Current Topics in Biophysics 2005, 29(1-2), 129-138

A TRITYL-BASED ASSAY FOR THE DETECTION OF SUPEROXIDE IN BIOLOGICAL SYSTEMS

VIJAY KUMAR KUTALA, PERIANNAN KUPPUSAMY

Center for Biomedical EPR Spectroscopy and Imaging, Davis Heart and Lung Research Institute
and Department of Internal Medicine, Ohio State University, Columbus, OH

Detection of superoxide radical is of critical importance for investigating the pathophysiological consequences resulting from altered cellular reactive oxygen homeostasis. In this study, we used the triarylmethyl (trityl) free radical, TAM Ox063, for the detection of superoxide by electron paramagnetic resonance (EPR) spectroscopy and spectrophotometry. TAM is paramagnetic (EPR active), highly soluble in water and exhibits a single sharp EPR peak in aqueous solutions. TAM reacts with superoxide with an apparent second order rate constant of 3.1×10^3 M^{-1} s^{-1}. The specific reactivity of TAM with superoxide, which leads to loss of EPR signal, was used for the determination of superoxide concentration in enzymatic and cellular model systems. We also investigated the feasibility of a spectrophotometric assay of superoxide by taking advantage of the changes in the optical spectrum of TAM on reaction with superoxide. In conclusion, the use of a trityl radical for a quantitative assay of superoxide offers an alternative approach with certain advantages over other methods.

INTRODUCTION

Oxygen free radicals such as superoxide, hydroxyl and alkyl peroxyl radicals have been implicated in a variety of physiological and pathophysiological processes (Sies, 1989). In biological systems, superoxide is considered to be the primary source which will lead to the formation of other radicals and a variety of down-stream oxidants. Hence, oftentimes, a reliable and accurate method to determine the concentration of the superoxide is preferred over methods that would determine the concentration of the other species. Up to now many methods for the quantification of superoxide have been proposed, such as spectrophotometric methods that use reduction of ferricytochrome *c* or nitroblue tetrazolium (NBT) by superoxide, chemiluminescence, fluorescence-based techniques, electrochemical and electron paramagnetic resonance (EPR) spectroscopy (Auclair, Torres & Hakim, 1978; Budd, Castilho & Nicholls, 1997; Li Zhu, Kuppusamy, Roubaud, Zweier & Trush, 1998; McCord & Fridovich, 1969; Roubaud, Sankarapandi, Kuppusamy, Tordo & Zweier, 1998). Among these, the most widely used cytochrome *c* reduction and lucigenin-amplifed chemiluminescence assays are considered reliable methods for the quantification of superoxide. EPR spin-trapping, however, remains the most direct and definitive method for the detection of superoxide radicals. Barbacanne et al compared the various methods of the detection of superoxide in cellular systems and suggested that ferricytochrome *c* reduction, EPR spectroscopy and hydroethidine-based fluorescence techniques are suitable for measuring extracellular superoxide generation (Barbacanne, Souchard, Darblade, Iliou, Nepveu, Pipy, Bayard & Arnal, 2000). The hydroethidine-based fluorescence assay can also be used to measure intracellular superoxide with a greater degree of sensitivity (Zhao, Kalivendi, Zhang, Joseph, Nithipatikom, Vasquez-Vivar & Kalyanaraman, 2003). However, these methods have certain limitations for measuring superoxide. In the lucigenin-based luminescence detection of superoxide, it has been demonstrated that the probe itself can redox-cycle resulting in artifactual production of superoxide (Fridovich, 1997) whereas the cytochrome *c* reduction assay is affected by the presence of oxidoreductases in cellular systems (Azzi, Montecucco & Richter, 1975; Thomson, Trujillo, Telleri & Radi, 1995). The EPR-spin-trap method requires the use of high concentrations (10-200 mM) of spin trap(s) that may perturb the redox balance in the biological system. Hence, there has been an avid interest in the development of new methods and/or probes for reliable determination of superoxide (Allouch, Roubaud, Lauricella, Bouteiller & Tuccio, 2003; Bottle, Hanson & Micallef, 2003; Dikalov, Dikalova & Mason, 2003; Dikalov, Tordo, Motten & Mason, 2002; Zhao *et al.*, 2003).

Recently, electrochemical methods have been employed to measure superoxide in biological systems with minimum disturbance to the sample

under estimation (Al-Ayash & Wilson, 1979). These include, a multilayer cytochrome *c* electrode which measures the reduction of cytochrome c by superoxide (Beissenhirtz, Scheller, Stocklein, Kurth, Mohwald & Lisdat, 2004) and third-generation superoxide anion sensor based on the SOD which is directly immobilized by sol-gel thin film on a gold electrode (Di, Bi & Zhang, 2004). However, the use of the cytochrome *c* electrode method for the determination of superoxide has limited due to interferences of ascorbate, glutathione and cellular reductases and SOD based electrode method measures only the extracellular superoxide.

In this study, we describe the application of a trityl free radical probe for the EPR and spectrophotometric detection of superoxide free radicals. Nearly a century after the first report of the stable triphenylmethyl radical by Gomberg (1900), a new class of triphenylmethyl-derived radicals was developed by Nycomed Innovation (Ardenkjaer-Larsen, Laursen, Leunbach, Ehnholm, Wistrand, Petersson & Golman, 1998) as contrast agents useful in magnetic resonance imaging. These radicals are characterized by a single sharp EPR peak in aqueous solutions. The derivatizations are structurally designed in such a way that the methyl carbon, the epicenter of the unpaired electron, is removed from the magnetic nuclei to a distance of at least 4 atom lengths by carbon, deuterium and sulfur. The idea was to remove all sources of inhomogeneous hyperfine contributions to obtain an extremely narrow EPR peak. This will enable the EPR detection of very low concentrations (about 10 nM) of these radical species in physiological media (Ardenkjaer-Larsen *et al*., 1998; Panagiotelis, Nicholson, Foster & Hutchison, 2001; Yong, Harbridge, Quine, Rinard, Eaton, Eaton, Mailer, Barth & Halpern, 2001). Earlier studies have shown that these trityl radicals are extremely useful as effective and sensitive probes for measurement of oxygen in several *in vitro* and *in vivo* studies (Golman, Petersson, Ardenkjaer-Larsen, Leunbach, Wistrand, Ehnholm & Liu. 2000; He, Deng, Li, Kuppusamy & Zweier, 2002; Krishna, English, Yamada, Yoo, Murugesan, Devasahayam, Cook, Golman, Ardenkjaer-Larsen, Subramanian & Mitchell, 2002; Kuppusamy, Wang, Chzhan & Zweier, 1997; Lurie, Li, Petryakov & Zweier, 2002, Murugesan, Cook, Devasahayam, Afeworki, Subramanian, Tschudin, Larsen, Mitchell, Russo & Krishna, 1997; Panagiotelis, Nicholson & Hutchison, 2001). The probe has also been used to obtain high resolution EPR images of biological samples by taking advantage of their narrow EPR peak (Kuppusamy *et al.*, 1997; Murugesan *et al*., 1997).

