartz cell (volume 180 μl, JEOL Co. Ltd, Tokyo). ESR measurements were made on a JES-FE2XG ESR spectrometer (JEOL, Tokyo), starting 40 seconds after the final mixing. Manganese oxide (MnO) was used as an internal standard for ESR signal intensity. Conditions for the measurement were as follows: magnetic field 3350 ± 5 mT; power 8.0 mW, response 0.3 sec; modulation 0.125 mT; temperature, room temperature; amplitude 2.5×10^3; sweep time, 2 min; magnetic field modulation frequency, 100 kHz.

Validation of SSA Assay Method

Equation (3) is valid only when the following two criteria are fulfilled; first, scavengers do not react with DMPO or DMPO-OOH and, secondly, scavengers do not interfere with the O_2^- generating system. To verify these criteria, the concentration of DMPO (1 mM) was monitored with optical absorbance at 227 nm[13] in the presence of 0.1 mM compounds. To exclude the possibility that these compounds interact directly with DMPO-OOH after it has been formed, the ESR signal intensity of DMPO-OH which was generated by the sonication of aqueous DMPO

TABLE 1
Xanthine oxidase activity in the presence of nitroxides

Compounds	Uric acid generation rate (μM/min)
Control	15.6 ± 0.3 (n = 6)
SLCNU	14.6 ± 0.2 (n = 3)
SLDTIC	15.0 ± 0.3 (n = 3)
TEMPO	14.6 ± 0.4 (n = 3)
trolox	11.3 ± 0.2 (n = 3)*

Xanthine oxidase activity expressed by uric acid generation rate (mean ± S.E.M.) in the presence of nitroxides or trolox (0. 1 mM). The concentrations of xanthine and xanthine oxidase were 0.1 mM and 0.1 unit/ml, respectively. There was no significant difference between the rate of control and nitroxides. *$p < 0.05$ in comparison with control.

solution (20 mM) was measured in the presence of 0.1 mM spin-labeled compounds. For this purpose, DMPO-OH was used as an alternative to DMPO-OOH since DMPO-OOH is unstable. XO activity in the presence of these compounds was also measured by measurement of uric acid formation. Xanthine (0.1 mM), DETAPAC (0.85 mM), and 0.1 mM of TEMPO, SLCNU, or SLDTIC were mixed in 0.1 M sodium phosphate buffer (pH 7.4) and the reaction was started by adding 0.1 unit/ml of XO. The generation of uric acid was monitored at 290 nm[14] ($\varepsilon_{290} = 9.6\,mM^{-1}\,cm^{-1}$). We also measured SSA using two different concentrations (69 mM and 690 mM) of DMPO to check the direct effect of these compounds on Eq. (1), since k_s should be independent of the DMPO concentration.

RESULTS

Validation Study for SSA Assay System

Experiments were performed in order to exclude the possibility that the spin-labeled compounds may directly react with DMPO or DMPO-OOH. It was proved that spin-labeled compounds did not react with DMPO because the concentration of DMPO (1 mM) as monitored by optical absorption at 227 nm, did not decrease by the addition of 0.1 mM of TEMPO, SLCNU, or SLDTIC. The ESR signal intensity of sonication-derived DMPO-OH was not significantly decreased by the addition of these spin-labeled compounds.

Since superoxide scavengers could exert a direct effect on the HX/XO superoxide generating system, we determined whether or not the spin-labeled compounds directly interfere with the O_2^- generating system. As shown in Table 1, nitroxide and spin-labeled compounds used this study did not show any direct inhibitory effect on the enzymic activity of xanthine oxidase estimated by the uric acid generation rate. As described below, k_s of these compounds was independent of the initial concentration of DMPO; indicating that these compounds do not react with DMPO or DMPO-OOH nor do they interfere with the superoxide generation system. Thus, this system is suitable for SSA measurements.

