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Recent developments in detection of superoxide radical anion and hydrogen peroxide: Opportunities, challenges, and implications in redox signaling☆

Balaraman Kalyanaraman [a,*], Micael Hardy [b], Radoslaw Podsiadly [c], Gang Cheng [a], Jacek Zielonka [a]

[a] Department of Biophysics and Free Radical Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA
[b] Aix-Marseille Université, CNRS, ICR UMR 7273, 13397 Marseille, France
[c] Institute of Polymer and Dye Technology, Lodz University of Technology, Stefanowskiego 12/16, 90-924 Lodz, Poland

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ABSTRACT

In this review, some of the recent developments in probes and assay techniques specific for superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are discussed. Over the last decade, significant progress has been made in $O_2^{\bullet-}$ and H_2O_2 detection due to syntheses of new redox probes, better understanding of their chemistry, and development of specific and sensitive assays. For superoxide detection, hydroethidine (HE) is the most suitable probe, as the product, 2-hydroxyethidium, is specific for $O_2^{\bullet-}$. In addition, HE-derived dimeric products are specific for one-electron oxidants. As red-fluorescent ethidium is always formed from HE intracellularly, chromatographic techniques are required for detecting 2-hydroxyethidium. HE analogs, Mito-SOX and hydropropidine, exhibit the same reaction chemistry with $O_2^{\bullet-}$ and one-electron oxidants. Thus, mitochondrial superoxide can be unequivocally detected using HPLC-based methods and not by fluorescence microscopy. Aromatic boronate-based probes react quantitatively with H_2O_2, forming a phenolic product. However, peroxynitrite and hypochlorite react more rapidly with boronates, forming the same product. Using ROS-specific probes and HPLC assays, it is possible to screen chemical libraries to discover specific inhibitors of NADPH oxidases. We hope that rigorous detection of $O_2^{\bullet-}$ and H_2O_2 in different cellular compartments will improve our understanding of their role in redox signaling.

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1. Introduction

Over the past few decades, we have been fortunate to work with organic chemists who synthesized spin traps, fluorescent and chemiluminescent probes, and compounds specifically targeted to mitochondria, as well as many cell biologists, cell signaling researchers, and molecular biologists. Our frequent interactions between synthetic chemists, physical chemists, and biologists have allowed us to identify relevant problems and develop pertinent probes and analytical techniques that can provide solutions to long-standing problems in free radical biology. Important novel probes for detecting reactive oxygen and nitrogen species are being developed in several laboratories here and around the world. Because of these advances, we are now in the position to simultaneously monitor different reactive oxygen and nitrogen species in biological systems, identify inhibitors of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox) by screening libraries of chemicals, and even detect reactive oxygen species (ROS) in a cell signaling milieu. In this review, the recent developments of probes and techniques to detect the specific products derived from the interaction between selected ROS and fluorescent probes *in vitro* and *in vivo*, and possibly under redox signaling conditions, are discussed.

2. Understanding the reaction chemistry of probes

Roger Tsien, who won the 2008 Noble Prize in chemistry for his discovery on genetically engineered green fluorescent proteins,

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* Corresponding author.
E-mail address: balarama@mcw.edu (B. Kalyanaraman).

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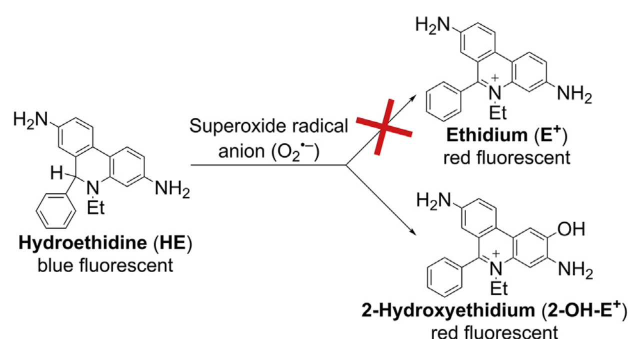

Fig. 1. Superoxide radical anion reacts with hydroethidine to produce 2-hydroxyethidium but not ethidium.

famously wrote, “Being able to do any chemistry was like being the one-eyed man in the kingdom of the blind” [1]. We realized that the free radical biology field would greatly benefit from a rigorous understanding of the chemistry of fluorescent probes, their reaction kinetics with ROS/reactive nitrogen species (RNS), the determination of stoichiometry and specific products, and development of rigorous assays and detection methods. Thus began our quest to understand the reaction mechanism of superoxide and the redox-sensitive fluorescent probe hydroethidine (HE). HE, a two-electron reduction product of ethidium (E^+), was thought to react specifically with the superoxide radical anion ($O_2^{\bullet-}$) and get oxidized back to E^+ [2]. However, we found that, in a pure superoxide-generating enzymatic system, HE was not oxidized to E^+ (Fig. 1); rather, a hydroxylated product that has fluorescence characteristics similar to that of E^+ was formed [3,4]. The structure of the hydroxylated product was determined to be 2-hydroxyethidium (2-OH-E^+, Fig. 1) [4].