In our efforts to characterize the reactivity of this compound toward a variety of biological oxidoreductants and its suitability for biological applications, we made some striking observations with respect to reactive oxygen species (ROS). In this manuscript, we report our results on the effect of various bio oxidoreductants, including ROS, on a triarylmethyl (TAM) radical, Ox063. We also investigated the spectrophotometric determination of superoxide by utilizing the specific absorbance of the reaction product of TAM with superoxide. We further compared the specificity, sensitivity and reliability of TAM for application in biological systems by comparing it to the standard cytochrome *c* assay. The results show that the new trityl radical-based EPR and spectrophotometric assay is comparable to the standard methods of superoxide determination and furthermore, both the methods were not affected by hydrogen peroxide, and other reducing agents which are common interfering substances in many assays for the measurement of superoxide generation.

MATERIALS AND METHODS

Chemicals

TAM OX063 was a gift from Nycomed Innovations Co (Malmö, Sweden). Ascorbate, glutathione, hydrogen peroxide, xanthine, diethylenetriaminepentaacetic acid (DTPA), phorbol-12-myristate-13-acetate (PMA), zymosan A, ferricytochrome *c*, and superoxide dismutase (SOD) were purchased from Sigma (St Louis, MO, USA). Catalase and xanthine oxidase were purchased from Boehringer Mannheim (Indianapolis, IN, USA). S-nitroso-N-acetyl-penicillamine (SNAP) was purchased from Calbiochem (La Jolla, CA, USA). All solutions were prepared in phosphate buffer (0.1 M, pH 7.4) in the presence of DTPA (0.1 mM). The buffers were pretreated with Chelex-100 resin (40 g/L) to remove adventitial metal ions.

EPR Spectroscopy

EPR spectra were recorded at room temperature using a Bruker ER 300 EPR spectrometer operating at X band with a TM 110 cavity. Solutions were taken in a quartz flat cell or in 50 µL borosilicate glass capillaries (Fisher Scientific, Pittsburgh, PA, USA). The following parameters were used in the data acquisition: modulation amplitude, 0.05 - 0.2 G; time constant, 40 ms; scan time, 10 s; microwave power, 2 mW; microwave frequency, 9.79 GHz; and modulation frequency 100 kHz. Data acquisition and processing were performed using custom-developed software.

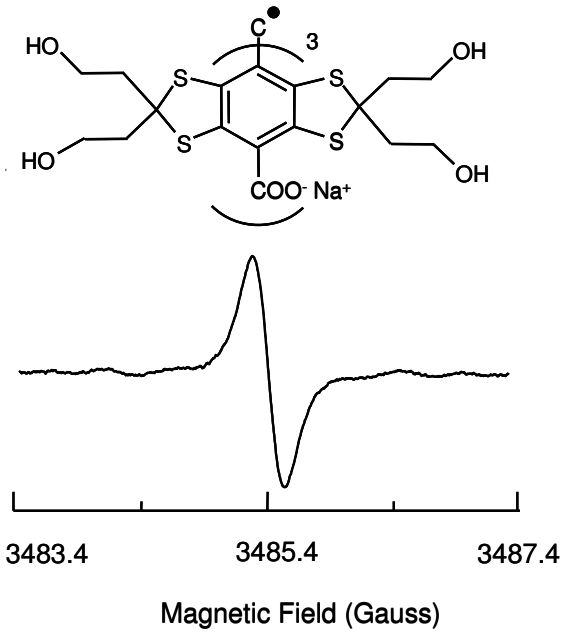

Fig. 1. Chemical structure and a typical EPR spectrum of TAM OX063. The spectrum was measured from an aerated solution of TAM (10 μM) in phosphate buffer at pH 7.4. The EPR data acquisition settings were: modulation amplitude, 0.05 G; microwave power, 2 mW; time constant, 40 msec; scan time, 10 s. The first derivative peak-to-peak width was 0.26 G.

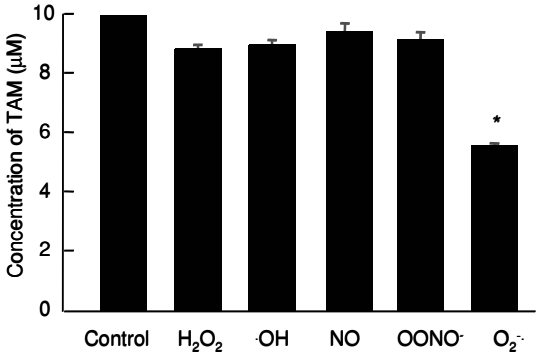

Fig. 2. Effect of various reactive oxygen species on TAM. The EPR spectra were measured at 30 min after mixing. The control solution consisted of TAM only. $O_2^{\cdot-}$ was generated using the X/XO system; The generation of the species and conditions are described in methods section for experimental conditions. *significantly different ($p < 0.05$) from control. The results show that TAM reacts specifically with superoxide resulting in 'spin-loss'.