Superoxide Scavenging Activity

Forty seconds after initiating the reaction by the addition of XO to the solution containing HX, DETAPAC and DMPO in PBS (pH 7.4), a clear ESR signal of

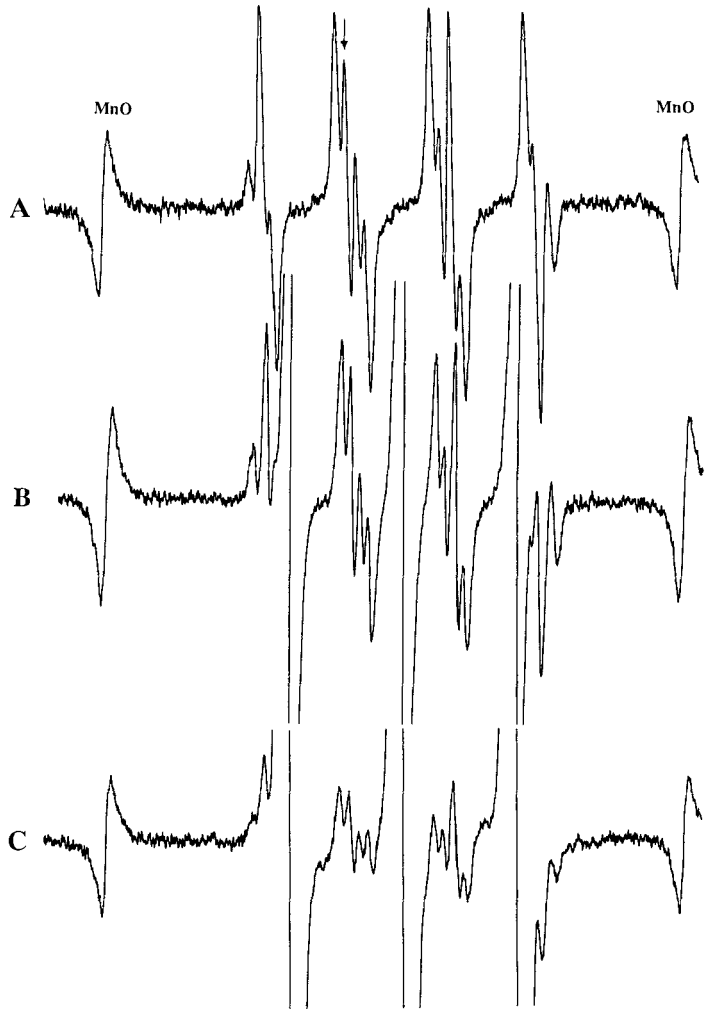

FIGURE 2 ESR spectra of DMPO-OOH spin-adduct in HX/XO system and DMPO 690 mM without SLCNU (A), with 10 μM SLCNU (B), and with 100 μM SLCNU (C). MnO was used as an internal standard.

DMPO-OOH adduct was recorded. The signal height was decreased in the presence of SLCNU, SLENU, SLPNU, SLDTIC, SLTA, SLTA$_1$, TEMPO, A-TEMPO and O-TEMPO in a dose dependent manner (5 μM ~ 100 μM). Typical spectral traces showing the inhibition of DMPO-OOH production by SLCNU were constructed by the superposition of DMPO-OOH and nitroxide triplet ESR signals (Figure 2). For the quantification of DMPO-OOH, one of the DMPO-OOH signal (indicated by the arrow in Figure 2) which is free of overlap of the nitroxide spectrum was used. The DMPO-OOH signal intensity was normalized with the intensity of the MnO peak, an internal standard. The signal intensity of DMPO-OOH decreased with the increase in SLCNU concentration. Triplets attributed to nitroxide,

SLCNU, increased with higher concentration of SLCNU as seen in the lower two traces of Figure 2.

The inhibition of DMPO-OOH signal intensity by the spin-labeled compounds SLCNU, SLDTIC and their precursors, TEMPO and A-TEMPO, is shown in Figure 3. All cases of these compounds showed excellent superoxide scavenging activities (SSA). IC_{50}, as roughly estimated from this figure, was found to range from 35 μM to 90 μM for 690 mM DMPO. For calculating k_s and IC_{50}, the regression analyses based on Eq. (3) were made; typical regression lines of SLCNU are shown in Figure 4A. SSA of these compounds calculated as IC_{50} and k_s are listed in Table 2. Our results show that all spin-labeled nitrosoureas, triazenes and their precursor nitroxides possessed a k_s comparable with that of ascorbic acid, a well-known superoxide scavenger, and about 300 times higher than that of trolox. CCNU and DTIC which are clinically-used nitrosourea and triazene, respectively, exhibited no SSA.

