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Hydroethidine- and Mito-SOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: Another inconvenient truth

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Abstract

Hydroethidine (or dihydroethidium) (HE) is the most popular fluorogenic probe used for detecting intracellular superoxide radical anion. The reaction between superoxide and HE generates a highly specific red fluorescent product, 2-hydroxyethidium (2-OH-E⁺). In biological systems, another red fluorescent product, ethidium (E⁺), is also formed, usually at a much higher concentration than 2-OH-E⁺. In this article, we have reviewed the methods to selectively detect the superoxide-specific product (2-OH-E⁺) and the factors affecting its levels in cellular and biological systems. The most important conclusion of the present review is that it is nearly impossible to assess the intracellular levels of the superoxide specific product, 2-OH-E⁺, using the confocal microscopy or other fluorescence-based microscopic assays and that it is essential to measure by HPLC the intracellular HE and other oxidation products of HE, in addition to 2-OH-E⁺, in order to fully understand the origin of red fluorescence. The chemical reactivity of mitochondria-targeted hydroethidine (Mito-HE, MitoSOX Red[®]) with superoxide is similar to the reactivity of HE with superoxide and therefore, all of the limitations attributed to the HE assay are applicable to Mito-HE (or Mito-SOX) as well.

Introduction

The discovery of the enzyme superoxide dismutase (SOD) has revolutionized our understanding of the role of reactive oxygen species (ROS) in biology and medicine (1-5). Once viewed as a deleterious byproduct of oxygen metabolism, superoxide radical anion (O₂^{•-}) is now considered to play a role in modulating signaling by reacting with •NO, a second messenger and as a precursor for H₂O₂, another second messenger. Despite tremendous progress in the understanding of the biological reactions and physiological signaling functions of superoxide, intracellular detection and quantitation of O₂^{•-} remains a challenge. Direct detection of intracellular superoxide is virtually impossible due to its short half-life and rapid intracellular scavenging. This has led to the development of many probes (e.g., luminol, nitro blue tetrazolium, hydroethidine) which can react with O₂^{•-}, producing easily detectable, relatively stable products that accumulate with time [6-10]. However, only a few of those probes yield products that are specific for superoxide. These probes include: lucigenin (a chemiluminescent probe, which forms N-methylacridone in excited state *via* an unstable dioxetane), hydroethidine (which forms a fluorescent product - 2-hydroxyethidium)

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and cyclic nitron spin traps (which form spin adducts exhibiting characteristic spectra of superoxide spin adducts detectable by electron paramagnetic resonance (EPR) spectrometry). As discussed in previous reviews, lucigenin undergoes redox-cycling (self-generating $O_2^{\bullet-}$) [11-13] and nitron spin traps react slowly with superoxide, forming a relatively unstable superoxide adduct [14-16]; consequently, hydroethidine (HE, also known as dihydroethidium (DHE), Figure 1) has become a probe of choice for the detection of intracellular superoxide. The method based on the detection of 2-hydroxyethidium was described as a “gold standard” for superoxide detection in cardiovascular research [7].

Over the last 20 years, hydroethidine has been used to detect superoxide produced in a variety of biological systems, ranging from intracellular organelles, whole cells to whole organs in live animals. Hydroethidine and its mitochondria-targeted analog (Mito-HE or Mito-SOX) have been used to detect $O_2^{\bullet-}$ formed during oxidative burst in leukocytes, pro- and antioxidative action of cellular endogenous compounds and xenobiotics, during apoptosis and anti- and pro-apoptotic action of drugs, neurosignaling, neurodegeneration and neuroprotection, ischemia and reperfusion, hypertension, mechanical stress induced oxidant production, vascular signaling and pathology, renal function and pathology, mitochondrial and radiation-induced damage. These studies provide evidence for the involvement of superoxide in many physiological and pathophysiological conditions and contribute to the understanding of the molecular mechanisms of many diseases, potentially allowing for development of new strategies for treatment. In almost all studies, the “red fluorescence” generated from HE, attributed to DNA-bound ethidium, was used as a marker of superoxide formation. In this review, we discuss the reactions of HE with superoxide and other oxidants, some of which generate oxidative red fluorescent product (different from that of the superoxide and HE reaction product) and other oxidative non-fluorescent dimeric products. In addition, we emphasize the need to monitor the levels of HE and its oxidation products in order to correctly interpret the 2-hydroxyethidium data obtained from HPLC-based studies.

Evolution of HE from a vital dye to superoxide probe

HE – product of reduction of E^+

Hydroethidine, a product of two-electron reduction of ethidium cation (E^+ , Figure 2), was initially synthesized by reacting ethidium bromide with sodium borohydride [17]. HE does not avidly bind to DNA as it lacks a positive charge and has a non-planar geometry of the phenanthridine moiety. Reduction of E^+ to HE was proposed as a way to release E^+ from DNA [17]. The ultraviolet-visible (UV-Vis) absorption spectrum of an aqueous solution of HE at neutral pH consists of several absorption bands in the range of 200 – 400 nm with the maxima at 224, 265 and 345 nm (Figure 3A), which can be utilized for quantitative analysis of the probe in solutions [17,18].