Generation of oxidants and nitric oxide

Superoxide radicals were generated by a xanthine/xanthine oxidase system (X/XO). The reaction was initiated by adding xanthine oxidase (0.02 U/mL) to a mixture of TAM (10 μM) and xanthine (0.5 mM) in phosphate buffer (pH 7.4, 0.1 M). The solutions were bubbled with 95 % oxygen for 30 s prior to addition of the enzyme. Ferrous ammonium sulfate (0.1 mM) and hydrogen peroxide (1 mM) were used to generate hydroxyl radicals. S-nitroso-N-acetylpenicillamine (SNAP, 1 mM) in phosphate buffer (pH 7.4, 0.1 M) at room temperature was used to generate nitric oxide (NO). Appropriate spin trapping EPR experiments were performed to verify the generation and flux of nitric oxide (Vanin, Liu, Samouilov, Stukan & Zweier, 2000). Unless otherwise mentioned, the reactions were carried out in the presence of TAM (10 μM) and the concentration of TAM was determined by double integration of the spectrum. Each experiment was conducted at least three times.

Isolation and activation of human neutrophils

Polymorphonuclear neutrophils (PMNs) were freshly isolated and used in the experiments. The PMNs were isolated from human blood using a percoll gradient centrifugation method (Fraticelli, Serrano, Bochner, Capogrossi & Zweier, 1996). The PMNs were suspended in a PBS solution containing DTPA (0.1 mM), glucose (1 mg/mL), and albumin (1 mg/mL) and counted using a hæmatocytometer (Olympus, Tokyo, Japan). The PMNs were stimulated by PMA or zymosan. A stock solution of PMA at 1 mg/mL was prepared in dimethylsulfoxide. Zymosan was prepared at a concentration of 2.5 mg/mL in Hanks' balanced salt solution (HBSS) and boiled for 30 min. After cooling at room temperature, the solution was centrifuged at 1000 rpm for 5 min. Fresh human serum was added to the washed pellet (final concentration 1.25 mg/mL) and incubated at 37°C for 1h before separating the serum by centrifugation (1000 rpm for 5 min). The opsonization was repeated and the zymosan was washed with HBSS. Opsonized zymosan at a concentration of 10 mg/mL was prepared in HBSS and the stock solution was kept at -70°C. A final concentration of 4×10^6 cells/mL in the presence of either stimulant, PMA (200 ng/mL) or opsonized zymosan (1 mg/mL) was used for superoxide generation. Control experiments were performed in presence of SOD (400 U/mL).

Spectrophotometric determination of superoxide generation

Superoxide radicals were generated by xanthine/xanthine oxidase system (X/XO). The reaction was initiated by adding XO (0.02 U/ml) to a mixture of TAM (100 μM) and X (0.5 mM) containing EDTA (0.1 mM) in Tris-HCl buffer (0.05 M, pH 7.4). The increase in the absorbance at 546 nm was monitored spectrophotometrically (CARY

50 BIO UV-Visible Spectrophotometer, Varian, Walnut Creek, CA) for 20 min. at room temperature. All the assays were performed in absence or presence of SOD (200 U/ml). For validation and comparison, a cytochrome *c* assay was also performed. Superoxide was generated by the action of XO (0.02 U/ml) on X (0.5 mM) in presence of catalase (500 U/ml), and EDTA (0.1 mM) in Tris-HCl buffer (0.05 M) at pH 7.4. The initial rates of superoxide generation were measured spectrophotometrically by following the SOD-inhibitable reduction of ferricytochrome *c* (75 μM) at 550 nm using an extinction coefficient of 21 mM^{-1} cm^{-1}.

Data analysis

All experiments were performed in triplicate with 4-5 independent measurements. The data were analyzed and compared using student's t-test for statistical significance.

RESULTS

Effect of bio oxidoreductants on the EPR spectrum of TAM

A typical EPR spectrum of TAM in an aerated phosphate buffer (pH 7.4, 0.1 M) is shown in Fig. 1. The effect of a variety of reactive species of common biological occurrence including ascorbate, glutathione, NADPH, hydrogen peroxide, superoxide ($O_2^{-\cdot}$), hydroxyl ($^{\cdot}OH$), NO, and peroxynitrite on TAM was investigated. Ascorbate (1 mM), glutathione (1 mM), or NADPH (1 mM) had no effect on the EPR signal intensity of TAM (10 μM) suggesting that TAM was stable in the presence of these species. Hydrogen peroxide (1 mM) did not cause significant effect on the signal intensity (Fig. 2). Hydroxyl radicals, generated by the Fenton reaction caused a small decrease (~10 %) of the TAM signal intensity. Superoxide caused a decrease of about 45 % under the experimental conditions as described in the Methods section. In separate experiments, it was established that the observed decrease was not due to either xanthine or xanthine oxidase alone (data not shown). Superoxide dismutase (SOD, 400 U/mL) completely inhibited the signal loss, suggesting that the spin-loss was solely caused by superoxide. Nitric oxide generated from NO donor, SNAP and peroxynitrite (1 mM in 0.1 M NaOH) prepared according to the procedure of Zou et al (Zou, Martin & Ullrich, 1997) had no effect on the EPR signal intensity of TAM. This observation, however, does not rule out the possibility of non-radical reaction of peroxynitrite with TAM which may not influence the EPR detection. These results suggest that superoxide can be detected in presence of common oxido-reductants in biological systems.

