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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

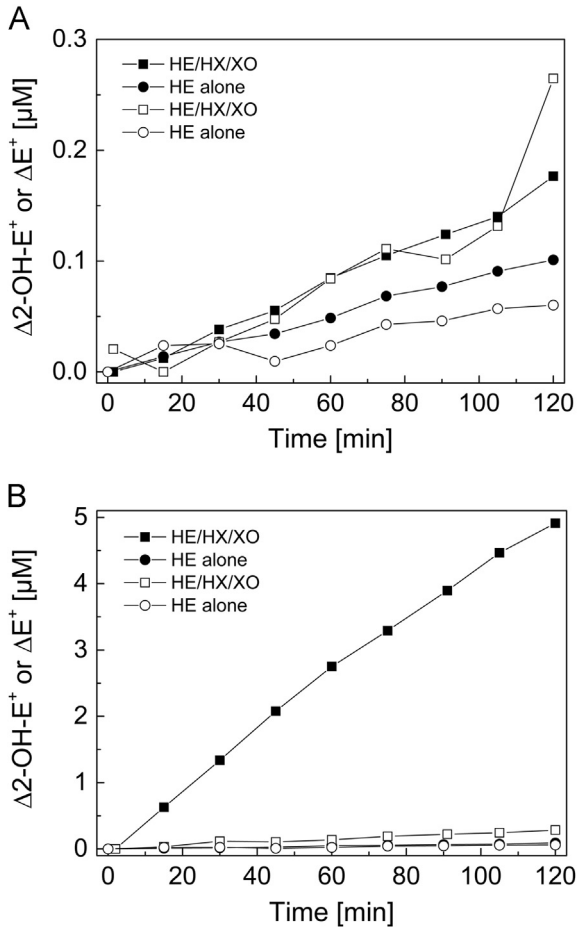


Fig. 2. Time courses of 2-OH-E^+ and E^+ formation monitored by HPLC at (A) low and (B) high fluxes of $O_2^{\bullet -}$. (A) The reaction mixture contained HE (60 μM) and dtpa (100 μM) in a phosphate buffer (50 mM; pH 7.4), in the presence or absence of HX (0.1 mM) and XO (0.005 mU/ml;$O_2^{\bullet -}$ 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.

