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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, Hall *et al.* reported that ethidium (E^+) is formed as a major product of hydroethidine or dihydroethidium (HE or DHE) 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 ethidium (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 (Zhao *et al*, *Free Radic Biol Med* 34:1359, 2003 and Dikalov *et al*, *Hypertension* 49:717, 2007) 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 (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.

Keywords

fluorescence lifetime; hydroethidine; dihydroethidium; 2-hydroxyethidium; ethidium

Introduction

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

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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) has 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 slice 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 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 following 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 fluorometric approaches have been used to detect 2-OH- E^+ in brain extracts (14–16). Thus, it has become crucial to reevaluate Hall *et al.* (11) overall conclusion 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] was used to generate $O_2^{\bullet-}$ in phosphate buffer (pH 7.4, 50 mM) in the presence of dtpa (100 μ M). The rate of formation of uric acid and superoxide was varied by varying the concentration of XO. The concentration of HE was 60 μ M. Superoxide formation was quantitated by measuring superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* (9, 17, 18). Authentic 2-OH- E^+ was prepared and purified as previously reported (17). Ethidium bromide was obtained from Sigma.

HE, 2-OH- E^+ and E^+ were separated using an Agilent 1100 HPLC system equipped with an UV-Vis absorption and fluorescence detectors, as described previously (19). Briefly, before the analysis, the C_{18} column (Phenomenex, Kinetex, 100 $\times$ 4.6 mm, 2.6 μ m) was equilibrated with acetonitrile/water mobile phase (10/90 v/v) containing trifluoroacetic acid (TFA, 0.1%). After injection of the sample (injection volume 50 μ l), 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 next 2.5 min.

The ultra-performance liquid chromatography system (UPLC Acquity, Waters Ltd.) equipped with a photo-diode array (PDA) spectrometer for UV-VIS absorption measurements was used to investigate the effects of HRP on the yield of 2-OH-E⁺. Separation was accomplished on a Waters UPLC column (Acquity UPLC BEH C₁₈ 1.7 μ m, 50 $\times$ 2.1 mm) maintained at 40°C and equilibrated with 32.5% CH₃OH [containing 0.1% (v/v) (TFA)] in 0.1% TFA aqueous solution. The compounds were separated by a linear increase in CH₃OH phase concentration from 32.5% to 57.5% using a flow rate 0.3 ml/min. The injection volumes and temperature for both the sample and standard solutions were 2 μ l and 23°C, respectively.

Results

Products derived from the reaction between O₂^{•-} and HE

Figure 1 shows the HPLC chromatograms of products formed from incubating HE in phosphate buffer containing 0.005 mU/ml and 1 mU/ml of 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. 1A). The intensity of the peak due to E⁺ also slightly increased over this incubation period. Figures 1B-D show the HPLC profiles observed in the presence of 0.005 mU/ml and 1 mU/ml XO, respectively. It is evident that even with the 200-fold decrease in XO concentration, 2-OH-E⁺ was still observed as the dominant HPLC peak. Increase in E⁺ peak intensity was relatively minor under low fluxes of O₂^{•-}, and at higher fluxes the E⁺ peak intensity was negligible, when compared to the 2-OH-E⁺ peak intensity. The quantitative analysis of the time-dependent increase in 2-OH-E⁺ and E⁺ in the presence of low levels of XO (1.25 nM/min) and in the absence of XO is shown in Fig. 2A. Although both 2-OH-E⁺ and E⁺ were elevated with time after the addition of XO to the incubation mixtures, only 2-OH-E⁺ and not E⁺ was inhibitable by SOD (Suppl. Fig. 1). Therefore we attribute E⁺ formation to O₂^{•-}-independent pathway(s). Figure 2B shows the quantitative analysis of the time-dependent increase in 2-OH-E⁺ and E⁺ using the highest (0.25 μ M/min) flux of O₂^{•-}. Clearly, 2-OH-E⁺ is the dominant product formed under those conditions.

The effect of varying O₂^{•-} flux on the stoichiometry of HE/O₂^{•-} reaction and HE-derived product formation

To further support our findings, we incubated HE with O₂^{•-} under varying fluxes of O₂^{•-} and tested the effect of O₂^{•-} flux on reaction stoichiometry. Figure 3A shows the chromatograms of HE oxidation products. These results indicate that the HPLC peak of 2-OH-E⁺ is dominant over a wide range of O₂^{•-} generation (20).

