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Evaluation of spin trapping agents and trapping conditions for detection of cell-generated reactive oxygen species

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Abstract

Electron paramagnetic resonance with spin trapping is a useful technique to detect reactive oxygen species, such as superoxide radical anion ($O_2^{\bullet-}$), a key species in many biological processes. We evaluated the abilities of four spin traps in trapping cell-generated $O_2^{\bullet-}$: 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BMPO), 2-diethoxyphosphoryl-2-phenethyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DEPPEPO), 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). Optimal experimental conditions for obtaining maximal signal intensity of $O_2^{\bullet-}$ adduct in a cellular system were first studied. The maximal intensities of BMPO, DEPMPO, and DMPO adducts were similar while DEPPEPO did not trap cell-generated $O_2^{\bullet-}$ induced by 1,6-benzo[*a*]pyrene quinone in a human mammary epithelial cell line (MCF-10A). BMPO and DEPMPO adducts were more stable, considering the stability of their maximal signal, than DMPO adduct in the tested cellular systems. In addition, we observed that $O_2^{\bullet-}$ spin adducts were reduced to their corresponding hydroxyl adducts in the cellular system. The selection of optimal spin trap in trapping cell-generated $O_2^{\bullet-}$ is discussed.

Keywords: BMPO; DMPO; DEPMPO; DEPPEPO; Spin trap; Superoxide radical anion; EPR; Cell

Reactive oxygen species have been implicated in the development of many diseases and aging, as well as having a role in many normal physiological processes. Particularly, superoxide radical anion ($O_2^{\bullet-}$) is most likely the initial reactive oxygen species generated in cells, from which other reactive species such as hydrogen peroxide and hydroxyl radical are derived. Many methods have been developed to assay $O_2^{\bullet-}$ in chemical, biochemical, cellular, and in vivo systems. These include cytochrome *c* reduction, chemiluminescence from lucigenin and related dyes, dihydroethidium oxidation, nitro blue tetrazolium reduction, aconitase inactivation, and electron paramagnetic resonance (EPR)[1] spectroscopy with spin trapping techniques. Among these methods, EPR spin trapping is a unique technique for detecting $O_2^{\bullet-}$ in various systems, providing the most direct and

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[1] *Abbreviations used:* EPR, electron paramagnetic resonance; BMPO, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; DEPPEPO, 2-diethoxyphosphoryl-2-phenethyl-3,4-dihydro-2*H*-pyrrole *N*-oxide; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

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specific measurement. The technique has been used widely and successfully in chemical, biochemical, and biological studies. EPR spin trapping technique has played a key role in elucidating important biological processes such as the $O_2^{\bullet-}$ formation from nitric oxide synthase [1,2], metal ion induced signal transduction [3], and peroxidation of mitochondrial cytochrome *c* oxidase [4].

In an effort to optimize EPR spin trapping techniques for biological uses, spin traps for $O_2^{\bullet-}$ have been continually developed in the last 30 years to provide better results in biological and cellular systems [5,6]. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) is a well-documented and widely used spin trap to detect $O_2^{\bullet-}$. However, the DMPO–superoxide adduct (DMPO–OOH) has a relatively short life with a half-life less than 1 min and can decompose or be metabolized to produce the hydroxyl adduct in the presence of metal ions [7]. Recently, several new $O_2^{\bullet-}$ spin traps have been developed by modifying DMPO, based on the theory that substitution of one of the methyl groups in DMPO with a strong electron-withdrawing diethoxyphosphoryl group will result in stabilization of the corresponding superoxide radical anion adduct. The synthesis of phosphonic acid derived spin trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) has been described by Frejaville et al. [8]. It was reported that DEPMPO had outstanding spin trapping properties and long-lived superoxide radical anion adduct (DEPMPO–OOH) with half-life 14.8 min, over 10 times higher than that of DMPO–OOH. In addition, DEPMPO–OOH does not decompose to DEPMPO–hydroxyl adduct (DEPMPO–OH) [8–10]. Most recently, 2-diethoxyphosphoryl-2-phenethyl-3,4-dihydro-2*H*-pyrrole-1-oxide (DEPPEPO) was developed to trap $O_2^{\bullet-}$. DEPPEPO has similar superoxide radical anion adduct half-life to DEPMPO but is more lipophilic [11]. 5-*tert*-Butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO) is also a modification of DMPO. By substituting a methyl group with a butoxyl group, BMPO has been synthesized and reported to have a more stable superoxide radical anion adduct over DMPO [12]. In addition, 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (EMPO) and ^{15}N-labeled EMPO have also been developed to trap $O_2^{\bullet-}$ [13].