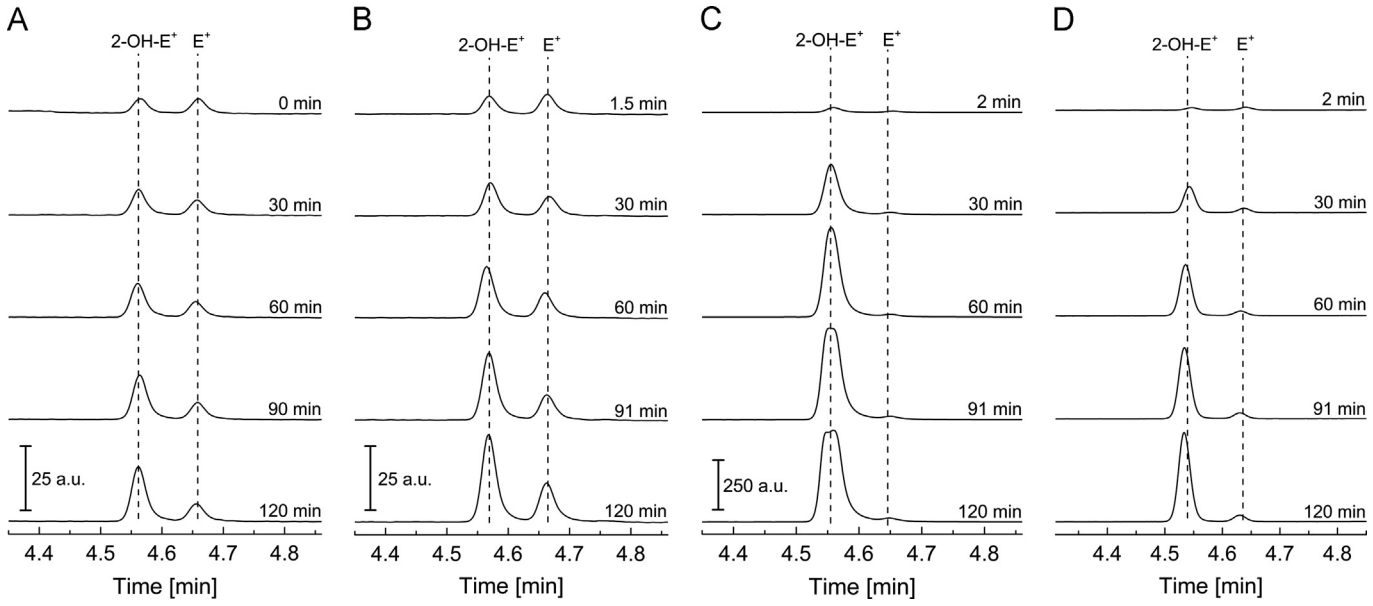


Fig. 1. HPLC chromatograms of products derived from HE/$O_2^{\bullet -}$ reaction. (A) Reaction mixtures contained HE (60 μM) and dtpa (100 μM) in phosphate buffer (50 mM; pH 7.4) in the absence of any additives; (B) in the presence of HX (0.1 mM) and XO (0.005 mU/ml; $O_2^{\bullet -}$ flux 1.25 nM/min); 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).

