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Original Contribution

Metabolic stability of superoxide and hydroxyl radical adducts of a cyclic nitrone toward rat liver microsomes and cytosol: A stopped-flow ESR spectroscopy study

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ABSTRACT

The metabolic stability of the spin adducts derived from the reaction of superoxide and hydroxyl radicals with 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BocMPO) in the presence of rat liver microsomes (RLM) and rat liver cytosol (RLC) was studied by using a stopped-flow device coupled to an electron spin resonance (ESR) spectrometer. The kinetics of the disappearance of the BocMPO-OH and BocMPO-OOH radicals could be followed by ESR spectroscopy with treatment of the ESR data by an appropriate computer program. The presence of cytosol led to a 60-fold decrease of the half-life of BocMPO-OOH with the intermediate formation of BocMPO-OH. This effect of cytosol was due to an ascorbate- and thiol-dependent reduction of BocMPO-OOH. RLC only led to a 5-fold decrease of the half-life of BocMPO-OH that was predominantly due to cytosolic ascorbate. RLM led to a 10-fold decrease of the BocMPO-OOH half-life that was mainly related to a direct reaction of the hydroperoxide function of BocMPO-OOH with cytochrome P450 Fe(III) (P450). Other ferric heme proteins, such as methemoglobin (metHb) and horseradish peroxidase (HRP), as well as hemin itself, exhibited a similar behavior. RLM and metHb showed a much weaker effect on BocMPO-OH half-life (2-fold decrease), whereas RLM in the presence of NADPH caused a greater decrease of the BocMPO-OH half-life ($\approx$5-fold). The effect of RLM without NADPH was mainly due to a direct reaction with microsomal P450, whereas the RLM- and NADPH-dependent effect was mainly due to flavin-containing reductases such as cytochrome P450 reductase. These data on the effects of liver subcellular fractions on the half-life of the BocMPO-OOH and the BocMPO-OH spin adducts highlight the role of heme as a biological cofactor involved in the disappearance of such spin adducts. They should be helpful for the design of new spin traps that would form more metabolically stable spin adducts *in vitro* and *in vivo*.

Introduction

Reactive oxygen species (ROS) are by-products of aerobic metabolism and it is now clear that superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) play critical roles in the genesis of various diseases [1–3]. These oxygen-derived species are involved in several pathological conditions including inflammation, neurodegenerative diseases, and carcinogenesis. Interestingly, they also play important roles as second messengers to activate various signaling pathways [4]. Superoxide is one of the most important ROS because it rapidly produces other ROS such as H_2O_2 and peroxynitrite, stimulates $HO^{\bullet}$ production through Fenton chemistry, and enhances the release of iron from various proteins [5–7]. Detection of superoxide is thus of crucial importance in the understanding of various biological events and a large number of methods have been developed to determine the amounts of this short-lived radical formed *in vitro* as well as *in vivo* [8–10]. Although there are many methods for identifying specific free radicals, spin trapping coupled with electron spin resonance (ESR) spectroscopy is a method of choice to characterize these species in chemical and biological systems [11–15].

In spin trapping experiments, short-lived free radicals react with diamagnetic compounds (spin traps) to form more stable radicals (spin adducts) that are detected by ESR spectroscopy. ESR spin trapping is the only physical method that can unambiguously detect and identify radicals in biological systems at room temperature. The reactivity of spin traps toward various radicals and the stability of the corresponding spin

Abbreviations: AscOx, ascorbic acid oxidase; BSA, bovine serum albumin; BocMPO, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; P450, cytochrome P450; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DPI, diphenyliodonium; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylene triamine pentaacetic acid; ESR, electron spin resonance; HRP, horseradish peroxidase; metHb, methemoglobin; metMb, metmyoglobin; NEM, *N*-ethylmaleimide; PB, phenobarbital; PCA, (2,2,5,5-tetramethyl)pyrrolidine-1-oxyl-3-carboxylic acid; RLC, rat liver cytosol; RLM, rat liver microsomes; ROS, reactive oxygen species; SOD, superoxide dismutase; $t_{1/2}$, half-life; TEMPO, (2,2,6,6-tetramethyl)piperidine-1-oxyl; X, xanthine; XO, xanthine oxidase.