Reports have suggested that DEPPEPO, DEPMPO, BMPO, and EMPO are "better" than DMPO in trapping $O_2^{\bullet-}$. However, most of the studies have been carried out in cell-free systems such as (hypo)xanthine/xanthine oxidase and irradiated xanthine. Swartz and co-workers [14] recently provided valuable information about the effect of various $O_2^{\bullet-}$ spin traps on cell viability. Still, there is a lack of systematic evaluation on their ability to detect cell-generated $O_2^{\bullet-}$ even though these new spin traps were developed with a goal of detecting cell-generated $O_2^{\bullet-}$. In addition, anecdotal observations in our laboratory suggest that use of optimal experimental parameters is critical for a successful $O_2^{\bullet-}$ trapping experiment. Thus, we designed experiments in the present study to: (1) determine the optimal trapping conditions (i.e., temperature, cell number, oxygen concentration, and spin trap concentration) for trapping cell-generated $O_2^{\bullet-}$; (2) compare the abilities of DMPO, BMPO, DEPMPO, and DEPPEPO in trapping $O_2^{\bullet-}$ in a cellular system.

Materials and methods

Chemicals

DEPMPO was provided by Dr. Jean-Louis Clément (Laboratoire SREP, UMR 6517 CNRS et Université d'Aix-Marseille I et III, France) and used without additional treatment. BMPO was synthesized by Dr. B. Kalyanaraman's group (Medical College of Wisconsin, WI). DMPO was from Alexis Biochemical (San Diego, CA) and used without additional treatment. DEPPEPO was provided by Dr. Y. Liu at Institute of Chemistry, Chinese Academy of Sciences, China. The structures of the four $O_2^{\bullet-}$ spin adducts are shown in Fig. 1. Diethylenetriamine pentaacetic acid (DTPA), xanthine, and xanthine oxidase were purchased from Sigma Chemical (St. Louis, MO). 1,6-Benzo[*a*]pyrene quinone (BPQ) was purchased from Midwest Research Institute (Kansas City, MO) at >99% purity and maintained as stock solutions in anhydrous tissue culture grade dimethyl sulfoxide (DMSO). Other chemicals were all obtained from Sigma Chemical (St. Louis, MO) unless indicated otherwise.

Cell culture

Human mammary epithelial cells, MCF-10A, were grown on Vitrogen-coated (Collagen, Palo Alto, CA)

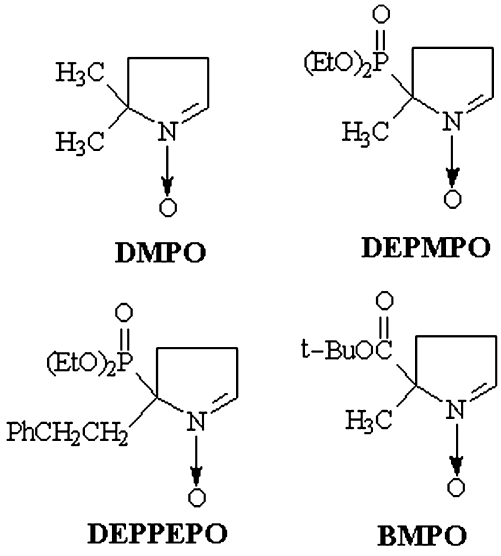

Fig. 1. Chemical structures of spin traps used in this study.