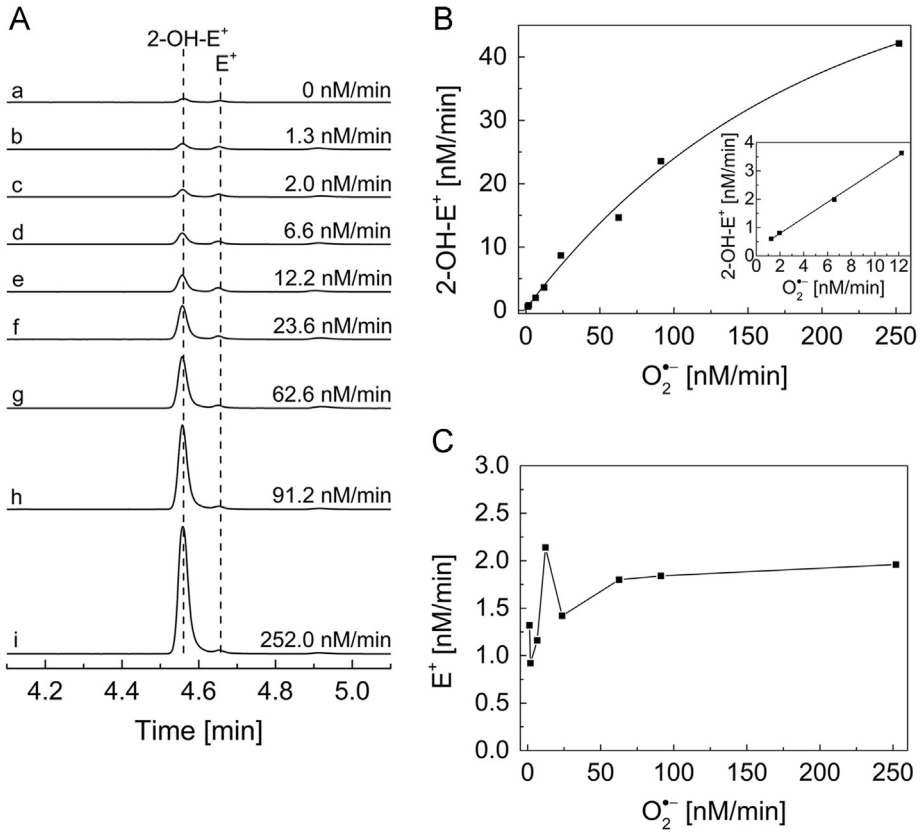


Fig. 3. The effect of varying $O_2^{\bullet -}$ fluxes on product formation from HE. (A) HPLC chromatograms were obtained after a 60-min incubation, using a fluorescence detector (λ_{ex} 490 nm, λ_{em} 596 nm), from reaction mixtures containing HE (60 μM), HX (0.1 mM), and dtpa (0.1 mM) in a phosphate buffer (50 mM; pH 7.4). The concentrations of XO used were as follows: 0 mU/ml (trace a), 0.005 mU/ml (trace b), 0.01 mU/ml (trace c), 0.03 mU/ml (trace d), 0.05 mU/ml (trace e), 0.1 mU/ml (trace f), 0.3 mU/ml (trace g), 0.5 mU/ml (trace h), and 1 mU/ml (trace i). The superoxide fluxes are as indicated. (B) The dependence of rate of 2-OH-E^+ formation on the flux of $O_2^{\bullet -}$. 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 $O_2^{\bullet -}$. (C) Same as (B) but E^+ was monitored. The amounts of 2-OH-E^+ and E^+ formed from the HE-alone control were taken into account (subtracted) in calculating the rates of 2-OH-E^+ and E^+ formation.

(Fig. 1A). The intensity of the peak due to E^+ also slightly increased over this incubation period. Figs. 1B–D show the HPLC profiles observed in the presence of 0.005 and 1 mU/ml XO. 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 $O_2^{\bullet -}$, and at higher fluxes the E^+ peak intensity was negligible, 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 (Supplementary Fig. 1). Therefore we attribute E^+ formation to an $O_2^{\bullet -}$-independent pathway(s). Fig. 2B shows the quantitative analysis of the time-dependent increase in 2-OH-E^+ and E^+ using the highest (0.25 μM/min) flux of $O_2^{\bullet -}$. Clearly, 2-OH-E^+ is the dominant product formed under those conditions.

The effect of varying $O_2^{\bullet -}$ flux on the stoichiometry of HE/$O_2^{\bullet -}$ reaction and HE-derived product formation

To further support our findings, we incubated HE with $O_2^{\bullet -}$ under varying fluxes of $O_2^{\bullet -}$ and tested the effect of $O_2^{\bullet -}$ flux on reaction stoichiometry. Fig. 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 $O_2^{\bullet -}$ generation [19].

The calculated stoichiometry between $O_2^{\bullet -}$ and 2-OH-E^+ using the lowest concentration of XO (0.005 mU/ml, corresponding to 1.25 nM $O_2^{\bullet -}$/min) indicates that two molecules of $O_2^{\bullet -}$ 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 $O_2^{\bullet -}$ and 2-OH-E^+ was significantly different, with nearly a 6:1 stoichiometry of $O_2^{\bullet -}$ to 2-OH-E^+ at the highest $O_2^{\bullet -}$ flux tested (Fig. 3B). In fact, the stoichiometry between $O_2^{\bullet -}$ and 2-OH-E^+ was variable, with increasing flux of $O_2^{\bullet -}$ (Fig. 3B, inset). Similar results have been published previously [6]. Previously, it was concluded that at low $O_2^{\bullet -}$ 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 of 2-OH-E^+ formed due to autoxidation of the probe. It is conceivable that the difference in data interpretation between the present work and that reported previously [6] is due to the omission of the contribution of HE autoxidation 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 an increased fraction of $O_2^{\bullet -}$ undergoing self-dismutation. The HE-derived radical was also

