Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 347 (2005) 144–151
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

An ultrasensitive fluorescent assay for the in vivo quantification of superoxide radical in organisms

Christos D. Georgiou [a,*], Ioannis Papapostolou [a], Nikolaos Patsoukis [a], Theodore Tsegenidis [b], Theodore Sideris [a]

[a] Department of Biology, Section of Genetics, Cell Biology, and Development, University of Patras, 26100 Patras, Greece
[b] Department of Chemistry, Section of Organic Chemistry, Biochemistry, and Natural Products, University of Patras, 26100 Patras, Greece

Received 25 August 2005
Available online 30 September 2005

Abstract

Superoxide radical is a very important parameter of oxidative stress involved in a variety of biological phenomena; therefore, its in vivo study is of utmost significance. However, its accurate detection is a challenge due to its short lifetime and its very low physiological concentration. All current assays are qualitative and nonspecific, and at best they are performed in vitro. The current dihydroethidine-based assay overcomes all these problems and introduces the following novelties. First, it measures the in vivo superoxide production in animals, plants, and microorganisms. Second, it is ultrasensitive and very simple in that it can measure superoxide radical as low as 1.5 pmol in biological samples as low as 5 mg. Third, the very high sensitivity of the assay renders possible, for the first time, the measurement of the actual rate of formation of superoxide radical under physiological and simulated nonphysiological conditions.

Keywords: Dihydroethidine; 2-Hydroxyethidium; Superoxide radical

Superoxide has been implicated in a variety of physiological processes such as aging, differentiation, development, reproduction, cell cycle, and apoptosis [1] as well as in pathological conditions such as cancer, atherosclerosis, hypertension, diabetes, ischemia/reperfusion injury, traumatic brain injury, acute lung injury, pulmonary fibrosis, cerebral ischemia, epilepsia, and impaired learning and memory functions [1–3].

Because of the immense biological significance of superoxide radical, many attempts have been made for its accurate detection, which has been problematic due to its short lifetime (a few seconds) and the presence of competing intra- and extracellular antioxidants. Extracellular superoxide has been detected in vitro by superoxide dismutase (SOD)[1]-inhibited cytochrome *c* reduction (reduced also by other cellular reductants such as GSH and ascorbate), aconitase inhibition (inhibited also by O_2, H_2O_2, NO, and $ONOO^-$), and nitroblue tetrazolium (NBT^{2+}) reduction (with the main pitfall of the assay being that NBT^{2+} can serve as both a generator and a detector of superoxide) [3,4]. Intracellular (and extracellular) detection of superoxide has been done in vitro with the chemiluminescent detector molecules lucigenin, luminol, and coelenterazine and has been done in vivo with electron paramagnetic resonance (EPR) spectroscopy. Lucigenin and luminol assays have the same drawback with the

* Corresponding author. Fax: +30 61 997840.
E-mail address: c.georgiou@upatras.gr (C.D. Georgiou).

[1] *Abbreviations used:* SOD, superoxide dismutase; NBT^{2+}, nitroblue tetrazolium; EPR, electron paramagnetic resonance; DHE, dihydroethidine; Et, ethidium; BAEC, bovine aortic endothelial cell; X, xanthine; XO, xanthine oxidase; HRP, horseradish peroxidase; A, adriamycin; T, tempol; DMSO, dimethyl sulfoxide; ACN, acetonitrile; P, paraquat; M, menadione; TFA, trifluoroacetic acid; HUVEC, human umbilical vein endothelial cell; ex/em, excitation/emission; ESI, electrospray ionization.

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.09.013

NBT^{2+} assay, whereas coelenterazine is nonspecific (reacts with $ONOO^-$ as well) [3,4]. EPR is currently the only analytical approach that permits the in vivo detection of superoxide, but it presents serious disadvantages such as adduct short stability, lack of specificity of spin trap, and cumbersome and expensive instrumentation [3].