We estimated SSA of these compounds through the slope in $I_o/I - 1$ vs. [S]/[D] plot shown in Figure 4A. The slopes obtained by the two different concentrations

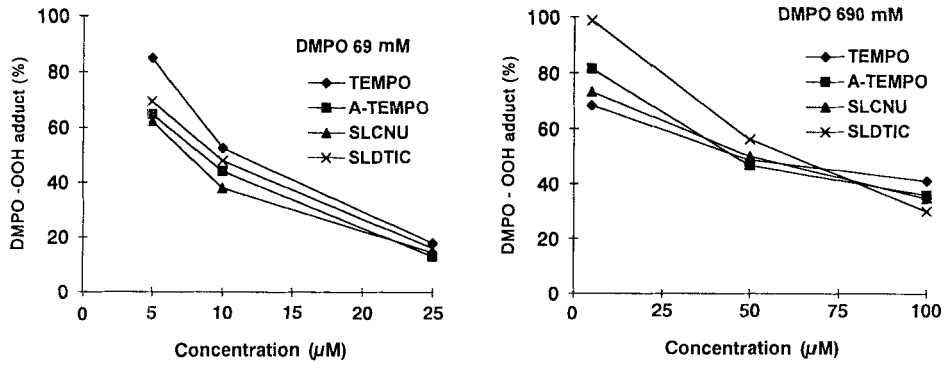

FIGURE 3 Inhibition of DMPO-OOH spin-adduct in HX/XO system by nitroxides. Two different concentrations of DMPO (69 mM and 690 mM) were used. Values represent the mean of three measurements.

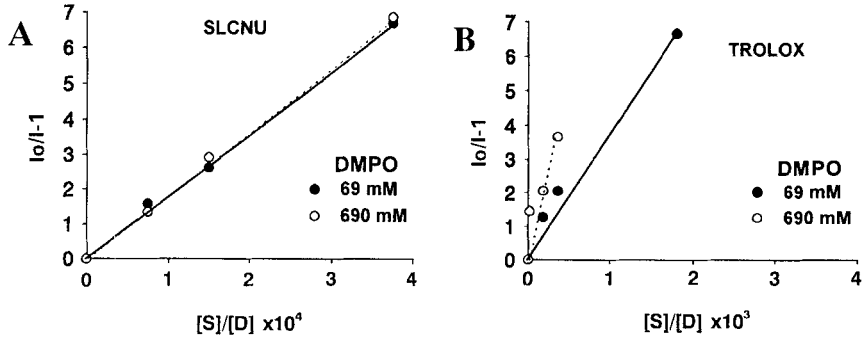

FIGURE 4 $I_o/I - 1$ vs. [S]/[D] plot for SLCNU (A) and trolox (B). Values represent the mean of three measurements. The regression lines for the determination of IC_{50} and k_s in Table 1 are also included.

TABLE 2
Superoxide scavenging activities of nitroxides, antitumor drugs, and antioxidants

Compounds	$IC_{50}(\mu M)$	k_s ($\times 10^5 M^{-1} sec^{-1}$)
TEMPO	41	5.0
A-TEMPO	36	5.8
O-TEMPO	51	4.0
SLCNU	51	4.0
SLENU	67	3.1
SLPNU	72	2.9
SLDTIC	64	3.2
SLTA	87	2.4
SLTA1	77	2.7
CCNU	∞	0
DTIC	∞	0
ascorbic acid	33	6.3
trolox	1230	0.16

Superoxide scavenging activity is expressed by IC_{50}, which is the drug concentration at 50% inhibition of the DMPO-OOH ESR signal, and k_s, which is a second-order rate constant for the reaction between the drug and superoxide. DMPO concentration for estimating IC_{50} and k_s was 690 mM. CCNU and DTIC exhibited no effect on the DMPO-OOH signal intensity.

of DMPO (69 mM and 690 mM) were identical, i.e. independent of the initial DMPO concentration. The agreement of the two slopes held true in all nitroxides and spin-labeled compounds. Therefore, k_s determined from this regression line likely indicates the true SSA. However, the slope for trolox (Figure 4B), which was used as a reference for superoxide scavengers, disclosed a clear dependence on the initial DMPO concentration. Moreover, trolox had a direct inhibitory effect on xanthine oxidase as shown in Table 1. Thus, k_s for trolox is overestimated in this system.