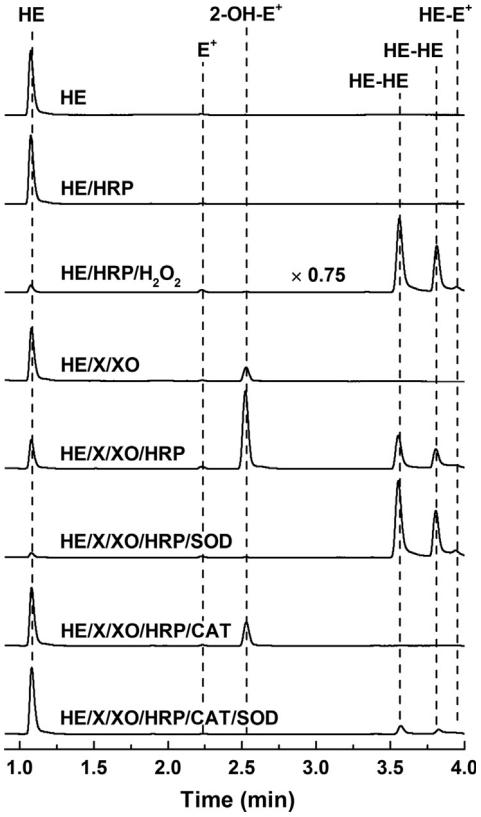


Fig. 4. The influence of HRP on the oxidation of hydroethidine in the xanthine/xanthine oxidase system. Chromatograms obtained from UPLC analyses of HE (60 μM) oxidation products formed in a superoxide-generating system containing X (2 mM) and XO (2.5 mU/ml, superoxide flux 1.1 ± 0.1 μM/min) in a phosphate 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 μM; SOD, 0.1 mg/ml; catalase (CAT), 100 U/ml. Experimental conditions: incubation time, 31 min; temperature, 23 °C. Analytical wavelength for UV–Vis detection, λ 270 ± 5 nm.

suggested to catalyze the dismutation of $O_2^{\bullet -}$ [20]. Under these conditions, E^+ formation was relatively insensitive to $O_2^{\bullet -}$ flux, with some variation at the 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 [9,16,21,22], 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 [21], 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]. Fig. 4 shows the UPLC chromatograms obtained from incubations containing X, XO, dtpa, and 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 nonspecific oxidation products (E^+ and dimers). As expected, SOD completely inhibited 2-OH-E^+ formation, with actual increase in the yield of nonspecific oxidation products. The dose–response effects of SOD on 2-OH-E^+ formation in the X/XO system in the presence and absence of HRP are shown in Figs. 5A–C. In the presence of 0.1 μg/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 Figs. 5D–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 HE (DHE) reaction with $O_2^{\bullet -}$ was determined to be 2-OH-E^+ [1–5,23,24]. Hall et al. suggested that lower $O_2^{\bullet -}$ levels resulting from low tissue oxygen levels under in vivo conditions preclude 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 ($k \approx 10^3\ M^{-1}\ s^{-1}$) [17]. However, the subsequent reaction between the HE radical and $O_2^{\bullet -}$ is fairly rapid ($k \approx 10^9\ M^{-1}\ s^{-1}$). In fact, Robinson et al. [22] 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 $O_2^{\bullet -}$. The dose–response effect of SOD on 2-OH-E^+ (Fig. 5) confirms that HE radical reaction with $O_2^{\bullet -}$ competes with SOD-catalyzed dismutation of $O_2^{\bullet -}$.

The present results show that over a range of $O_2^{\bullet -}$ flux generation, the major product formed during oxidation of HE in the 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 $O_2^{\bullet -}$ to 2-OH-E^+ stoichiometry for the reaction between HE and $O_2^{\bullet -}$ to be 2:1 (two molecules of $O_2^{\bullet -}$ required to form one molecule of 2-OH-E^+) under conditions generating lower fluxes of $O_2^{\bullet -}$. Based on these results, we reaffirm our original conclusion that 2-OH-E^+, but not E^+, is the product of HE/$O_2^{\bullet -}$ reaction over a wide range of $O_2^{\bullet -}$ fluxes.

