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Commentary

On the use of fluorescence lifetime imaging and dihydroethidium to detect superoxide in intact animals and ex vivo tissues: A reassessment

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ABSTRACT

Recently, D.J. Hall et al. reported that ethidium (E^+) is formed as a major product of hydroethidine (HE) or dihydroethidium reaction with superoxide ($O_2^{\bullet -}$) in intact animals with low tissue oxygen levels (*J. Cereb. Blood Flow Metab.* 32:23–32, 2012). The authors concluded that measurement of E^+ is an indicator of $O_2^{\bullet -}$ formation in intact brains of animals. This finding is in stark contrast to previous reports using in vitro systems showing that 2-hydroxyethidium, not ethidium, is formed from the reaction between $O_2^{\bullet -}$ and HE. Published in vivo results support the in vitro findings. In this study, we performed additional experiments in which HE oxidation products were monitored under different fluxes of $O_2^{\bullet -}$. Results from these experiments further reaffirm our earlier findings (H. Zhao et al., *Free Radic. Biol. Med.* 34:1359, 2003). We conclude that whether in vitro or in vivo, E^+ measured by HPLC or by fluorescence lifetime imaging is not a diagnostic marker product for $O_2^{\bullet -}$ reaction with HE.

We reported nearly a decade ago that 2-hydroxyethidium (2-OH-E^+), and not ethidium (E^+), is the only product of the reaction between superoxide ($O_2^{\bullet -}$) and hydroethidine (HE) or dihydroethidium (DHE) in enzymatic and cellular systems [1,2]. Subsequently, this finding has been confirmed in several other laboratories around the world [3–10]. However, Hall et al. [11] have recently reported that under in vivo conditions—with the oxygen concentration in most tissues typically low, between 40 and 50 mm Hg—the product of $O_2^{\bullet -}$ reaction with HE is E^+ and not 2-OH-E^+ as reported in our previous work [1,2]. The authors [11] indicated that the previous studies in cell culture and tissue slices were performed at ambient (21%) oxygen—"a condition under which artifactual oxidation of DHE rapidly occurs"—and that the lower concentration of tissue oxygen in vivo decreased the likelihood of two sequential encounters with $O_2^{\bullet -}$, precluding 2-OH-E^+ formation and promoting E^+ (denoted as ox-DHE) fluorescence. The authors concluded that under low oxygen tension (i.e., under low levels of $O_2^{\bullet -}$), DHE is oxidized by $O_2^{\bullet -}$ to E^+ in mouse brain [11].

On the other hand, increased $O_2^{\bullet -}$ production in fetal brains after reperfusion–reoxygenation was recently reported, as evidenced by enhanced 2-OH-E^+ detection by HPLC [12]. In other studies, chromatographic techniques were used to detect 2-OH-E^+ in mouse brain tissue [6,13]. There also exist other reports wherein fluorimetric approaches have been used to detect 2-OH-E^+ in brain extracts [8,14,15]. Thus, it has become crucial to reevaluate the overall conclusion of Hall et al. [11] and to provide new insight for future research that could potentially mitigate the nonspecific oxidation of DHE to E^+ in cells and tissues.

In this reevaluation, we show that, using a wide range of DHE-to-$O_2^{\bullet -}$ ratios, the only product formed from DHE is 2-OH-E^+ and not E^+. These results further corroborate that 2-OH-E^+ is the only product of DHE oxidation by $O_2^{\bullet -}$ and that the proposed mechanism as reported by Hall et al. [11], for DHE and $O_2^{\bullet -}$ forming E^+ as the predominant product under in vivo conditions, is incorrect and not mechanistically authenticated under well-defined in vitro conditions.

Materials and methods

Hypoxanthine (or xanthine)/xanthine oxidase (HX (or X)/XO) were used to generate $O_2^{\bullet -}$ in phosphate buffer (pH 7.4, 50 mM)

Abbreviations:: 2-OH-E^+, 2-hydroxyethidium; DE, deuteroethidine; DHE, dihydroethidium; dtpa, diethylenetriaminepentaacetate; E^+, ethidium; HE, hydroethidine; HX, hypoxanthine; X, xanthine; XO, xanthine oxidase; HRP, horseradish peroxidase; TFA, trifluoroacetic acid

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in the presence of dtpa (100 μM). The rate of formation of uric acid and superoxide was varied by varying the concentration of XO. The concentration of HE was 60 μM. Superoxide formation was quantitated by measuring superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* [9,16,17]. Authentic 2-OH-E^+ was prepared and purified as previously reported [16]. Ethidium bromide was obtained from Sigma.