Underlying Mechanism of the Superoxide Scavenging Reaction of Nitroxides

To deduce the detailed reaction between these compounds and superoxide, the ESR signal intensities of nitroxide were continuously monitored using the SSA measurement system. Figures 5A and 5B show typical ESR spectra SLCNU in the absence and presence of HX/XO superoxide generating system, respectively. In all cases, the signal intensity of nitroxide rapidly decreased in the complete system for measuring SSA but remained unchanged in a pure buffer system. Both XO and HX were necessary for the decrease in the signal intensity, indicating that superoxide is responsible for the decreased concentration of nitroxide. However, the nitroxide signal stopped decreasing at 1 min after reaction initiation and exhibited a plateau level of nitroxide for 5 minutes even though the generation of superoxide remained stable. This suggested that the decrease in nitroxide is due to the reduction of nitroxide to hydroxylamine rather than the destruction of nitroxide. It is also suggested that the hydroxylamine can be reoxidized by superoxide and participates in scavenging superoxide, leaving a plateau level of nitroxide.

To clarify these findings, we tried to restore nitroxide by inhibiting xanthine oxidase with allopurinol (10 mM), blocking superoxide by superoxide dismutase (100 Unit/ml), and reoxidizing with $K_3[Fe(CN)_6]$ (2 mM), H_2O_2 (3%), or air in alkaline solution (0.1 N NaOH). The signal intensity of nitroxide was partially recovered by inhibiting xanthine oxidase (Figure 5C) and completely restored by the

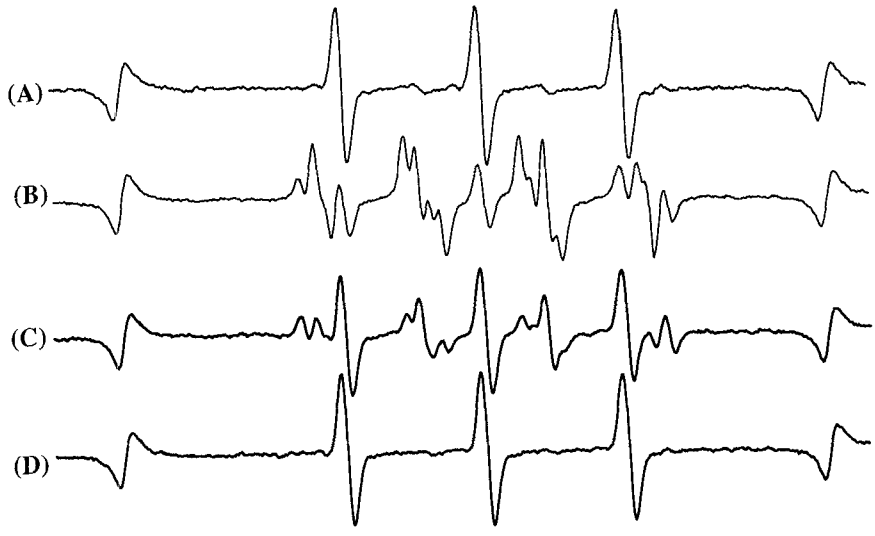

FIGURE 5 ESR spectra of 25 μM SLCNU in PBS (pH 7.4). A) in the absence of HX/XO system. B) in the presence of HX/XO system and DMPO 69 mM, 1 min after reaction initiation. C) allopurinol 10 mM were added to (B) 5 min after reaction initiation. D) superoxide dismutase (SOD) 100 unit/ml and 0.1 N NaOH were successively added to (C).