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 [23,25]. Two-electron oxidation of HE could involve cytochrome *c* oxidase [25] 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 the formation of HE dimers to probe nonradical- 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 [26]. As the C–H bond is involved in the rate-determining step in the hydride transfer mechanism, one would expect a large

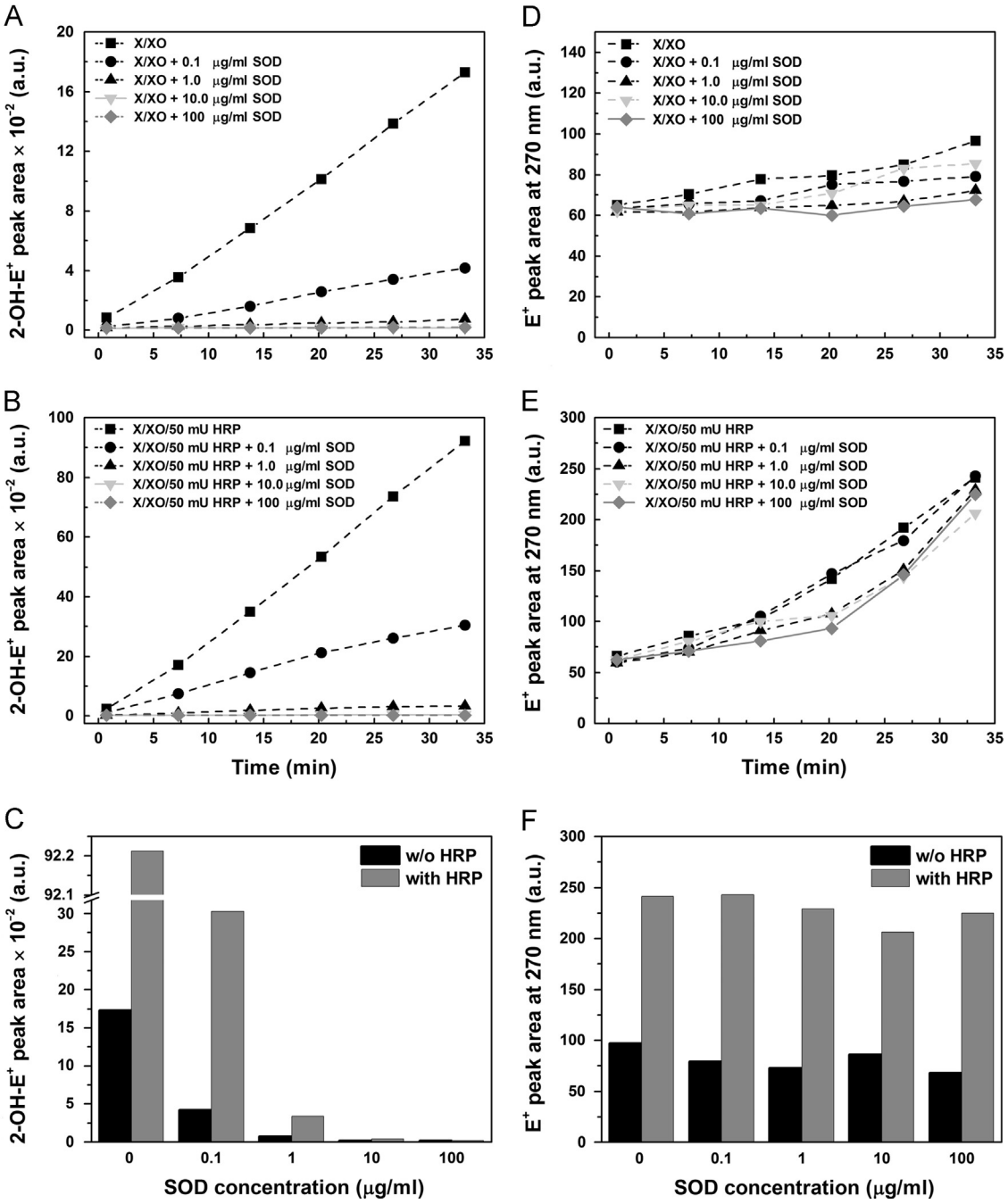
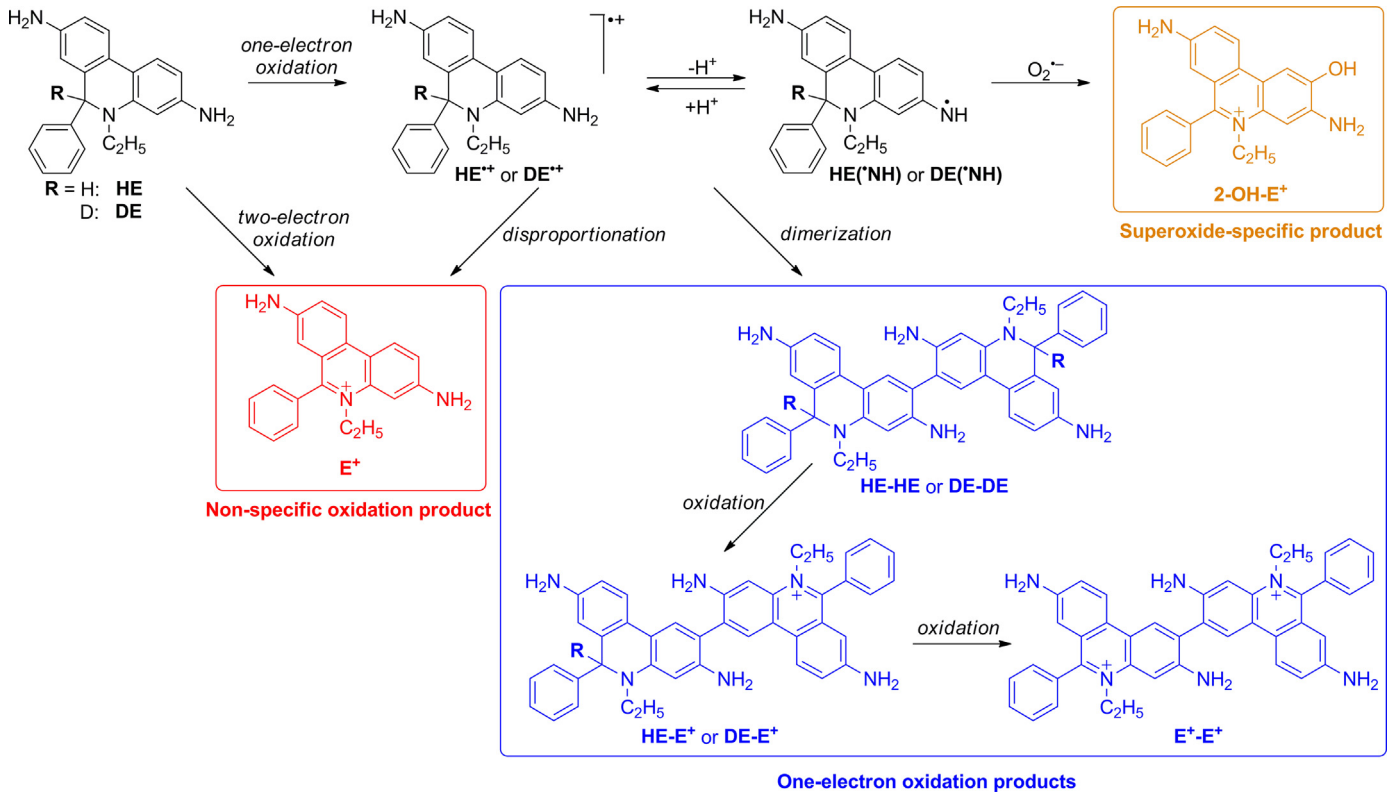