HE, 2-OH-E^+, and E^+ were separated using an Agilent 1100 HPLC system equipped with UV–Vis absorption and fluorescence detectors, as described previously [18]. Briefly, before the analysis, the C_{18} column (Phenomenex, Kinetex, 100 × 4.6 mm, 2.6 μm) was equilibrated with an acetonitrile/water mobile phase (10/90 v/v) containing trifluoroacetic acid (TFA; 0.1%). After injection of the sample (injection volume 50 μl), the acetonitrile fraction in the mobile phase was increased as follows: from 10 to 50% over 5 min, from 50 to 100% over the next 2 min, and kept at this level over the next 2.5 min.

The ultraperformance liquid chromatography system (UPLC Acquity, Waters Ltd.) equipped with a photodiode array spectrometer for UV–Vis absorption measurements was used to investigate the effects of horseradish peroxidase (HRP) on the yield of 2-OH-E^+. Separation was accomplished on a Waters UPLC column (Acquity UPLC BEH C_{18}, 1.7 μm, 50 × 2.1 mm) kept at 40 °C and equilibrated with 32.5% CH_3OH (containing 0.1% (v/v) TFA) in 0.1% TFA aqueous solution. The compounds were separated by a linear increase in CH_3OH phase concentration from 32.5 to 57.5% using a flow rate of 0.3 ml/min. The injection volumes and temperature for both the sample and the standard solutions were 2 μl and 23 °C, respectively.

Results

Products derived from the reaction between $O_2^{\bullet -}$ and HE

Fig. 1 shows the HPLC chromatograms of products formed from incubating HE in phosphate buffer containing 0.005 and 1 mU/ml XO and in the absence of XO. In the absence of added XO, there was an increase in 2-OH-E^+ formation over a period of 120 min

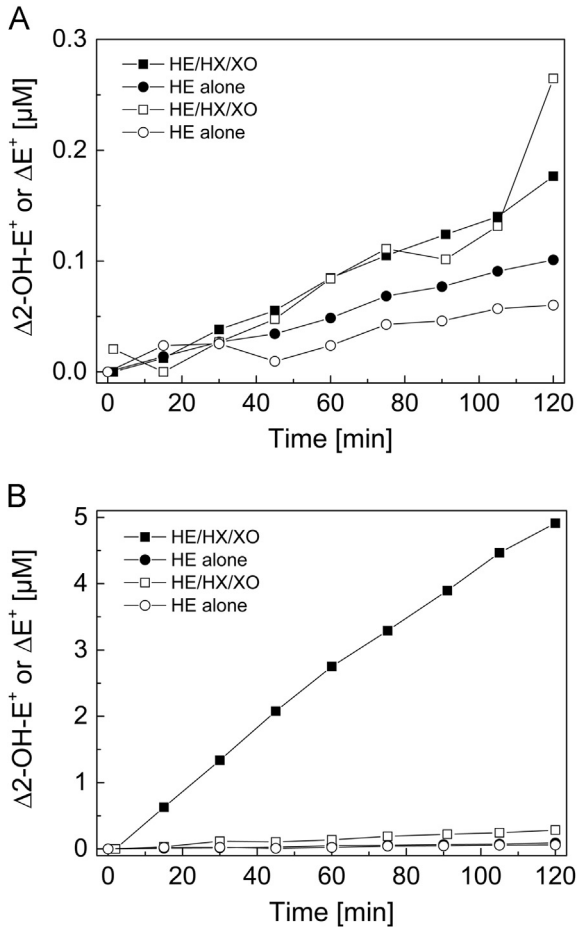

Fig. 2. Time courses of 2-OH-E^+ and E^+ formation monitored by HPLC at (A) low and (B) high fluxes of $O_2^{\bullet -}$. (A) The reaction mixture contained HE (60 μM) and dtpa (100 μM) in a phosphate buffer (50 mM; pH 7.4), in the presence or absence of HX (0.1 mM) and XO (0.005 mU/ml;$O_2^{\bullet -}$ flux 1.25 nM/min). (B) Same as (A) but the concentration of XO was increased to 1 mU/ml ($O_2^{\bullet -}$ flux 0.25 μM/min). The same HE-alone control was used in (A) and (B) for both analytes, 2-OH-E^+ and E^+. Solid symbols and open symbols denote 2-OH-E^+ and E^+, respectively.

