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Original article

Quantification of light-induced miniSOG superoxide production using the selective marker, 2-hydroxyethidium

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ABSTRACT

Genetically-encoded photosensitizers produce reactive oxygen species (ROS) in response to light. Transgenic expression of fusion proteins can target the photosensitizers to specific cell regions and permit the spatial and temporal control of ROS production. These ROS-generating proteins (RGPs) are widely used for cell ablation, mutagenesis and chromophore-assisted light inactivation of target proteins. However, the species produced by RGPs are unclear due to indirect measures with confounding interpretations. Recently, the RGP mini "Singlet Oxygen Generator" (miniSOG) was engineered from *Arabidopsis thaliana* phototropin 2. While miniSOG produces singlet oxygen ($^1O_2$), the contribution of superoxide ($O_2^{\bullet-}$) to miniSOG-generated ROS remains unclear. We measured the light-dependent $O_2^{\bullet-}$ production of purified miniSOG using HPLC separation of dihydroethidium (DHE) oxidation products. We demonstrate that DHE is insensitive to $^1O_2$ and establish that DHE is a suitable indicator to measure $O_2^{\bullet-}$ production in a system that produces both $^1O_2$ and $O_2^{\bullet-}$. We report that miniSOG produces both $^1O_2$ and $O_2^{\bullet-}$, as can its free chromophore, flavin mononucleotide. miniSOG produced $O_2^{\bullet-}$ at a rate of ~4.0 μmol $O_2^{\bullet-}$/min/μmol photosensitizer for an excitation fluence rate of 5.9 mW/mm^2 at 470 ± 20 nm, and the rate remained consistent across fluences (light doses). Overall, the contribution of $O_2^{\bullet-}$ to miniSOG phenotypes should be considered.

1. Introduction

Photosensitizers produce reactive oxygen species (ROS) in response to light [1]. Reactive-oxygen-species-generating proteins, or RGPs, are a class of genetically-encoded photosensitizers [1]. These include SuperNova [2], KillerRed [3], KillerOrange [4], and miniSOG [5]. A RGP has the capability to generate different types of ROS including superoxide ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$). mini "Singlet Oxygen Generator" (miniSOG) is unique in that it generates a relatively large quantum yield of $^1O_2$ [5]. This ROS production is attributed to its chromophore flavin mononucleotide (FMN), a well-known $^1O_2$-generating photosensitizer [6,7]. miniSOG has been used for a variety of applications, such as electron microscopy [5], cell death [8,9], mutagenesis [10] and target protein inactivation [11,12]. While miniSOG was successful for these applications, the ROS responsible remained unknown as the $^1O_2$ yield was debated [13,14].

The disparity between $^1O_2$ yields with different detection methods led to the hypothesis that $O_2^{\bullet-}$ may be a species produced by miniSOG [13]. Pimenta *et al.* measured $O_2^{\bullet-}$ production using the fluorescence of dihydroethidium (DHE) oxidation products, a nonspecific measure of $O_2^{\bullet-}$. The fluorescence of DHE oxidation products can be the result of the $O_2^{\bullet-}$ specific product, 2-OHE$^+$, and the nonspecific product, E$^+$. These resulting DHE oxidation products are indistinguishable via fluorescence alone, and require HPLC separation to measure $O_2^{\bullet-}$ production specifically [15,16]. Moreover, although based on fluorescence, there are conflicting results and no clear consensus on whether or not $^1O_2$ can react with DHE to form E$^+$ [17–19]. Overall, our goal was to clarify the impact of $^1O_2$ on DHE-oxidation products and confirm if miniSOG generates $O_2^{\bullet-}$ by measuring the formation of the $O_2^{\bullet-}$ specific DHE oxidation product, 2-OHE$^+$.

Thus, we measured $O_2^{\bullet-}$ generated by miniSOG using HPLC separation of the DHE oxidation products to specifically detect $O_2^{\bullet-}$. We characterized the measurement system using Rose Bengal, a chemical photosensitizer that generates both $O_2^{\bullet-}$ and $^1O_2$ [20–22]. Detailed DHE oxidation product analysis demonstrates that $^1O_2$ does not react with DHE. Under conditions where miniSOG makes $^1O_2$, it also produces $O_2^{\bullet-}$ at a flux that is consistent across fluences (light doses).