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adducts are thus critical challenges in the development of this technique in biological systems (Scheme 1). The cyclic nitrone 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, Scheme 1) has been commonly used to trap $O_2^{\bullet-}$, but its DMPO-OOH adduct spontaneously (nonenzymatically) decays with a very short half-life close to 1 min at pH 7.0 [16–19]. This drawback and the difficult purification of DMPO have restricted its use. In comparison with DMPO, the spin trap diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO, Scheme 1) offers some advantages as the DEPMPO-OOH adduct exhibits a longer half-life in phosphate buffer, pH 7.0, and does not spontaneously decompose to the corresponding DEPMPO-OH adduct [20–22]. 5-*Tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BocMPO, Scheme 1) is another new cyclic nitrone. Superoxide spin trapping by BocMPO is slower than by DEPMPO, and the BocMPO-OOH adduct exhibits a slightly shorter half-life than the DEPMPO-OOH adduct [23–25]. However, the corresponding superoxide and hydroxyl spin adducts exhibit clearly distinct and characteristic ESR spectra and the ESR signal of the BocMPO-OOH adduct is simpler than that of the DEPMPO-OOH adduct [20–25]. Furthermore, BocMPO is easily isolated as a pure white solid that is stable for days at room temperature and thus offers several advantages for applications in biological systems.

The detection of free radicals *in vitro* in complex biological media or *in vivo* in small animals by spin trapping experiments requires that the corresponding spin adducts are formed in high enough steady-state concentrations to be detected by ESR spectroscopy or imaging [12,26]. This will depend on the reactivity of the spin trap toward the free radical of interest and on the half-life of the spin adduct in the biological medium. In simple buffers at pH 7.4, the half-lives of $HO^{\bullet}$ spin adducts are between minutes and hours whereas the half-lives of $O_2^{\bullet-}$ adducts are much smaller [12,18,20,23]. In biological media, these half-lives should be considerably decreased because of chemical and enzymatic reactions [12,26]. However, very few data are currently available on the "metabolic stability" of the $HO^{\bullet}$ spin adducts in the presence of various hepatic subcellular fractions containing the usual xenobiotic-metabolizing enzymes [27]. Moreover, to our knowledge, no data have been reported so far on the "metabolic stability" of the more unstable $O_2^{\bullet-}$ adducts under those conditions.

By contrast, a great number of metabolic studies have been performed on stable nitroxide radicals, such as (2,2,6,6-tetramethyl) piperidine-1-oxyl (TEMPO) or (2,2,5,5-tetramethyl)pyrrolidine-1-oxyl-3-carboxylic acid (PCA) radicals (Scheme 2) that are used as biochemical probes of redox metabolism, as contrast agents for magnetic resonance imaging, and for *in vivo* ESR imaging, as well as antioxidants [28–33]. Contrary to spin adducts, those nitroxide radicals are almost indefinitely stable in the solid state and highly stable in simple buffers.

In an effort to study the "metabolic stability" of $O_2^{\bullet-}$ and $HO^{\bullet}$ spin adducts that exhibit much shorter half-lives than stable nitroxides, we have used a stopped-flow device coupled to an ESR spectrometer to follow the effects of various hepatic subcellular fractions on the half-lives of these spin adducts. This article describes first results on the effects of rat liver microsomes (RLM) and rat liver cytosol (RLC) on the adducts of BocMPO with $O_2^{\bullet-}$ (BocMPO-OOH) and $HO^{\bullet}$ (BocMPO-OH).