However, in superoxide-generating systems that contain trace levels of redox-active metal ions (iron) or peroxidases, E^+ was formed as a nonspecific product (Fig. 2). In addition to E^+, several dimeric products (nonfluorescent), such as E^+-E^+, that are characteristic of a radical-radical dimerization mechanism were formed (Fig. 2) [5]. Because the reaction of HE radical cation with superoxide is rapid (estimated rate constant ~10^9 $M^{-1}s^{-1}$), one-electron oxidation of HE may significantly improve its efficiency in competing for $O_2^{\bullet-}$ with other targets of superoxide, including intracellular superoxide dismutase (SOD). Thus, the presence of one-electron oxidants may increase the yield of 2-OH-E^+, as the HE-derived radical is a common intermediate for superoxide (or its protonated form, hydroperoxyl radical [$HO_2^{\bullet}$]) and other one-electron oxidants [6,7]. As the one-electron oxidants will also lead to formation of the dimeric products, complete profiling of HE oxidation products is necessary for proper interpretation of the changes in intracellular levels of 2-OH-E^+ [8]. Recently, an additional chlorination product of hydroethidine (2-chloroethidium) has been identified as a product of the reaction between HE and hypochlorous acid, and proposed as a specific marker of myeloperoxidase activity in the *in vitro* and *in vivo* settings [9,10].

3. Rigorous identification of ROS and RNS using the fluorescent probes

The proper use of molecular probes for detection and characterization of reactive oxidizing and nitrating species requires a detailed knowledge of the chemistry, reaction kinetics, stoichiometry, and product(s) formed from the reaction between ROS/RNS and fluorescent or chemiluminescent probes. Clearly, accurate interpretation of the fluorescence data requires sufficient knowledge of the ROS/RNS reaction chemistry with the fluorescent dyes. Sometimes, determination of minor, but specific, products can provide major new information regarding the identity of the ROS/RNS species [11–13].

4. Intracellular levels of the fluorescent probes

With very few exceptions, fluorogenic probes for ROS are not present in sufficient concentrations in cells to efficiently compete with other targets of superoxide or hydrogen peroxide. This may be regarded as an advantage, as in such cases the probe is not expected to significantly disturb the system, providing a way for biorthogonal ROS detection. It has, however, two important consequences for ROS measurements: (i) only a fraction of $O_2^{\bullet-}$ or H_2O_2 is detected and, therefore, the assay is semi-quantitative at best, and (ii) changes in intracellular concentration of the probe will affect its effectiveness in competition with other targets, resulting in different yields of the products detected. Therefore, it is critically important to monitor intracellular probe levels for an accurate interpretation of the changes in the amounts of the oxidation product(s).

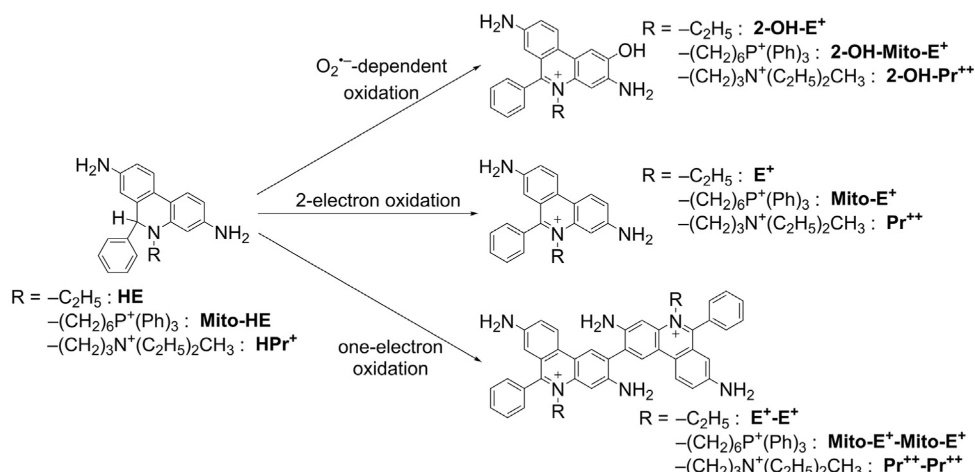

Fig. 2. Products formed from hydroethidine (HE), Mito-SOX (Mito-HE), and hydropropidine (HPr^+) as a function of oxidant identity/oxidation mechanism.

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