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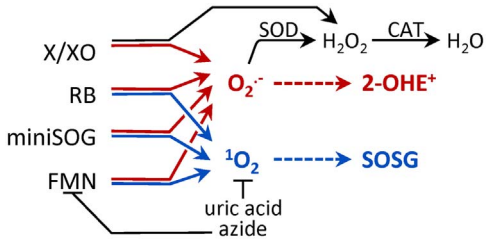

Scheme 1. *Overview of ROS detection methods.* ROS generators were used to produce superoxide ($O_2^{\bullet-}$) and/or singlet oxygen ($^1O_2$). 2-hydroxyethidum (2-OHE$^+$) is a $O_2^{\bullet-}$-selective marker, while $^1O_2$ was detected using Singlet Oxygen Sensor Green (SOSG). Superoxide dismutase (SOD) converts $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2), which can be removed by catalase (CAT) to water. Both uric acid and azide quench $^1O_2$, while azide additionally quenches the triplet state of FMN. Abbreviations: X (Xanthine), XO (Xanthine oxidase), RB (Rose Bengal), FMN (flavin mononucleotide).

2. Materials and methods

2.1. Singlet oxygen detection using singlet oxygen sensor green

$^1O_2$ was measured using singlet oxygen sensor green (SOSG; Molecular Probes). SOSG has a weak blue fluorescence but upon reaction with $^1O_2$ exhibits a strong green fluorescence [23]. SOSG (1 μM) baseline fluorescence was measured in a cuvette containing a photosensitizer, Rose Bengal (RB, 2.5 μM; Sigma) or Deuteroporphyrin (DP, 2.5 μM; Frontier Scientific), and SOSG buffer (SB; 120 mM KCl, 25 mM sucrose, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM EGTA, 10 mM HEPES, 0.1 mM DPTA, pH 7.3). Where indicated, 20 mM azide or 800 units/mL superoxide dismutase (SOD, Sigma) was present (as illustrated in Scheme 1). Temperature was held constant at 25 °C. The fluorescence (Ex 488 nm; Em 525 nm; slit width 5 nm) was recorded for 1 min with constant stirring before and after illumination (560 ± 20 nm, 10.6 mW/mm^2) for 0–30 min. The change in fluorescence intensity (post minus pre-illumination) was calculated. Experiments using FMN (10 μM) or purified miniSOG (10 μM) were illuminated at 470 ± 20 nm (5.9 mW/mm^2). To avoid spectral overlap with FMN, SOSG fluorescence was excited at 504 nm (Em 525 nm; slit width 5 nm). Structures of Rose Bengal and FMN are shown in Supplemental Fig. 1.

Light intensity was measured using a calibrated thermopile detector (818P-010–12, Newport Corporation, Irvine, CA) connected to an optical power meter (1916-R, Newport Corporation). For all experiments in which the illumination was varied, the intensity of light or fluence rate (watts/area) was held constant. The total fluence (joules/area) was altered by varying the illumination time.

2.2. Quantification of xanthine/xanthine oxidase superoxide production using cytochrome c

Xanthine oxidase (XO) catalyzes the oxidation of xanthine (X) to uric acid. During this reaction XO generates $O_2^{\bullet-}$ or H_2O_2 via either a 1 or 2 electron reduction, respectively. The rate of X/XO $O_2^{\bullet-}$ formation was measured as the rate of SOD-sensitive cytochrome *c* reduction. Briefly, at ambient O_2, XO (0.1 units/mL) and X (1 mM) were added to a 0.1 cm cuvette containing cytochrome *c* (800 mM). The rate of cytochrome *c* reduction was monitored at 550 nm and calculated using an extinction coefficient of 18.7 M^{-1} cm^{-1} in the presence or absence of SOD (800 units/mL) [24]. The X/XO reaction predominately generates H_2O_2 in a pH and oxygen concentration-dependent manner [25]. The percentage of $O_2^{\bullet-}$ generated by X/XO was calculated by dividing the $O_2^{\bullet-}$ generation by the total electron flux of X to uric acid by XO, as previously described by Kelley *et al.* [25].