The calculated stoichiometry between O₂^{•-} and 2-OH-E⁺ using the lowest concentration of XO (0.005 mU/ml, corresponding to 1.25 nM O₂^{•-}/min) indicates that two molecules of O₂^{•-} are needed to form one molecule of 2-OH-E⁺ (Fig 3B, *inset*). In the presence of high concentrations of XO, the calculated stoichiometry between O₂^{•-} and 2-OH-E⁺ was significantly different, with nearly a 6:1 stoichiometry of O₂^{•-} to 2-OH-E⁺ at the highest O₂^{•-} flux tested (Fig. 3B). In fact, the stoichiometry between O₂^{•-} and 2-OH-E⁺ was found to be variable, with increasing flux of O₂^{•-} (Fig. 3B, *inset*). Similar results have been published previously (6). Previously, it was concluded that at low O₂^{•-} fluxes, the

stoichiometry reaches 1:1 ($O_2^{\bullet-}$ to 2-OH- E^+) (6), which is contrary to the present data. For determining the correct stoichiometry, we subtracted the amount 2-OH- E^+ formed due to autooxidation of the probe. It is conceivable that the difference in data interpretation between the present work and that reported previously (6) is due to omission of the contribution of HE autooxidation to the yield of 2-OH- E^+ , which becomes significant at low fluxes of $O_2^{\bullet-}$.

We attribute the decrease in the relative yield of 2-OH- E^+ with increasing fluxes of $O_2^{\bullet-}$ to increased fraction of $O_2^{\bullet-}$ undergoing self-dismutation. HE-derived radical was also suggested to catalyze the dismutation of $O_2^{\bullet-}$ (21). Under these conditions, E^+ formation was relatively insensitive to $O_2^{\bullet-}$ flux, with some variation at lowest fluxes tested. As the conversion of HE to E^+ was SOD-insensitive, we attribute E^+ formation to mechanisms other than the direct reaction between $O_2^{\bullet-}$ and HE.

Stimulation of HE-derived product formation in the presence of HRP

As has been suggested earlier (9, 17, 22, 23), the 2-OH- E^+ formation from HE/ $O_2^{\bullet-}$ reaction occurs in two steps: the formation of HE radical or radical cation from a hydrogen or electron abstraction reaction, followed by the recombination reaction with a second molecule of $O_2^{\bullet-}$. This is consistent with the 2:1 stoichiometry determined between $O_2^{\bullet-}$ and 2-OH- E^+ , as described above. As the recombination rate ($O_2^{\bullet-}$ reaction with HE radical species) is expected to be rapid (22), we surmised that the yield of 2-OH- E^+ can be increased in the presence of higher steady-state concentrations of HE radical derived from a peroxidatic oxidation mechanism (9). Figure 4 shows the UPLC chromatograms obtained from incubations containing X, XO, dtpa, and horseradish peroxidase (HRP) enzyme. In the presence of HRP and H_2O_2 , dimeric products (e.g. HE-HE) and E^+ were the major oxidation products of HE. However, addition of HRP to HE and X/XO dramatically enhanced 2-OH- E^+ formation (Fig. 4). Catalase reversed the stimulatory effect of HRP on 2-OH- E^+ and completely inhibited the formation of non-specific oxidation products (E^+ and dimers). As expected, SOD completely inhibited 2-OH- E^+ formation, with actual increase in the yield of non-specific oxidation products. The dose response effects of SOD on 2-OH- E^+ formation in X/XO system in the presence and absence of HRP are shown in Figure 5 (A–C). In the presence of 0.1 $\mu\text{g/ml}$ SOD, the 2-OH- E^+ formed was still considerably higher in the presence of HRP. The amount of E^+ formed under these conditions is shown in Figure 5(D–F). These results indicate that SOD has little or no effect on E^+ formation.

Discussion

Hall *et al.* reported that DHE is oxidized by $O_2^{\bullet-}$ to E^+ but not 2-OH- E^+ in intact mouse brain (11). This is contrary to what has been reported by many investigators in cellular and enzymatic systems wherein the major product of DHE or HE reaction with $O_2^{\bullet-}$ was determined to be 2-OH- E^+ (1–5, 24, 25). Hall *et al.* suggested that lower $O_2^{\bullet-}$ levels resulting from low tissue oxygen levels under *in vivo* conditions precludes the sequential reaction between $O_2^{\bullet-}$ and HE forming 2-OH- E^+ (11). Published results indicate that the initial reaction rate between $O_2^{\bullet-}$ and HE to form the HE radical is relatively slow ($k \approx 10^3 \text{ M}^{-1}\text{s}^{-1}$) (18). However, the subsequent reaction between the HE radical and $O_2^{\bullet-}$ is fairly