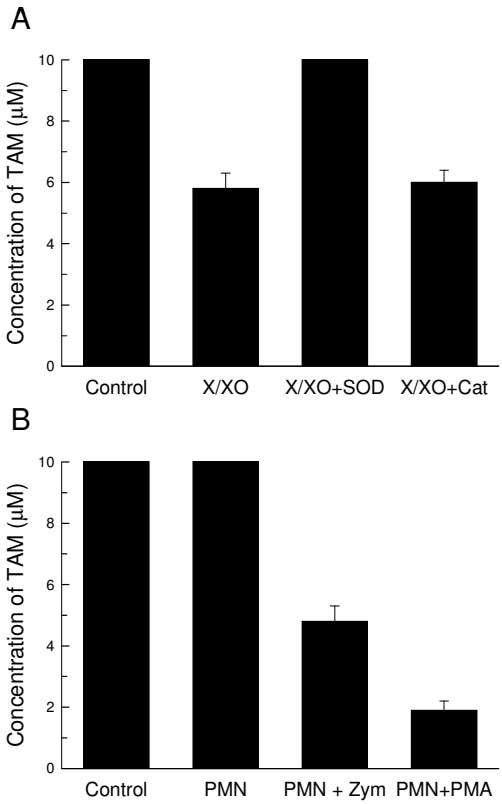

Fig. 3. Effect of superoxide flux on TAM. The reaction was monitored by measuring the EPR spectrum of TAM. (A) Effect of superoxide generated by X/XO. The reaction mixture consisted of the following: TAM, 10 μM; xanthine 0.5 mM; xanthine oxidase, 0.02 U/mL; SOD, 400 U/mL; and catalase, 400 U/mL. The results reflect the concentration of TAM at 20 min after mixing. (B) Effect of superoxide generated by stimulated PMNs. The reaction was monitored by measuring the EPR spectrum of TAM in presence of resting PMNs and cells activated by zymosan or PMA. The reaction mixture consisted of the following: TAM, 10 μM; PMN, 4×10^6 cells/mL; opsonized zymosan, 1 mg/mL and PMA, 200 ng/mL. The results reflect the concentration of TAM at 45 min after mixing.

TAM as a probe for detection of superoxide

A steady flux of superoxide, generated by X/XO system, caused a continuous decay of the EPR signal of TAM. Figure 3A shows the concentration of TAM measured at 20 min. after incubation with X/XO. The decay was almost abolished by SOD (400 U/mL). In order to eliminate the effect of hydrogen peroxide that was generated in the X/XO system, subsequent experiments were performed in the presence of catalase (400 U/ml). The magnitude of the spectral decay in the presence of catalase was similar to that observed in the absence of

catalase suggesting the ineffectiveness of catalase. This observation also suggests that hydrogen peroxide does not influence the decay of the TAM signal.

To evaluate the possible application of TAM as a probe for detection of superoxide in biological systems, we used a cellular system consisting of stimulated PMNs, which produce superoxide radicals through the NADPH oxidase system. Resting neutrophils, did not affect the EPR signal intensity of TAM (Figure 3B). But, when the neutrophils were stimulated with opsonized zymosan or PMA, a continuous decay of the signal was observed. After 45 min. of incubation with PMNs + PMA, the signal intensity of TAM was reduced by 75% compared to that observed with the resting neutrophils. However, zymosan stimulation showed a 50% loss of the signal intensity of TAM during the same time period.

Effect of superoxide on the UV-visible absorption of TAM

The UV-visible absorption spectrum of TAM in Tris-HCl buffer showed an absorption maximum at 464 nm (Figure 4). Upon reaction with superoxide, generated by X/XO, there was a time-dependent decrease in the absorbance at 464 nm and a concomitant increase at 546 nm. The change in the absorption spectrum was completely inhibited by SOD (200 U/ml) suggesting the formation of the new absorbance peak was caused by superoxide. In order to confirm the specificity of TAM for superoxide formed from X/XO system, separate experiments were performed with the inclusion of SOD (200 U/ml) in the reaction mixture. The results showed that SOD completely inhibited the absorbance at 546 nm confirming the specificity of TAM for superoxide.

Comparison with Cytochrome c assay of superoxide

In order to compare the cytochrome *c* method with the present method, we also measured the superoxide generation using cytochrome *c* (75 μM) under similar experimental conditions. The initial rate of change in absorbance was linear to superoxide concentrations (data not shown). Under these conditions, the flux of superoxide generation as measured by the cytochrome *c* was 2.74 μM/min using the molar extinction coefficient of reduced cytochrome *c* ($\varepsilon = 21$ $mM^{-1}cm^{-1}$) (van Gelder, 1962). Superoxide production measured with the TAM assay using the molar extinction coefficient of TAM product ($\varepsilon = 22$ $mM^{-1}cm^{-1}$) under similar flux of superoxide was 2.17 μM/min. The interference of various biological oxidizing and reducing agents such as hydrogen peroxide, glutathione, and ascorbate was studied on the assay of superoxide using TAM and compared with that using cytochrome *c* (Figure 5). Hydrogen peroxide, up to 500 μM did not show any effect on the detection of superoxide with TAM, whereas it showed interfer-

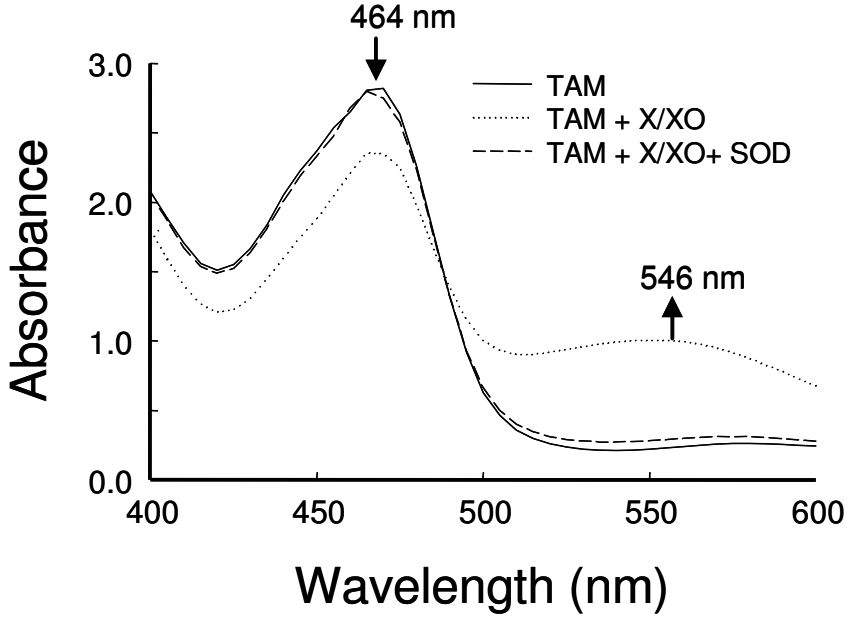

Fig. 4. Effect of superoxide on the UV-visible absorption spectrum of TAM. TAM (100 μM) was incubated with X (1.0 mM), XO (0.04 U/ml), and EDTA (100 μM) in 50 mM Tris-HCl buffer (pH 7.4) at 22 °C. The reaction was initiated by the addition of the XO. The measurements were performed in presence of SOD (200 U/ml) and measured 20 min after mixing. TAM is characterized by absorption at 464 nm, which on reaction with superoxide decreases with time. The new absorption maximum at 546 nm is presumably due to the product of the reaction. SOD completely inhibited the conversion.

ence in the cytochrome *c* reduction assay. This effect of hydrogen peroxide was completely abolished by catalase (500 U/ml). Glutathione (500 μM) did not show any effect on the assay with both TAM and cytochrome *c*, but ascorbate did show interference at a 50 μM concentration in either the TAM and cytochrome *c* methods.