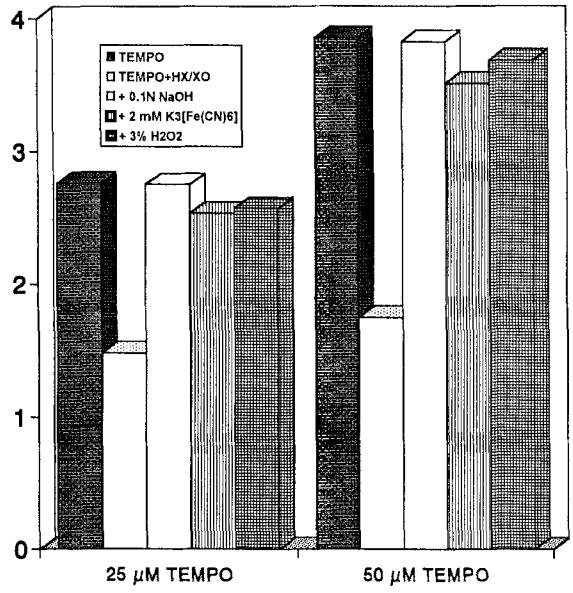

FIGURE 6 Restoration of ESR signal intensity of TEMPO by reoxidizers in HX/XO system, allopurinol 10 mM, and superoxide dismutase (SOD) 100 unit/ml. ■ TEMPO: the signal intensity of TEMPO in the absence of HX/XO system. □ TEMPO + HX/XO: the signal intensity of TEMPO in the presence of HX/XO system, 1 min after reaction initiation. Other bars indicate the signal intensity of TEMPO after the successive addition of allopurinol, SOD, and reoxidizers. The addition of these reagents was performed 5 min after the HX/XO reaction was initiated. Values represent the mean of three measurements.

reoxidation (Figure 5D). Figure 6 also shows the change in the signal intensities of TEMPO (25 μM or 50 μM) under the same procedures. The spin loss was completely reversed after the air reoxidation in 0.1 N NaOH. The other oxidizers also completely restored the signal intensities. These results demonstrated that the decrease in the nitroxide concentration is due to the one-electron reduction of nitroxide to hydroxylamine by superoxide. The steady state concentrations of TEMPO and A-TEMPO in HX/XO system were 58% and 31% of the initial concentration, respectively, and were almost independent of the initial concentration.

DISCUSSION

Nitroxides are widely used as spin probes and spin labels in biology and medicine. It has been shown that some five-membered cyclic nitroxides possess SOD-like activity.[9,15] On the other hand, the ESR signal of six-membered ring nitroxides such as TEMPO is not influenced by superoxide.[9] The results of the present study, using the spin trap DMPO demonstrated that the six-membered cyclic nitroxides TEMPO, A-TEMPO and O-TEMPO have high SSA (IC_{50} and k_s) which is compatible with that of reference superoxide scavengers such as ascorbic acid. These nitroxides were found to be responsible for the excellent SSA of spin labeled nitrosoureas SLCNU, SLENU, SLPNU and triazenes SLDTIC, SLTA, $SLTA_1$ in which nitroxide is incorporated since the clinically used nitrosourea CCNU and triazene DTIC exhibited no SSA. These results were obtained in an optimal system for the measurement of SSA, in which direct and indirect interference between nitroxides and the spin-labeled compounds and the superoxide generating system were excluded.

Regarding the mechanism of nitroxide reduction, Finkelstein *et al.*[16] reported in 1984 that superoxide can reduce certain nitroxide free radicals to their corresponding hydroxylamines in the presence of sulfhydryl-containing compounds. Very recently it has been confirmed by Mitchell *et al.* that superoxide can reduce OXANO to OXANOH and that superoxide is also capable of oxidizing OXANOH to OXANO, which shows the presence of redox cycling as expressed by the following equations:[9]

$$N{-}O\cdot + \cdot O_2^- + H^+ \xrightarrow{k_r} N{-}OH \quad (5)$$

$$N{-}OH + \cdot O_2^- + H^+ \xrightarrow{k_o} N{-}O\cdot + H_2O_2 \quad (6)$$

where k_r and k_o are second-order rate constants for the reduction of nitroxide and oxidation of hydroxylamine by superoxide, respectively. If this redox cycling between nitroxide and hydroxylamine also occurs in the present spin-labeled compounds, the concentrations of both nitroxide [N—O·] and hydroxylamine [N—OH] is constant and the ratio of nitroxide to hydroxylamine ([N—O·]/[N—OH]) is equal to k_o/k_r. Since it was proved that the spin loss by the reaction between nitroxides and superoxide could be completely reversed by reoxidation and the concentration of nitroxide remained constant for 5 minutes under constant superoxide generation after the initial rapid decrease, it is concluded that nitroxides scavenge superoxide by redox cycling between nitroxide and corresponding hydroxylamine. Thus, hydroxylamine as well as nitroxide take part in scavenging superoxide, which is responsible for the excellent SSA of nitroxides, and the second-order rate constant (k_s) listed in Table 1 for nitroxides is from the contribution of both

nitroxide and hydroxylamine. Since the steady state concentrations of TEMPO and A-TEMPO were 58% and 31% of the initial concentration, respectively, the ratios of rate constants (k_o/k_r) are calculated to be 1.4 and 0.45, respectively. This indicates that the contribution of nitroxide and corresponding hydroxylamine to SSA is different in each nitroxide.