Fig. 5. The dose-dependent effect of SOD on 2-OH-E^+ and E^+ formation. Reaction mixtures contained HE (60 μM), dtpa (0.1 mM), X (2 mM), XO (2.5 mU/ml, $O_2^{\bullet -}$ flux 1.2 ± 0.1 μM/min), HRP (50 mU/ml, if used), and various concentrations of SOD (0.1–100 μg/ml, as indicated) in a phosphate buffer (50 mM; pH 7.4). (A) Time course of 2-OH-E^+ formation in the X/XO system. (B) Time course of 2-OH-E^+ formation in the 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 in both the presence and the absence of HRP. Analytical wavelength for UV–Vis detection was λ 270 ± 5 nm. (D) The formation of E^+ in the X/XO system. (E) The formation of E^+ in the X/XO system in the presence of HRP. (F) End-point measurement of E^+ formed after 33 min of incubation in both the presence and the absence of HRP. Experimental conditions: incubation time, 33 min; temperature, 23 °C. Analytical wavelength for UV–Vis detection, λ 270 ± 5 nm.

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 a 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 $O_2^{\bullet -}$ reaction with HE forms a diagnostic marker product, 2-OH-E^+, but not E^+. The present data indicate that in the presence of varying $O_2^{\bullet -}$ fluxes, the only SOD-sensitive product formed from the reaction between HE and $O_2^{\bullet -}$ is 2-OH-E^+. Whether the experiment is in vitro or in vivo, it is



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.

erroneous to conclude that E^+ formed from HE is indicative of $O_2^{\bullet -}$ levels.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2013.10.816.

References

[1] Zhao, H.; Kalivendi, S.; Zhang, H.; Joseph, J.; Nithipatikom, K.; Vásquez-Vivar, J.; Kalyanaraman, B. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic. Biol. Med.* **34:**1359–1368; 2003.

[2] Zhao, H.; Joseph, J.; Fales, H. M.; Sokoloski, E. A.; Levine, R. L.; Vásquez-Vivar, J.; Kalyanaraman, B. Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proc. Natl. Acad. Sci. USA* **102:**5727–5732; 2005. ([Erratum: Proc. Natl. Acad. Sci. USA 102:9086, 2005.]).

[3] Dikalov, S.; Griendling, K. E.; Harrison, D. G. Measurement of reactive oxygen species in cardiovascular studies. *Hypertension* **49:**717–727; 2007.

[4] Laurindo, F. R.; Fernandes, D. C.; Santos, C. X. Assessment of superoxide production and NADPH oxidase activity by HPLC analysis of dihydroethidium oxidation products. *Methods Enzymol.* **441:**237–260; 2008.

[5] Maghzal, G. J.; Stocker, R. Improved analysis of hydroethidine and 2-hydroxyethidium by HPLC and electrochemical detection. *Free Radic. Biol. Med.* **43:**1095–1096; 2007.

[6] Georgiou, C. D.; Papapostolou, I.; Grintzalis, K. Superoxide radical detection in cells, tissues, organisms (animals, plants, insects, microorganisms) and soils. *Nat. Protoc.* **3:**1679–1692; 2008.

[7] Robinson, K. M.; Janes, M. S.; Beckman, J. S. The selective detection of mitochondrial superoxide by live cell imaging. *Nat. Protoc.* **3:**941–947; 2008.

[8] Kanamori, A.; Catrinescu, M. M.; Kanamori, N.; Mears, K. A.; Beaubien, R.; Levin, L. A. Superoxide is an associated signal for apoptosis in axonal injury. *Brain* **133:**2612–2625; 2010.

[9] Zielonka, J.; Vásquez-Vivar, J.; Kalyanaraman, B. Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. *Nat. Protoc.* **3:**8–21; 2008.

[10] Back, P.; Matthijssens, F.; Vanfleteren, J. R.; Braeckman, B. P. A simplified hydroethidine method for fast and accurate detection of superoxide in isolated mitochondria. *Anal. Biochem.* **423:**147–151; 2012.

[11] Hall, D. J.; Han, S. H.; Chepetan, A.; Inui, E. G.; Rogers, M.; Dugan, L. L. Dynamic optical imaging of metabolic and NADPH oxidase-derived superoxide in live mouse brain using fluorescence lifetime unmixing. *J. Cereb. Blood Flow Metab.* **32:**23–32; 2012.

[12] Drobyshevsky, A.; Luo, K.; Derrick, M.; Yu, L.; Du, H.; Prasad, P. V.; Vasquez-Vivar, J.; Batinic-Haberle, I.; Tan, S. Motor deficits are triggered by reperfusion–reoxygenation injury as diagnosed by MRI and by a mechanism involving oxidants. *J. Neurosci.* **32:**5500–5509; 2012.