DISCUSSION

The results showed that major biological oxidoreductants including glutathione, ascorbate and hydrogen peroxide had no effect on TAM. However, the TAM EPR signal was found to decay significantly in the presence of superoxide radicals. The results demonstrated that TAM was an efficient spin probe for the detection of the generation of superoxide in enzymatic or cellular systems. TAM can undergo two types of chemical reactions: (1) spin-loss reactions leading to conversion of the EPR active paramagnetic molecule to EPR silent diamagnetic molecule; (2) reactions wherein the paramagnetism is preserved while changes are made elsewhere in the molecule. In the later case, the resultant molecule will still be a radical and may give rise to an EPR spectrum that may be similar to or different from that of TAM. The reactions leading to spin-loss can be conveniently followed using EPR spectroscopy.

In the spectrophotometric method, the reaction between the trityl radical and superoxide gave a stable product with a distinct absorption in the visible spectrum (546 nm). TAM, upon reaction with superoxide, formed a characteristic product which showed an absorbance maximum at 546 nm and the absorbance linearly increased with time and superoxide concentration (Kutala, Parinandi, Zweier & Kuppusamy, 2004). The increase in absorbance in the presence of X/XO was specifically due to superoxide as confirmed by its complete inhibition by SOD. The data on the signal decay of TAM in the presence of activated PMNs reflected the known ability of the stimulants. Opsonized zymosan was reported to be a less efficient stimulant of superoxide production than PMA (Roubaud, Sankarapandi, Kuppusamy, Tordo & Zweier, 1998).

The exact nature of the reaction between TAM and superoxide is yet not clear. We observed a faster decay of the signal at a lower pH suggested that $HOO^{\cdot}$ rather than $O_2^{-\cdot}$ ($HOO^{\cdot}/O_2^{-\cdot}$ pK_a= 4.8) was involved in the reaction, as it was previously observed in the case of stable nitroxides (Krishna, Russo, Mitchell, Goldstein, Dafni & Samuni. 1996). The involvement of a neutral protonated

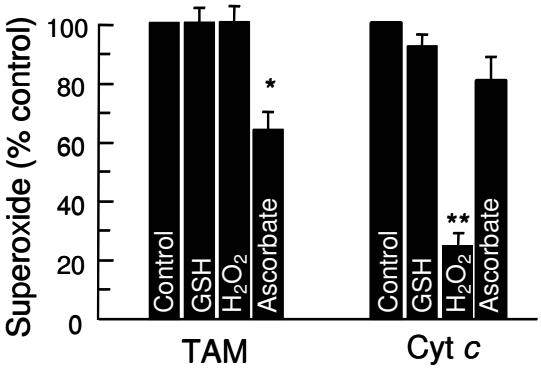

Fig. 5. Effect of GSH, H_2O_2, and ascorbate on the superoxide determination with TAM and cytochrome c (cyt c). The assay mixture for TAM consisted of 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM EDTA, 0.5 mM X, 0.02 U/ml XO, and 0.1 mM TAM and for cytochrome c assay the mixture contained 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM EDTA, 0.5 mM X, 0.02 U/ml XO, and cytochrome c (75 μM). The concentrations of GSH and H2O2 were both 500 μM and that of ascorbate was 50 μM. All results are from experiments carried out in duplicate or triplicate and each time point was the Mean ± SD of four independent measurements. *p < 0.05 vs control; **p<0.01 vs control.

form of superoxide is also necessary due to the fact that the TAM molecule is negatively charged. Since the product formed in the reaction of TAM with superoxide is EPR silent, the reaction may involve a simple radical-radical combination leading to a diamagnetic adduct as shown in reaction (Figure 6):

$$[R_3C]^{3-} + {}^{\bullet}OOH \longrightarrow [R_3C - OOH]^{3-} \qquad (6)$$

We tested the presence of the peroxide in the reaction mixture using a FOX assay (Gay, Collins & Gebicki, 1999). We estimated that the concentration of hydroperoxide was ~1 μM, which strongly favors the radical-radical combination reaction leading to the formation of hydroperoxide.

TAM was found to react very fast with superoxide with a rate constant of 3.1×10^3 M^{-1} s^{-1}, as determined using a competitive kinetics approach (Rizzi, Samouilov, Kumar Kutala, Parinandi, Zweier & Kuppusamy, 2003). This rate constant, as those measured generally for hydroxylamine oxidation (Dikalov, Skatchkov & Bassenge. 1997; Rosen, Finkelstein & Rauckman, 1982), is several orders of magnitude larger than those previously determined for superoxide spin-trapping by various nitrones (*e.g.* compared at pH 7.4 to 30 M^{-1} s^{-1} for DMPO) (Finkelstein, Rosen & Rauckman, 1980). The poor efficiency of spin-trapping is usually counter-balanced by increased spin-trap concentra-