rapid ($k \approx 10^9 \text{ M}^{-1}\text{s}^{-1}$). In fact, Robinson *et al.* suggested that HE-derived radical may be produced intracellularly by other oxidants (peroxidases or other one-electron oxidants), but 2-OH- E^+ is ultimately formed from the rapid reaction between the HE radical and $\text{O}_2^{\bullet-}$ (23). The dose-response effect of SOD on 2-OH- E^+ (Fig. 5) confirms that HE radical reaction with $\text{O}_2^{\bullet-}$ competes with SOD-catalyzed dismutation of $\text{O}_2^{\bullet-}$.

The present results show that over a range of $\text{O}_2^{\bullet-}$ flux generation, the major product formed during oxidation of HE in X/XO system is 2-OH- E^+ . Any increase in E^+ was SOD-independent and most probably was the product of H_2O_2 produced and/or contamination of XO solution with metal ions. In addition, we determined the $\text{O}_2^{\bullet-}$ to 2-OH- E^+ stoichiometry for the reaction between HE and $\text{O}_2^{\bullet-}$ reaction to be 2:1 (two molecules of $\text{O}_2^{\bullet-}$ required to form one molecule of 2-OH- E^+) under conditions generating lower fluxes of $\text{O}_2^{\bullet-}$. Based on these results, we reaffirm our original conclusion that 2-OH- E^+ , but not E^+ , is the product of HE/ $\text{O}_2^{\bullet-}$ reaction over a wide range of $\text{O}_2^{\bullet-}$ flux.

HE is a versatile redox probe that can undergo both one-electron and two-electron oxidations (Scheme 1). The two-electron oxidation product, E^+ , is almost always generated intracellularly. As shown, E^+ can be produced via a one-electron oxidation (HE radical intermediate) or by a hydride transfer mechanism. Peroxidation (cytochrome *c* heme, myeloperoxidase) could be involved in the one-electron oxidation of HE (24, 26). Two-electron oxidation of HE could involve cytochrome *c* oxidase (26) and mitochondrial dehydrogenases. One-electron oxidation of HE results in the formation of characteristic dimeric products, whereas the two-electron oxidation of HE with chloranil forming E^+ is devoid of HE dimer formation. Thus, it is necessary to analyze formation of HE dimers in order to probe non-radical or radical-mediated E^+ formation from HE. In this regard, the deuterated DE analog (C-H bond in HE is changed to a C-D bond) (Scheme 1) may offer some advantages (27). As the C-H bond is involved in the rate determining step in the hydride transfer mechanism, one would expect a large kinetic isotope effect with the deuterated analog during oxidation of HE to E^+ . As 2-OH- E^+ formation from the deuterated analog should not be affected, the use of deuterated HE probe (i.e., DE in Scheme 1) in cellular systems should advance our understanding of the mechanism of intracellular oxidation of HE. Future studies on cells using both HE and DE should be performed in a hypoxic chamber (4% O_2) so as to mimic the *in vivo* conditions.

Conclusions

Results presented in this study further corroborate our original finding (1) that $\text{O}_2^{\bullet-}$ reaction with HE forms a diagnostic marker product, 2-OH- E^+ , but not E^+ . The present data indicate that in the presence of varying $\text{O}_2^{\bullet-}$ fluxes, the only SOD-sensitive product formed from the reaction between HE and $\text{O}_2^{\bullet-}$ is 2-OH- E^+ . Whether *in vitro* or *in vivo* experiment, it is erroneous to conclude that E^+ formed from HE is indicative of $\text{O}_2^{\bullet-}$ levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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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Highlights

- 2-OH- E^+ but not E^+ is formed from HE reaction with varying fluxes of $O_2^{\bullet-}$
- Peroxidase enhanced the yield of E^+ and 2-OH- E^+ in HE/ $O_2^{\bullet-}$ reaction
- Whether *in vivo* or *in vitro*, E^+ is not a specific product of HE/ $O_2^{\bullet-}$ reaction

Figure 1. HPLC chromatograms of products derived from HE/ $O_2^{\bullet-}$ reaction

(A) Reaction mixtures contained HE (60 μ M), dtpa (100 μ M) 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); (C) in the presence of HX (0.1 mM) and XO (1 mU/ml; $O_2^{\bullet-}$ flux = 0.25 μ M/min). The HPLC traces were collected using the fluorescence detector (λ_{exc} = 490 nm, λ_{emi} = 596 nm) in (A), (B) and (C). (D) Same as (C) but the HPLC traces were collected by an UV/Vis absorption detector (λ = 500 nm).