[13] Georgiou, C. D.; Papapostolou, I.; Patsoukis, N.; Tsegenidis, T.; Sideris, T. An ultrasensitive fluorescent assay for the in vivo quantification of superoxide radical in organisms. *Anal. Biochem.* **347:**144–151; 2005.

[14] Faropoulos, K.; Chroni, E.; Assimakopoulos, S. F.; Mavrakis, A.; Stamatopoulou, V.; Toumpeki, C.; Drainas, D.; Grintzalis, K.; Papapostolou, I.; Georgiou, C. D.; Konstantinou, D. Altered occluding expression in brain capillaries induced by obstructive jaundice in rats. *Brain Res.* **1325:**121–127; 2010.

[15] Konstantinou, D.; Mavrakis, A.; Grintzalis, K.; Papapostolou, I.; Assimakopoulos, S. F.; Chroni, E.; Georgiou, C. Quantification of superoxide radical in the brain of rats with experimentally induced obstructive jaundice. *Neurochem. Res.* **33:**1101–1105; 2008.

[16] Zielonka, J.; Zhao, H.; Xu, Y.; Kalyanaraman, B. Mechanistic similarities between oxidation of hydroethidine by Fremy's salt and superoxide: stopped-flow optical and EPR studies. *Free Radic. Biol. Med.* **39:**853–863; 2005.

[17] Michalski, R.; Zielonka, J.; Hardy, M.; Joseph, J.; Kalyanaraman, B. Hydropropidine: a novel, cell-impermeant fluorogenic probe for detecting extracellular superoxide. *Free Radic. Biol. Med.* **43:**135–147; 2013.

[18] Zielonka, J.; Zielonka, M.; Sikora, A.; Adamus, J.; Hardy, M.; Ouari, O.; Dranka, B. P.; Kalyanaraman, B. Global profiling of reactive oxygen and nitrogen species in

biological systems: high-throughput real-time analyses. *J. Biol. Chem.* **287:** 2984–2995; 2011.

[19] Kalyanaraman, B.; Darley-Usmar, V.; Davies, K. J.; Dennery, P. A.; Forman, H. J.; Grisham, M. B.; Mann, G. E.; Moore, K.; Roberts, L. J.; Ischiropoulos, I. I. H. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radic. Biol. Med.* **52:**1–6; 2012.

[20] Benov, L.; Sztejnberg, L.; Fridovich, I. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radic. Biol. Med.* **25:**826–831; 1998.

[21] Zielonka, J.; Sarna, T.; Roberts, J. E.; Wishart, J. F.; Kalyanaraman, B. Pulse radiolysis and steady-state analyses of the reaction between hydroethidine and superoxide and other oxidants. *Arch. Biochem. Biophys.* **456:**39–47; 2006.

[22] Robinson, K. M.; Janes, M. S.; Pehar, M.; Monette, J. S.; Ross, M. F.; Hagen, T. M.; Murphy, M. P.; Beckman, J. S. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. *Proc. Natl. Acad. Sci. USA* **103:** 15038–15043; 2006.

[23] Zielonka, J.; Kalyanaraman, B. Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. *Free Radic. Biol. Med.* **48:**983–1001; 2010.

[24] Georgiou, C. D.; Papapostolou, I.; Patsoukis, N.; Tsegenidis, T.; Sideris, T. An ultrasensitive fluorescent assay for the in vivo quantification of superoxide radical in organisms. *Anal. Biochem.* **347:**1144–1151; 2005.

[25] Zielonka, J.; Srinivasan, S.; Hardy, M.; Ouari, O.; Lopez, M.; Vasquez-Vivar, J.; Avadhani, N. G.; Kalyanaraman, B. Cytochrome c-mediated oxidation of hydroethidine and mito-hydroethidine in mitochondria: identification of homo- and heterodimers. *Free Radic. Biol. Med.* **44:**835–846; 2008.

[26] Kundu, K.; Knight, S. F.; Lee, S.; Taylor, W. R.; Murthy, N. A significant improvement of the efficacy of radical oxidant probes by the kinetic isotope effect. *Angew. Chem. Int. Ed.* **49:**6134–6138; 2010.