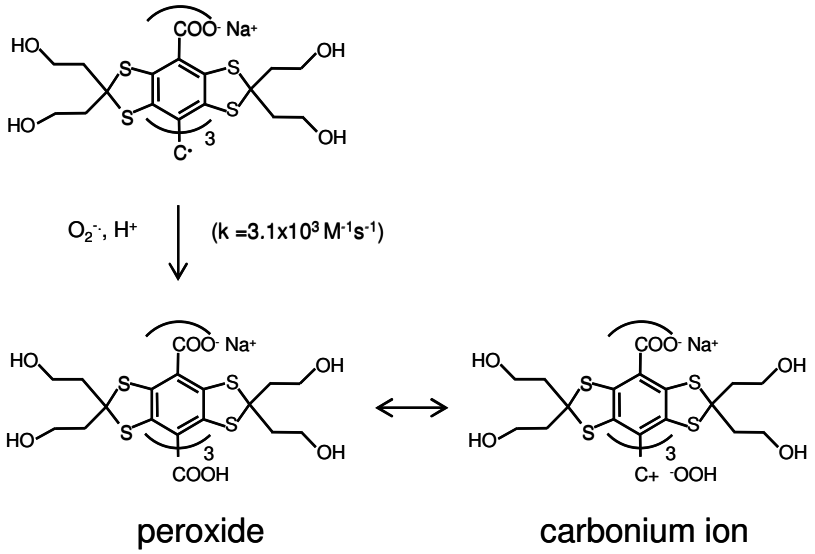

Fig. 6. Suggested mechanism of reaction of TAM with superoxide. The trityl radical on reaction with superoxide forms a hydroperoxide, which may exist in the form of carbonium ion.

tion. The trapping efficiency of TAM at the 10 μM concentration level was three times lower than that of DEPMPO (at 10 mM), but this was an additional advantage of TAM, which should allow the measurement of superoxide radicals under nonperturbing conditions by maintaining the cells homeostasis.

Conventional EPR detection techniques for superoxide and related radicals employ non-radical (diamagnetic) molecules that result in the production of radical (paramagnetic) spin-adducts. The spin-adducts exhibit characteristic spectra enabling the identification and possible quantitation of the reactive oxygen free radicals (Frejaville, Karoui, Tuccio, Le Moigne, Culcasi, Pietri, Lauricella & Tordo, 1995; Roubaud *et al.*, 1997; Zhang, Joseph, Vasquez-Vivar, Karoui, Nsanzumuhire, Martasek, Tordo & Kalyanaraman, 2000). This method is generally preferred for two reasons: (i) the appearance and growth of an EPR signal positively indicates the presence and generation of radicals and (ii) the method could enable the identification of the radical species trapped. The spin-trapping method, however, requires the use of large concentrations of the trapping agent. Further complications arise due to instability of the spin-adduct and interconversion reactions between the adducts. While the multiple peaks due to hyperfine coupling of the free electron with nitrogen, proton or phosphorus of the adduct molecule facilitate identification of the trapped species, they also decrease significantly the sensitivity of detection. In the present study we used a radical probe, which had severable desirable properties. The probe gave a single sharp line with a width of 0.26 G, which was much smaller when compared to about 1.0-1.5 G obtained with the spin-adducts. This means that the working concentration of the probe can be reduced three orders of magnitude, for example, 10 μM compared to the >10 mM concentration of spin-traps. This low concentration combined with the fact that the probe is non-toxic should enable measurements of radicals at virtually unperturbed conditions.

The use of TAM as an EPR probe for superoxide detection in biological systems may also have certain advantages over the more recently reported hydroxylamines, which are shown to be oxidized by superoxide to paramagnetic nitroxides. Unlike nitroxides, TAM is very stable toward bioreduction. For example, the TAM signal remained constant in the presence of biological reductants, such as ascorbate and glutathione. For nitroxides, known to be reduced back to their hydroxylamines has been shown to affect their use in biological systems (Belkin, Mehlhorn, Hideg, Hankovsky & Packer, 1987; Krishna, Grahame, Samuni, Mitchell & Russo, 1992; Zhang, Goldstein & Samuni, 1999) A main drawback in using stable nitroxide radical probes is that they behave as superoxide dismutase mimics capable of modifying the superoxide radical concentration in cells (Afzal, Brasch, Nitecki & Wolff, 1984).

In this study, we also compared the results of superoxide detection by the TAM method with another spectrophotometric method namely, a cytochrome *c* reduction assay under identical conditions. Results obtained with the TAM method were comparable to those obtained from the cytochrome *c* method. However, the sensitivity of the

determination of superoxide concentration by the TAM assay was less at lower superoxide concentration (<1.0 μM/min). This difference may be due to the differences in the rate constant of TAM and cytochrome *c*. The rate constants of TAM and cytochrome *c* under these experimental conditions were 3.1×10^3 M^{-1} s^{-1} (Rizzi *et al*., 2003) and 5.0×10^5 M^{-1} s^{-1} (Butler, Koppenol & Margoliash, 1982) respectively. Under optimal conditions, the superoxide production measured by the TAM assay was 2.17 μM/min. and by the cytochrome *c* was 2.74 μM/min. This suggests that the sensitivity of determination of superoxide production by the TAM assay was greater at higher superoxide concentrations and was comparable to the cytochrome *c* assay. In this study, we also found that the TAM assay is not influenced by the presence of strong oxidizing agents such as hydrogen peroxide, which is an interfering substance in all the spectrophotometric methods including the cytochrome *c* assay. The results also showed that TAM was stable in the presence of a major biological reductant such as glutathione while ascorbate and Fe^{2+} showed interference in superoxide determination by both the TAM and cytochrome *c* assays. The effect of ascorbate on cytochrome *c* reduction was also reported previously (Al-Ayash & Wilson, 1979).

TAM measures extracellular superoxide generation as it cannot pass through the cellular membranes due to the triple-negative charge on the molecule. However, other trityl radicals which can be transported into the cell with stability in an intracellular oxidant/reductant environment and greater sensitivity to superoxide should be systematically and carefully evaluated for use in intracellular determination of superoxide generation. In addition to the utilization of the paramagnetic property of TAM that enables its use in EPR spectroscopy, the visible spectral property of TAM offers a convenient and suitable spectrophotometric assay to measure superoxide. Thus, the assays can be performed independently or can be used as complementary techniques.