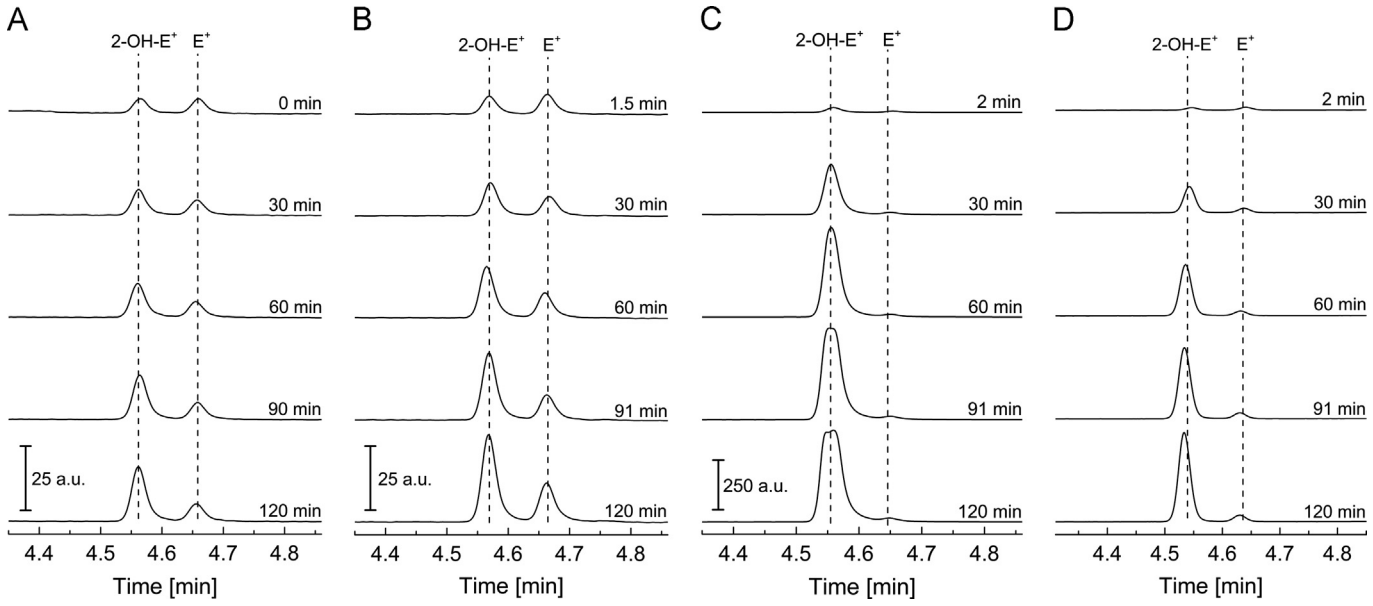

Fig. 1. HPLC chromatograms of products derived from HE/$O_2^{\bullet -}$ reaction. (A) Reaction mixtures contained HE (60 μM) and dtpa (100 μM) in phosphate buffer (50 mM; pH 7.4) in the absence of any additives; (B) in the presence of HX (0.1 mM) and XO (0.005 mU/ml; $O_2^{\bullet -}$ flux 1.25 nM/min); and (C) in the presence of HX (0.1 mM) and XO (1 mU/ml; $O_2^{\bullet -}$ flux 0.25 μM/min). The HPLC traces were collected using the fluorescence detector (λ_{ex} 490 nm, λ_{em} 596 nm) in (A), (B), and (C). (D) Same as (C) but the HPLC traces were collected by a UV–Vis absorption detector (λ 500 nm).