2.3. Superoxide detection using HPLC separation of DHE oxidation products

2.3.1. Xanthine/xanthine oxidase (X/XO) system

$O_2^{\bullet-}$ was measured using dihydroethidium (DHE; Thermo Fisher Scientific) followed by HPLC separation of the resulting oxidation products [16]. Upon oxidation, DHE forms red fluorescent products, a $O_2^{\bullet-}$ specific product, 2-OHE$^+$, and a nonspecific product, E$^+$, which must be separated by HPLC due to overlapping fluorescence spectra [16,26]. Structures of DHE, 2-OHE$^+$ and E$^+$ are shown in Supplemental Fig. 1. In 1 mL of PBS containing DPTA (DPBS; 7.78 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.1 mM DPTA, pH 7.4 at 37 °C) and DHE (50 μM), 0.1 units/mL XO and 1 mM X were added to generate $O_2^{\bullet-}$. Where indicated, 800 units/mL SOD was present (as illustrated in Scheme 1). The solution was incubated at 37 °C for 0–20 min (as indicated) to generate increasing amounts of $O_2^{\bullet-}$, after which the reaction was stopped with an equal volume of acidified methanol (200 mM $HClO_4$ in methanol). The solution was incubated at −20 °C for 30 min and then centrifuged at 17,000 × *g*, 4 °C for 20 min. Next, an equal volume of sample was combined with 1 M $K^+PO_4^-$ (pH 2.6). Again, the sample was incubated at −20 °C for 30 min and then centrifuged at 17,000 × *g* for 10 min. Samples were separated on a Polar-RP column (Phenomenex, 150 × 2 mm; 4 μm) run on Shimadzu HPLC with fluorescence detection (RF-20A). From 0–15 min the detector was on low sensitivity (channel 1: Ex: 358 nm, Em: 440 nm; channel 2: Ex: 490 nm, Em: 596 nm). After 15 min, the sensitivity switched to high (channel 1: Ex: 490 nm, Em: 567 nm; channel 2 remained constant) with a constant flow rate of 0.1 mL/min. Two mobile phases were used (A, 10% ACN with 0.1%TFA in water; B, 60% ACN with 0.1%TFA in water) using the following gradient: 0 min, 40% B; 5 min, 40% B; 25 min, 100% B; 30 min, 100% B; 35 min, 40% B; 40 min, 40% B. Concentrations of DHE, 2-OHE$^+$ and E$^+$ were measured using standard curves. DHE and E$^+$ standards were commercially available. The 2-OHE$^+$ was made using X/XO generated $O_2^{\bullet-}$. The resulting mixture was separated using HPLC and the fraction containing 2-OHE$^+$ was collected and lyophilized to a dry powder. 2-OHE$^+$ was confirmed using mass spectrometry (URMC Mass Spectrometry Resource Laboratory; m/z = 330.16; the intervals between isotopic peaks = 1.0).

2.3.2. Photosensitizers

Superoxide production from photosensitizers was measured as described above with the following modifications. DHE (50 μM) and RB or DP (2.5 μM) were illuminated (560 ± 20 nm, 17 mW/mm^2) in assay buffer (120 mM KCl, 25 mM sucrose, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM EGTA, 10 mM HEPES, 0.1 mM DPTA, pH 7.3) for 0–5 min. As a control, one cuvette was also incubated in the dark. Where indicated, 20 mM azide or 800 units/mL SOD or 4200 units/mL of catalase (CAT, 1 mg/mL; from bovine liver or from Corynebacterium glutamicum as indicated, Sigma) or hydrogen peroxide (H_2O_2, Sigma) was present. The procedure was repeated with 10 μM free FMN (Sigma) or purified miniSOG. Samples were illuminated at 470 ± 20 nm (5.9 mW/mm^2).

2.4. miniSOG purification

Recombinant miniSOG with an N-terminal histidine tag was expressed and purified from BL21DE3pLYS cells. Briefly, the coding region of miniSOG (courtesy of Drs. Roger Tsien and Yishi Jin, University of California, San Diego) was inserted in pRSET B using *Bam*HI and *Eco*RI. The plasmid was electroporated into competent BL21pLyS cells and plated on ampicillin plates (100 μg/mL). A single colony was grown to OD_{600} of 0.5 and miniSOG expression was induced using IPTG (100 μM). Cells were grown in the dark and lysed after 4 h of expression with lysis buffer (3% SDS, 50 mM Tris, pH 8.0) and protease inhibitors (Pierce). His-tagged miniSOG was then allowed to bind to Ni-NTA agarose beads (Qiagen) overnight; fluorescence was monitored to ensure protein binding. miniSOG was eluted with 100 μM imidazole and