Figure 2. Time courses of 2-OH-E⁺ and E⁺ formation monitored by HPLC at low (A) and high (B) fluxes of O₂^{•-}
 (A) The reaction mixture contained HE (60 μM), 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; flux = 1.25 nM/min). (B) Same as (A) but the concentration of XO was increased to 1 mU/ml (O₂^{•-} flux = 250 nM/min). The same “HE alone” control was used in panel (A) and (B) for both analytes, 2-OH-E⁺ and E⁺. Solid symbols and open symbols denote 2-OH-E⁺ and E⁺, respectively.

Figure 3. The effect of varying O₂^{•-} fluxes on product formation from HE

(A) HPLC chromatograms were obtained after a 60-min incubation, using a fluorescence detector ($\lambda_{\text{exc}} = 490 \text{ nm}$, $\lambda_{\text{emi}} = 596 \text{ nm}$) from reaction mixtures containing HE (60 μM), HX (0.1 mM), dtpa (0.1 mM) in a phosphate buffer (50 mM; pH = 7.4). The concentrations of XO used: 0 mU/ml (a), 0.005 mU/ml (b), 0.01 mU/ml (c), 0.03 mU/ml (d), 0.05 mU/ml (e), 0.1 mU/ml (f), 0.3 mU/ml (g), 0.5 mU/ml (h), and 1 mU/ml (i). The superoxide fluxes are as indicated. (B) The dependence of rate of 2-OHE⁺ formation on the flux of O₂^{•-}. Reaction mixtures contained HE (60 μM), dtpa (0.1 mM), HX (0.1 mM), and various concentrations of XO (0.005 – 1 mU/ml) in a phosphate buffer (50 mM; pH = 7.4). *Inset*: the rate of 2-OH-E⁺ formation at lowest fluxes of O₂^{•-}. (C) Same as (B) but E⁺ was monitored. The amount of 2-OH-E⁺ and E⁺ formed from “HE alone” control was taken into account (subtracted) in calculating the rates of 2-OH-E⁺ and E⁺ formation.

Figure 4. The influence of HRP on the oxidation of hydroethidine in xanthine/xanthine oxidase system

Chromatograms obtained from UPLC analyses of HE (60 μ M) oxidation products formed in a superoxide generating system containing X (2 mM), XO (2.5 mU/ml, superoxide flux 1.1 ± 0.1 μ M/min) in a phosphate buffer (50 mM, pH 7.4) with dtpa (0.1 mM) in the presence and absence of HRP (50 mU/ml). All control experiments were made as indicated: HRP (50 mU/ml), H_2O_2 (500 μ M), SOD (0.1 mg/ml), CAT (100 U/ml). Experimental conditions: incubation time (31 min), temperature (23°C). Analytical wavelength for UV-VIS detection $\lambda = 270 \pm 5$ nm.

Figure 5. The dose dependent effect of HRP on 2-OH-E⁺ and E⁺ formation

Reaction mixtures contained HE (60 µM), dtpa (0.1 mM), X (2 mM), XO (2.5 mU/ml, O₂^{•-} flux 1.2 ± 0.1 µM/min), HRP (50 mU/ml, if used), and various concentration of SOD (0.1 – 100 µg/ml, as indicated) in a phosphate buffer (50 mM; pH = 7.4). (A) Time course of 2-OH-E⁺ formation in X/XO system. (B) Time course of 2-OH-E⁺ formation in X/XO system in the presence of HRP. (C) End-point measurement of 2-OH-E⁺ formed after 33 min of incubation at 23 °C both in the presence and absence of HRP. Analytical wavelength for UV-VIS detection was $\lambda = 270 \pm 5$ nm. (D) The formation of E⁺ in X/XO system. (E) The

formation of E^+ in X/XO system in the presence of HRP. (F) End-point measurement of E^+ formed after 33 min of incubation both in the presence and absence of HRP. Experimental conditions: incubation time (33 min), temperature (23°C). Analytical wavelength for UV-VIS detection $\lambda = 270 \pm 5$ nm.

Scheme 1.

Proposed one-electron and two-electron oxidation pathways of HE. Note that oxygen is not involved in the one-electron or two-electron oxidation process.