Free Radical Biology and Medicine 71 (2014) 281–290

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Detection of superoxide production in stimulated and unstimulated living cells using new cyclic nitrone spin traps

Kahina Abbas [a], Micael Hardy [b], Florent Poulhès [b], Hakim Karoui [b], Paul Tordo [b], Olivier Ouari [b], Fabienne Peyrot [a,c,*]

[a] Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques (UMR CNRS 8601), Université Paris Descartes, Sorbonne Paris Cité, 75006 Paris, France
[b] Aix-Marseille Université, CNRS, ICR UMR 7273, 13397 Marseille Cedex 20, France
[c] ESPE de l'Académie de Paris, Université Paris Sorbonne, 75016 Paris, France

ARTICLE INFO

Article history:
Received 30 December 2013
Received in revised form
11 March 2014
Accepted 17 March 2014
Available online 21 March 2014

Keywords:
Cyclic nitrones
ESR spectroscopy
EPR spectroscopy
Spin trapping
Superoxide
Hydroxyl radical
RAW macrophages
Free radical

ABSTRACT

Reactive oxygen species (ROS), including superoxide anion and hydrogen peroxide (H_2O_2), have a diverse array of physiological and pathological effects within living cells depending on the extent, timing, and location of their production. For measuring ROS production in cells, the ESR spin trapping technique using cyclic nitrones distinguishes itself from other methods by its specificity for superoxide and hydroxyl radical. However, several drawbacks, such as the low spin trapping rate and the spontaneous and cell-enhanced decomposition of the spin adducts to ESR-silent products, limit the application of this method to biological systems. Recently, new cyclic nitrones bearing a triphenylphosphonium (Mito-DIPPMPO) or a permethylated β-cyclodextrin moiety (CD-DIPPMPO) have been synthesized and their spin adducts demonstrated increased stability in buffer. In this study, a comparison of the spin trapping efficiency of these new compounds with commonly used cyclic nitrone spin traps, i.e., 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and analogs BMPO, DEPMPO, and DIPPMPO, was performed on RAW 264.7 macrophages stimulated with phorbol 12-myristate 13-acetate. Our results show that Mito-DIPPMPO and CD-DIPPMPO enable a higher detection of superoxide adduct, with a low (if any) amount of hydroxyl adduct. CD-DIPPMPO, especially, appears to be a superior spin trap for extracellular superoxide detection in living macrophages, allowing measurement of superoxide production in unstimulated cells for the first time. The main rationale put forward for this extreme sensitivity is that the extracellular localization of the spin trap prevents the reduction of the spin adducts by ascorbic acid and glutathione within cells.

Reactive oxygen species (ROS), including superoxide radical ($O_2^{\bullet -}$) and its disproportionation product H_2O_2, are by-products of normal aerobic metabolism of the cell. Under physiological conditions, their concentration is kept finely under control by antioxidant enzymes, such as superoxide dismutase (SOD) or catalase. This basal level is involved in cell signaling and regulation of physiological processes such as differentiation, proliferation, and apoptosis [1,2]. Inflammatory response to pathogens also relies on the production of $O_2^{\bullet -}$, H_2O_2, $NO^{\bullet}$, and hypochlorous acid (HOCl) by macrophages and neutrophils, leading to bacterial killing. However, excessive production or a failure in antioxidant defenses results in the accumulation of oxidative damage to cell constituents (proteins, DNA, carbohydrates, and lipids). These events of oxidative stress have been witnessed at the onset and evolution of many diseases, including cancer, cardiovascular pathologies, and neurological disorders [3–5].

Sensitive and specific detection of ROS, and especially of $O_2^{\bullet -}$ as a parent species for other ROS, is an ongoing focus in biomedical research. Spin trapping of superoxide with cyclic nitrones coupled