Experimental procedures

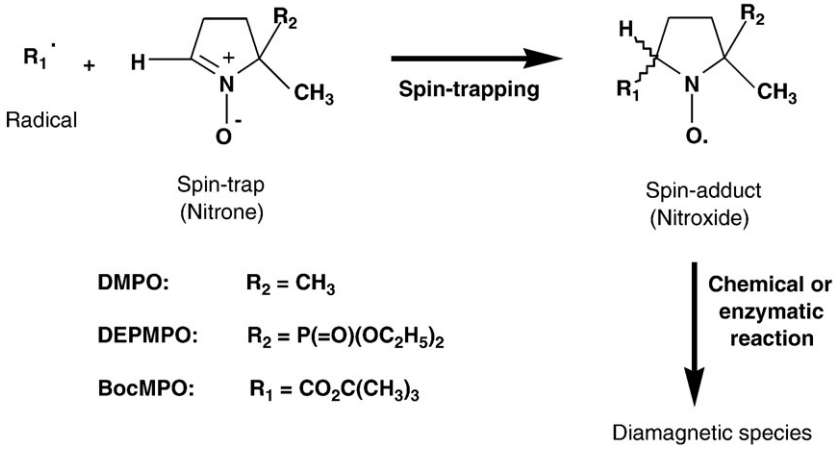
Reagents

BocMPO was synthesized as a pure white solid according to a previously published protocol [23]. Purity was assayed by ^{1}H NMR and HPLC coupled with mass spectrometry and was above 98%. NADPH and NADH were purchased from Boehringer (Mannheim, Germany). Xanthine (X), xanthine oxidase (XO), superoxide dismutase (SOD), cytochrome b_5, cytochrome *c*, catalase, methemoglobin (metHb), metmyoglobin (metMb), horseradish peroxidase (HRP), diethylene triamine pentaacetic acid (DTPA), diphenyliodonium chloride (DPI), clotrimazole, TEMPO, ascorbate oxidase (AscOx), and metyrapone were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). All other chemicals and solvents were of the highest grade commercially available. Ultrapure water (ELGA, Antony, France, resistivity: 18.2 MΩ. cm) was used for the preparation of 0.1 M phosphate buffer (pH 7.4) containing 1 mM DTPA. H_2O_2 concentration was determined using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$ [34].

Preparation of rat liver microsomes (RLM) and cytosol (RLC)

Male Sprague Dawley rats (200–250 g, Charles River, L'Arbresle, France) were provided laboratory chow and water *ad libitum*. After 7 days of adaptation, animals were treated either with phenobarbital (PB) (20 mg kg^{-1}, in corn oil, ip for 4 days), β-naphthoflavone or dexamethasone (each 50 mg kg^{-1}, in corn oil, ip for 4 days). Control animals were treated with corn oil only (0.5 mL, ip, for 4 days). Liver cytosols and microsomes were prepared by differential centrifugation as previously reported [35] and stored at -80 °C until use.

Protein concentrations were determined by the Bradford assay with bovine serum albumin (BSA) as standard [36]. Cytochrome P450 (P450) contents were determined by the method of Omura and Sato [37]. Determination of XO activity was performed by quantitation of superoxide generation measured using the initial rates of reduction of ferric cytochrome *c* to ferrous cytochrome *c*. Cuvettes (total volume 150 µL) contained 0.1 M phosphate buffer, pH 7.4, 1 mM DTPA, 500 µM X, 100 U/mL catalase, 100 µM cytochrome *c*, and variable amounts of XO. Spectra were recorded on a Uvikon 942 spectrophotometer (Kontron Biotech) at 37 °C and a $\Delta\varepsilon_{550nm}$ value of 21,000 $M^{-1}\ cm^{-1}$ was used.



Scheme 1. Spin trapping of free radicals (such as $HO^{\cdot}$ and $O_2^{\bullet-}$) by cyclic nitrones and structures of DMPO, DEPMPO, and BocMPO mentioned in the text.