SUMMARY AND CONCLUSION

A simple and convenient method for the determination of superoxide using TAM is reported. TAM reacts with superoxide with an apparent second order rate constant of 3.1×10^3 M^{-1} s^{-1} to give a diamagnetic product. The specific reactivity of TAM with superoxide, which leads to the loss of EPR signal, was utilized to detect the generation of superoxide in enzymatic (xanthine/xanthine oxidase) and cellular (stimulated neutrophils) model systems. The reaction product shows a new distinct absorption maximum at 546 nm, which can be used for spectrophotometric assay of superoxide. The results demonstrated that the TAM assay is comparable to the cytochrome *c* assay for the determination of superoxide concentration in biological systems and the assay is not affected by hydrogen peroxide. The use of TAM for detection of superoxide offers unique advantages namely, the utilization of a very low concentration of the probe and its stability against bioreduction.

Acknowledgment

We thank Nycomed Innovations (Malmö, Sweden) for providing the TAM Ox063 compound. This work was supported by National Institutes of Health Grant CA-78886.

List of Abbreviations

DMPO - 5,5-dimethyl-1-pyrroline-*N*-oxide
DTPA - diethylenetriaminepentaacetic acid
EPR – Electron paramagnetic resonance
PMA - phorbol-12-myristate-13-acetate
PMN – Polymorphonuclear neutrophil
SOD - superoxide dismutase
TAM - Triarylmethyl
X/XO - xanthine/xanthine oxidase

REFERENCES

Afzal V., Brasch R. C., Nitecki D. E. & Wolff S., (1984). Nitroxyl spin label contrast enhancers for magnetic resonance imaging. Studies of acute toxicity and mutagenesis. *Invest Radiol*, 19, 549-552.

Al-Ayash A. I. & Wilson M. T., (1979). The mechanism of reduction of single-site redox proteins by ascorbic acid. *Biochem J*, 177, 641-648.

Allouch A., Roubaud V., Lauricella R., Bouteiller J. C. & Tuccio B., (2003). Preparation and use as spin trapping agents of new ester-nitrones. *Org Biomol Chem*, 1, 593-598.

Ardenkjaer-Larsen J. H., Laursen I., Leunbach I., Ehnholm G., Wistrand L. G., Petersson J. S. & Golman K., (1998). EPR and DNP properties of certain novel single electron contrast agents intended for oximetric imaging. *J Magn Reson*, 133, 1-12.

Auclair C., Torres M. & Hakim J., (1978). Superoxide anion involvement in NBT reduction catalyzed by NADPH-cytochrome P-450 reductase: a pitfall. *FEBS Lett*, 89, 26-28.

Azzi A., Montecucco C. & Richter C., (1975). The use of acetylated ferricytochrome c for the detection of superoxide radicals produced in biological membranes. *Biochem Biophys Res Commun*, 65, 597-603.

Barbacanne M. A., Souchard J. P., Darblade B., Iliou J. P., Nepveu F., Pipy B., Bayard F. & Arnal J. F., (2000). Detection of superoxide anion released ex-

tracellularly by endothelial cells using cytochrome c reduction, ESR, fluorescence and lucigenin-enhanced chemiluminescence techniques. *Free Radic Biol Med*, 29, 388-396.

Beissenhirtz M. K., Scheller F. W., Stocklein W. F., Kurth D. G., Mohwald H. & Lisdat F., (2004). Electroactive cytochrome c multilayers within a polyelectrolyte assembly. *Angew Chem Int Ed Engl*, 43, 4357-4360.

Belkin S., Mehlhorn R. J., Hideg K., Hankovsky O. & Packer L., (1987). Reduction and destruction rates of nitroxide spin probes. *Arch Biochem Biophys*, 256, 232-243.

Bottle S. E., Hanson G. R. & Micallef A. S., (2003). Application of the new EPR spin trap 1,1,3-trimethylisoindole N-oxide (TMINO) in trapping HO. and related biologically important radicals. *Org Biomol Chem*, 1, 2585-2589.

Budd S. L., Castilho R. F. & Nicholls D. G., (1997). Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells. *FEBS Lett*, 415, 21-24.

Butler J., Koppenol W. H. & Margoliash E., (1982). Kinetics and mechanism of the reduction of ferricytochrome c by the superoxide anion. *J Biol Chem*, 257, 10747-10750.

Di J., Bi S. & Zhang M., (2004). Third-generation superoxide anion sensor based on superoxide dismutase directly immobilized by sol-gel thin film on gold electrode. *Biosens Bioelectron*, 19, 1479-1486.

Dikalov S., Skatchkov M. & Bassenge E., (1997). Quantification of peroxynitrite, superoxide, and peroxyl radicals by a new spin trap hydroxylamine 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine. *Biochem Biophys Res Commun*, 230, 54-57.

Dikalov S., Tordo P., Motten A. & Mason R. P., (2003). Characterization of the high resolution ESR spectra of the methoxyl radical adducts of 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO). *Free Radic Res*, 37, 705-712.

Dikalov S. I., Dikalova A. E. & Mason R. P., (2002). Noninvasive diagnostic tool for inflammation-induced oxidative stress using electron spin resonance spectroscopy and an extracellular cyclic hydroxylamine. *Arch Biochem Biophys*, 402, 218-226.

Finkelstein E., Rosen G. M. & Rauckman E. J., (1980). Spin trapping of superoxide and hydroxyl radical: practical aspects. *Arch Biochem Biophys*, 200, 1-16.

Fraticelli A., Serrano C. V., Jr., Bochner B. S., Capogrossi M. C. & Zweier J. L., (1996). Hydrogen peroxide and superoxide modulate leukocyte adhesion molecule expression and leukocyte endothelial adhesion. *Biochim Biophys Acta*, 1310, 251-259.

Frejaville C., Karoui H., Tuccio B., Le Moigne F., Culcasi M., Pietri S., Lauricella R. & Tordo P., (1995). 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: a new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centered radicals. *J Med Chem*, 38, 258-265.

Fridovich I., (1997). Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *J Biol Chem*, 272, 18515-18517.

Gay C., Collins J. & Gebicki J. M., (1999). Hydroperoxide assay with the ferric-xylenol orange complex. *Anal Biochem*, 273, 149-155.