Abbreviations: BMPO, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; CAT, catalase; CD-DIPPMPO, 6-monodeoxy-6-mono-4-[(5-diisopropoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide)-ethylenecarbamoyl-(2,3-di-*O*-methyl)hexakis-(2,3,6-tri-*O*-methyl)]-β-cyclodextrin; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DIPPMPO, 5-diisopropoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DOSY NMR, diffusion order spectroscopy nuclear magnetic resonance; DPI, diphenyliodonium chloride; DTPA, diethylene triamine pentaacetic acid; ESI–HRMS, electrospray ionization–high-resolution mass spectrometry; ESR, electron spin resonance; Mito-DIPPMPO, (4*R**,5*R**)-5-(diisopropyloxyphosphoryl)-5-methyl-4-[({[2-(triphenylphosphonio)ethyl]carbamoyl}oxy)methyl]pyrroline *N*-oxide bromide; NEM, *N*-ethylmaleimide; NOX2, NADPH oxidase 2; P450, cytochrome P450; PEG, polyethylene glycol; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SKF 525A, *N*,*N*-diethylaminoethyl 2,2-diphenylvalerate hydrochloride; SOD, superoxide dismutase; TEMPO, (2,2,6,6-tetramethyl)piperidine-1-oxyl; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; TOMER, 2-diethoxyphosphoryl-2,5,5-trimethylpyrrolidin-1-oxyl; X, xanthine; XO, xanthine oxidase

* Corresponding author at: Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques (UMR CNRS 8601), Université Paris Descartes, Sorbonne Paris Cité, 45 rue des Saints-Pères, 75006 Paris, France. Fax: +33 142 86 83 87.
E-mail address: fabienne.peyrot@parisdescartes.fr (F. Peyrot).

http://dx.doi.org/10.1016/j.freeradbiomed.2014.03.019
0891-5849/

to ESR detection was introduced in the 1970s [6]. The principle of this method relies on the reaction of the short-lived radical of interest with a diamagnetic molecule, the spin trap, to form a persistent aminoxyl (nitroxide) radical, called the spin adduct. Ideally, the ESR spectrum of the adduct is characteristic of the initially trapped radical, which confers high specificity to this method over other techniques based on fluorescent and chemiluminescent probes or on hydroxylamine spin probes [7].

DMPO (Scheme 1) was the first cyclic nitrone spin trap successfully used for detecting ROS on chloroplasts [8] and then on intact cells, i.e., stimulated neutrophils and macrophages in the late 1970s and early 1980s [9–12]. However, severe limitations occur with DMPO: the low spin trapping rate, which makes high spin trap concentrations (50–100 mM) mandatory to compete with superoxide disproportionation, and the short half-life of the superoxide adduct (DMPO–OOH, less than 1 min at physiological pH), whose decomposition is accompanied by the formation of the hydroxyl adduct (DMPO–OH). Moreover, metabolic processes in biological systems further reduce the stability of the adducts and enhance the conversion of superoxide to hydroxyl adducts [13]. Control experiments with SOD are required to distinguish between superoxide and hydroxyl radical trapping.

Electron-withdrawing groups at position 5, as in DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide) [14], DIPPMPO (5-diisopropoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide) [15], BMPO (5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide) [16], or EMPO (5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide) [17] (Scheme 1), were shown to improve the stability of the spin adducts in buffer. Several DEPMPO derivatives bearing a triphenylphosphonium group (Mito-DEPMPO [18,19] and Mito-DIPPMPO) or a permethylated β-cyclodextrin moiety (CD-DEPMPO [20] and CD-DIPPMPO, Scheme 1) were also synthesized and their superoxide adducts were shown to be very persistent. The half-lifetime values of the spin adducts depend significantly on the experimental conditions used, and thus the reported values may vary. Recently, in phosphate buffer at pH 7.4, we estimated the half-lives of the superoxide adducts of Mito-DEPMPO, Mito-DIPPMPO, CD-DEPMPO, and CD-DIPPMPO at 50 ± 4, 65 ± 10, 83 ± 8, and 109 ± 10 min, respectively, compared to 38 ± 3 min for DEPMPO [21]. Some selectivity of superoxide versus hydroxyl radical trapping was observed in vitro when the cyclic nitrone was substituted by a triphenylphosphonium group or a permethylated β-cyclodextrin moiety [21].