Dihydroethidine (DHE) has been used as a superoxide probe since 1990 and shown to be specific for this radical [5,6]. Later on, it was found that DHE could also be oxidized by a variety of cellular reactants such as cytochrome *c*, mitochondrial cytochrome, hemoglobin, myoglobin, hypochlorous acid, and H_2O_2 via the nonspecific peroxidases horseradish and myeloperoxidase [7–9]. The resulting oxidation products presented serious interference problems because they were found to have fluorescence emission peaks near the initially thought ethidium (Et) superoxide–DHE oxidation product and the recently clarified 2-OH-ethidium product [10,11]. Additional interfering problems arose from the enhanced fluorescence of all known DHE oxidation products due to their interaction with DNA when the superoxide–DHE oxidation product was detected by flow cytometry or fluorescence microscopy. Moreover, quantification of superoxide production using DHE initially was shown to be inaccurate due to its capacity to enhance the rate of superoxide dismutation; thus, the initial rate of superoxide formation was underestimated because there was not a detailed investigation of its stoichiometry [7]. Moreover, at that time it was assumed falsely that the product of the oxidation of DHE by superoxide radical was Et.

Despite the fact that DHE can be oxidized to various products, superoxide radical is the only oxidant that gives a characteristic mono-oxygenated oxidation product with DHE, namely 2-OH-ethidium. Currently, 2-OH-ethidium has been identified [10] and detected only in vitro [in bovine aortic endothelial cell (BAEC) cultures and small mouse aorta segments] after isobutanol or methanol extraction (of unknown percentage recovery required to assess its concentration accurately) and HPLC quantification. Moreover, the rate of 2-OH-ethidium formation has not corresponded to the rate of superoxide production [10,12]. The current work deals with all of the above problems by introducing a DHE-based assay that measures, for the first time, superoxide production in vivo (in whole organisms such as mouse, spinach, and yeast) via an established (in this study) stoichiometric relationship of this radical with 2-OH-ethidium.

Materials and methods

Reagents

Xanthine (X) was obtained from Serva (Heidelberg, Germany). Xanthine oxidase (XO), DHE, Et (bromide), horseradish peroxidase (HRP), myeloperoxidase, catalase (from bovine liver), adriamycin (A), tempol (T), DNA type III (from salmon testes), cytochrome *c*, hemoglobin, myoglobin, sodium dithionite, and Dowex 50X-8 (mesh 400) were obtained from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), acetone, chloroform, diethyl ether, acetonitrile (ACN), absolute methanol, paraquat (P), menadione (M), hydrogen peroxide, sodium cyanide, hypochlorous acid, and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany). Hydrophobic Oasis HLB 1 cm^3 (30 mg) extraction cartridges were obtained from Waters (Milford, MA, USA). All reagents and solvents used were of the highest purity.

Superoxide assay

Step 1: organism treatment

Superoxide radical was measured in vivo in the following experimental model organisms. *Saccharomyces cerevisiae* (strain L1494, kindly provided by Dennis Synetos, School of Medicine, University of Patras, Greece) was grown in 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) D-glucose up to the early log phase. Mouse organs (excised from 3-month-old type BalbC mice from Theagenion Anticancer Hospital, Thessaloniki, Greece) were kindly provided by Nikolaos Matsokis and Nikolaos Panagopoulos (Department of Biology, University of Patras). Mature spinach leaves were purchased at the local market in bundles with roots in moistened sponge and were used the same day. Superoxide radical was also measured in vitro in the following cell cultures. Human umbilical vein endothelial cells (HUVECs) were kindly provided by Christodoulos Flordellis and Nikolaos Tsopanoglou (Department of Pharmacology, School of Medicine, University of Patras) and grown as described elsewhere [13]. $CD3^+$ T cells were kindly provided by Panagiota Matsouka and Konstantina Floratou (Department of Hematology, School of Medicine, University of Patras) and grown as described elsewhere for $CD4^+$ T cells [14] with 5% (w/v) fetal bovine serum in the growth medium. Human cell treatment procedures were in accordance with the Hospital Bioethics Committee of the University of Patras, and informed consent was obtained from all subjects. All animal procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Greek Animal Care and Use Committee. Mouse organ tissue was homogenized with a glass–glass Potter–Elvehjem homogenizer in 1:1 tissue wet weight:volume ice-cold phosphate buffer (50 mM, pH 7.8, containing 10 mM sodium cyanide as inhibitor of nonspecific peroxidases). $CD3^+$ T cells, yeast, and HUVECs were mixed with 0.1 ml phosphate buffer and freeze-thawed three times with liquid nitrogen (necessary for yeast cells). Before homogenization, yeast cells (or any other organisms with cell walls immersed in the DHE incubation medium) were rapidly washed with 1:1 DHE incubation medium:10 N HCl volume, followed by 3 volumes of distilled water, to remove any bound 2-OH-ethidium formed extracellularly. Spinach leaves were mixed at 5:1 plant wet weight:phosphate buffer