Golman K., Petersson J. S., Ardenkjaer-Larsen J. H., Leunbach I., Wistrand L. G., Ehnholm G. & Liu K., (2000). Dynamic in vivo oxymetry using overhauser enhanced MR imaging. *J Magn Reson Imaging*, 12, 929-938.

Gomberg M., (1900). An instance of trivalent cation: Triphenylmethyl. *J Am Chem Soc*, 22, 757-771.

He G., Deng Y., Li H., Kuppusamy P. & Zweier J. L., (2002). EPR/NMR co-imaging for anatomic registration of free-radical images. *Magn Reson Med*, 47, 571-578.

Krishna M. C., English S., Yamada K., Yoo J., Murugesan R., Devasahayam N., Cook J. A., Golman K., Ardenkjaer-Larsen J. H., Subramanian S. & Mitchell J. B., (2002). Overhauser enhanced magnetic resonance imaging for tumor oximetry: coregistration of tumor anatomy and tissue oxygen concentration. *Proc Natl Acad Sci U S A*, 99, 2216-2221.

Krishna M. C., Grahame D. A., Samuni A., Mitchell J. B. & Russo A., (1992). Oxoammonium cation intermediate in the nitroxide-catalyzed dismutation of superoxide. *Proc Natl Acad Sci U S A*, 89, 5537-5541.

Krishna M. C., Russo A., Mitchell J. B., Goldstein S., Dafni H. & Samuni A., (1996). Do nitroxide antioxidants act as scavengers of O2-. or as SOD mimics? *J Biol Chem*, 271, 26026-26031.

Kuppusamy P., Wang P., Chzhan M. & Zweier J. L., (1997). High resolution electron paramagnetic resonance imaging of biological samples with a single line paramagnetic label. *Magn Reson Med*, 37, 479-483.

Kutala V. K., Parinandi N. L., Zweier J. L. & Kuppusamy P., (2004). Reaction of superoxide with trityl radical: implications for the determination of superoxide by spectrophotometry. *Arch Biochem Biophys*, 424, 81-88.

Li Y., Zhu H., Kuppusamy P., Roubaud V., Zweier J. L. & Trush M. A., (1998). Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem*, 273, 2015-2023.

Lurie D. J., Li H., Petryakov S. & Zweier J. L., (2002). Development of a PEDRI free-radical imager using a 0.38 T clinical MRI system. *Magn Reson Med*, 47, 181-186.

McCord J. M. & Fridovich I., (1969). The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. *J Biol Chem*, 244, 6056-6063.

Murugesan R., Cook J. A., Devasahayam N., Afeworki M., Subramanian S., Tschudin R., Larsen J. A., Mitchell J. B., Russo A. & Krishna M. C., (1997). In vivo imaging of a stable paramagnetic probe by pulsed-radiofrequency electron paramagnetic resonance spectroscopy. *Magn Reson Med*, 38, 409-414.

Panagiotelis I., Nicholson I., Foster M. A. & Hutchison J. M., (2001). T*1e and T*2e maps derived in vivo from the rat using longitudinally detected electron spin resonance phase imaging: application to abdomi-

nal oxygen mapping. *Magn Reson Med*, 46, 1223-1232.

Panagiotelis I., Nicholson I. & Hutchison J. M., (2001). Electron spin relaxation time measurements using radiofrequency longitudinally detected ESR and application in oximetry. *J Magn Reson*, 149, 74-84.

Rizzi C., Samouilov A., Kumar Kutala V., Parinandi N. L., Zweier J. L. & Kuppusamy P., (2003). Application of a trityl-based radical probe for measuring superoxide. *Free Radic Biol Med*, 35, 1608-1618.

Rosen G. M., Finkelstein E. & Rauckman E. J., (1982). A method for the detection of superoxide in biological systems. *Arch Biochem Biophys*, 215, 367-378.

Roubaud V., Sankarapandi S., Kuppusamy P., Tordo P. & Zweier J. L., (1998). Quantitative measurement of superoxide generation and oxygen consumption from leukocytes using electron paramagnetic resonance spectroscopy. *Anal Biochem*, 257, 210-217.

Roubaud V., Sankarapandi S., Kuppusamy P., Tordo P. & Zweier J. L., (1997). Quantitative measurement of superoxide generation using the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide. *Anal Biochem*, 247, 404-411.

Sies H., (1989). Relationship between free radicals and vitamins: an overview. *Int J Vitam Nutr Res Suppl*, 30, 215-223.

Thomson L., Trujillo M., Telleri R. & Radi R., (1995). Kinetics of cytochrome c2+ oxidation by peroxynitrite: implications for superoxide measurements in nitric oxide-producing biological systems. *Arch Biochem Biophys*, 319, 491-497.

van Gelder B S. E., (1962). The extinction coefficient of cytochrome c. *Biochim Biophys Acta*, 58, 593-595.

Vanin A. F., Liu X., Samouilov A., Stukan R. A. & Zweier J. L., (2000). Redox properties of iron-dithiocarbamates and their nitrosyl derivatives: implications for their use as traps of nitric oxide in biological systems. *Biochim Biophys Acta*, 1474, 365-377.

Yong L., Harbridge J., Quine R. W., Rinard G. A., Eaton S. S., Eaton G. R., Mailer C., Barth E. & Halpern H. J., (2001). Electron spin relaxation of triarylmethyl radicals in fluid solution. *J Magn Reson*, 152, 156-161.

Zhang H., Joseph J., Vasquez-Vivar J., Karoui H., Nsanzumuhire C., Martasek P., Tordo P. & Kalyanaraman B., (2000). Detection of superoxide anion using an isotopically labeled nitrone spin trap: potential biological applications. *FEBS Lett*, 473, 58-62.

Zhang R., Goldstein S. & Samuni A., (1999). Kinetics of superoxide-induced exchange among nitroxide antioxidants and their oxidized and reduced forms. *Free Radic Biol Med*, 26, 1245-1252.

Zhao H., Kalivendi S., Zhang H., Joseph J., Nithipatikom K., Vasquez-Vivar J. & Kalyanaraman B., (2003). Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med*, 34, 1359-1368.

Zou M., Martin C. & Ullrich V., (1997). Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol Chem*, 378, 707-713.