In previous studies [21,22], we analyzed the resistance of superoxide adducts to decomposition processes involving microsomal and cytosolic enzymes responsible for the metabolism of xenobiotics. We emphasized the major roles of heme and hemeproteins, together with those of biological reductants such as ascorbic acid and glutathione, in the conversion of the superoxide and hydroxyl adducts of all cyclic nitrones to ESR-silent compounds. By contrast, only CD-substituted spin traps appeared highly stable in the presence of microsomal proteins, being prevented from entering the cytochrome P450 (P450) active site by steric constraints.

Here we evaluated the spin trapping efficiency of the two new cyclic nitrones, Mito-DIPPMPO and CD-DIPPMPO, in comparison to four established spin traps (DMPO, BMPO, DEPMPO, and DIPPMPO) in the context of superoxide production by RAW macrophages. Treatment of RAW macrophages with PMA induces the activation of protein kinase C, which performs the phosphorylation of a critical residue in NADPH oxidase (NOX2) in the macrophage membrane [23]. This enzyme produces superoxide as part of the normal inflammatory process.

Moreover, we investigated the possibility of detecting superoxide production in unstimulated cells with these new spin traps—a challenging objective because many articles describe spin trapping results on stimulated cells [9–12,24–28] but no production has ever been measured in unstimulated cells with cyclic nitrone spin traps using continuous-wave ESR. Only rapid-scan ESR recently enabled detection with BMPO of superoxide produced by unstimulated *Enterococcus faecalis* [29]. We also analyzed the causes of the enhanced stability of the corresponding superoxide adducts in the presence of cells.

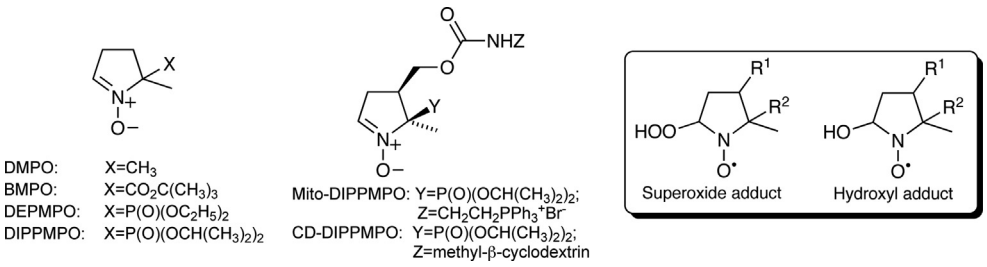
Experimental procedures

Reagents

BMPO and DIPPMPO were synthesized according to previously published protocols [15,16]. 2-Diethoxyphosphoryl-2,5,5-trimethylpyrrolidin-1-oxyl (TOMER) was synthesized according to Le Moigne et al. [30]. Purity was determined by NMR and HPLC coupled with mass spectrometry and was above 98%. Mito-DIPPMPO and CD-DIPPMPO were prepared using the procedures described for diethoxyphosphoryl analogs [18–20]. Their NMR and ESI–HRMS characterizations were previously published [21]. DEPMPO was obtained from Radical Vision (Marseille, France). Tris(ethylenediamine)nickel(II) chloride 2-hydrate ($Ni(en)_3^{2+}$) was prepared according to M. Vanduijn et al. [31]. Diethylene triamine pentaacetic acid (DTPA), xanthine (X), xanthine oxidase (XO), catalase (CAT), catalase conjugated with polyethylene glycol (CAT–PEG), superoxide dismutase, superoxide dismutase conjugated with polyethylene glycol (SOD–PEG), (2,2,6,6-tetramethyl) piperidine-1-oxyl (TEMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), diphenyliodonium chloride (DPI), *N*,*N*-diethylaminoethyl 2,2-diphenylvalerate hydrochloride (SKF 525A), *N*-ethylmaleimide (NEM), and Dulbecco's phosphate-buffered saline (PBS; pH 7.1–7.5) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).

Culture of RAW macrophages

Macrophages (RAW 264.7 cells), originally purchased from the American Type Culture Collection (CRL-9609), were a kind gift from Dr. Jean-Claude Drapier (CNRS UPR 2301, Gif-sur-Yvette, France). Cells were cultured in Dulbecco's modified Eagle medium (DMEM AQmedia, Sigma–Aldrich) supplemented with 5% fetal



Scheme 1. Structures of the spin traps under study and general structures for superoxide and hydroxyl adducts.