High antitumor activity and low toxicity have already been shown for some newly synthesized spin-labeled nitrosoureas and triazenes.[6,7] The beneficial effects are likely attributable to the antioxidant effect of the incorporated nitroxide, which is derived efficiently from the redox cycling reaction.

References

1. S.K. Carter, F.M. Schabel, L.E. Broder and T.P. Johnston (1972) 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and other nitrosoureas in cancer treatment. A review. *Advances in Cancer Research*, **16**, 273–332.
2. Y.F. Shealy (1970) Synthesis and biological activity of 5-aminoimidazoles and 5-triazenoimidazoles. *Journal of Pharmaceutical Science*, **59**, 1533–1558.
3. Z. Raikov, D. Todorov, M. Ilarionova, G. Demirov, Ts. Tsanova and D. Kafalieva (1985) Synthesis and study of spin labeled nitrosoureas. *Cancer Biochemistry and Biophysics*, **7**, 343–348.
4. Z. Raikov, V. Gadzheva, M. Koch and G. Kolar (1993) Synthesis of spin labeled triazenes. *Organic Preparations and Procedures International*, **25**, 473–477.
5. V.G. Gadzheva and Z.D. Raikov (1991) SLCNCH. *Drug Data Report*, **13**, 344.
6. G. Sosnovsky and S.W. Li (1985) In the research for new anticancer drugs, XII. Synthesis and biological evaluation of spin labeled nitrosoureas. *Life Sciences*, **36**, 1479–1483.
7. G. Sosnovsky, S.W. Li and V.M. Rao (1987) In the search for new anticancer drugs. XXI. Spin labeled nitrosoureas. *Zeitschrift fur Naturforschung*, **42C**, 921–931.
8. N. Emanuel, N. Konovalova and R. Djachkovskaja (1976) Toxicity, antitumor activity and pharmacokinetics of spin-labeled Thio-TEPA analogues. *Cancer Treatment Reports*, **60**, 1605–1609.
9. J.B. Mitchell, A. Samuni, M.C. Krishna, W.G. De Graff, M.S. Ahn, U. Samuni, A. Russo (1990) Biologically active metal-independent superoxide dismutase mimics. *Biochemistry*, **29**, 2802–2807.
10. E. Rosancev and R. Zhdanov (1981) Paramagnetic models of biological active compounds. Nauka, Moskva.
11. L. Pronai, Y. Ichikawa, K. Ichimori, H. Nakazawa and S. Arimori (1990) Hydroxyl radical-scavenging activity of slow-acting anti-rheumatic drugs. *Journal of Clinical Biochemistry and Nutrition*, **9**, 17–23.
12. E. Finkelstein, G.M. Rosen, and E.J. Rauckman (1980) Spin trapping of superoxide and hydroxyl radical: Practical aspects. *Archives of Biochemistry and Biophysics*, **200**, 1–16.
13. A. Samuni, C.D.V. Black, C.M. Krishna, H.L. Malech, E.F. Bernstein and A. Russo (1988) Hydroxyl radical production by stimulated neutrophils reappraised. *The Journal of Biological Chemistry*, **263**, 13797–13801.
14. G.G. Roussos (1967) Xanthine oxidase from bovine small intestine. *Methods in Enzymology*, **12A**, 5–16.
15. A. Samuni, C.M. Krishna, P. Riesz, E. Finkelstein and A. Russo (1988) A novel metal-free low molecular weight superoxide dismutase mimic. *The Journal of Biological Chemistry*, **263**, 17921–17924.
16. E. Finkelstein, G. Rosen and E. Rauckman (1984) Superoxide-dependent reduction of nitroxides by thiols. *Biochemica et Biophysica Acta*, **802**, 90–98.

Accepted by Prof. B. Halliwell