HE as a vital dye

HE has been initially used as a vital dye because it is cell-permeable [19-23]. The “blue fluorescence” after incubation of live cells with HE was observed predominantly in the cytoplasm of various tumor cells [19]. Depending on the cell line, HE was distributed uniformly over the cytoplasm or was localized in small spots of bluish-white fluorescence. Moreover, the extent of HE uptake and accumulation may be dependent not only on the dye concentration and incubation time, but also on the metabolic state of the cells [19]. HE was used in the investigation of antiproliferative compounds [24-26] and in other biological studies as a vital stain [27-31].

HE as a probe for cellular redox status

The “blue fluorescence” detected in cells following incubation with HE was attributed to the unreacted HE whereas the “red fluorescence” in cells was attributed to the oxidized form of HE, namely the ethidium cation that was bound to the nuclear DNA [19,26]. Because of its charge, the ethidium cation binds to DNA and remains trapped inside the cells [19,22,32]. The “red fluorescence” staining of the cells observed upon incubation with HE [33-39] was used to follow the effector-target interaction [21,40-43] and to visualize the DNA structures in intact cells [44]. However, it was also shown that E^+ was rapidly cleared by doxorubicin-resistant fibrosarcoma cells [20]. The extent of the conversion of HE into E^+ was attributed to the redox state of the cells. It was proposed that HE oxidation is dependent upon the availability of $NADP^+$ coenzyme and can be used to distinguish between the hypoxic and normoxic cells [45]. It was also shown that the intensity of “red fluorescence” of the cells is dependent on HE uptake and cell oxygenation [45]. An additional factor affecting the rate of intracellular oxidation of HE to E^+ was the cell type. For example, the extent of HE oxidation was greater in monocytes as compared to neutrophils [46].

HE as a probe for intracellular ROS

The intracellular oxidation of HE to E^+ was used to visualize the reactive oxygen species (ROS) generated upon stimulation of macrophages and monocytes [25,47-49]. Incubation of the cells with xanthine and xanthine oxidase (generating both superoxide and hydrogen peroxide) and HE caused an increase in the fluorescence intensity that was inhibitable by SOD and catalase. It was suggested that HE can react both with superoxide and H_2O_2 [25,47,48]. Reports also indicate that HE can be oxidized by other oxidants, including $ONOO^-$ and the Fenton's reagent, forming a fluorescent product, suggesting that HE is not specific for detecting the superoxide radical anion [50]. These reports concluded that HE is a non-specific probe for ROS [51,52]. However, further research, as outlined below, indicated that this appears not to be the case.

HE is a specific probe for superoxide

Several reports indicated that HE does not react readily with H_2O_2 and was oxidized intracellularly to E^+ , most likely by superoxide [46,53,54]. Support for this conclusion came from experiments using the SOD linked to polyethylene glycol (PEG-SOD) [55] or high concentrations of extracellularly added SOD [56]. The increase in the HE-derived fluorescence observed after treatment of the cells with H_2O_2 was attributed to intracellular stimulation of superoxide production by H_2O_2 [53,54,57-59]. Based on these results, the HE-derived red fluorescence became widely used for the detection and quantitation of intracellularly generated superoxide [60-68].

2-Hydroxyethidium – the product of the reaction between HE and superoxide

We first reported that the reaction between HE and superoxide formed a product that was structurally different from the ethidium cation, E^+ [69,70]. The mass spectrometric analysis indicated that an oxygen atom was incorporated into ethidium cation. Results also showed that this particular product was unique to superoxide and that other oxidants (peroxynitrite, hydrogen peroxide, and hydroxyl radical) did not form the same product upon reaction with HE. Soon after this discovery, other investigators confirmed the formation of the same product in cells, as determined by high performance liquid chromatography (HPLC) analysis of the extracts of cells and blood vessels incubated with HE [71,72]. Detailed nuclear magnetic resonance (NMR) and mass spectral (MS) analyses of the product indicated that an oxygen atom was attached at the carbon atom position 2 of the phenanthridine ring resulting in the formation of the product, 2-hydroxyethidium (2-OH- E^+ , Figure 4) [72,73]. Several

studies have since confirmed that 2-OH-E⁺ is the only product of the reaction of HE with superoxide generated by xanthine/xanthine oxidase [74-76].

Targeting HE to mitochondria

In order to detect superoxide generated in the mitochondria, the chemical structure was modified by conjugating HE to the triphenylphosphonium moiety (Fig. 1). The attachment of the triphenylphosphonium moiety to HE increased the accumulation of the compound (Mito-HE, Figure 1) into mitochondria [77]. The reaction between Mito-HE and superoxide yielded the corresponding hydroxylated ethidium analog (hydroxy-Mito-ethidium), similar to that formed from the reaction between HE and superoxide [73,77]. Based on NMR analyses, two different structures were proposed: hydroxyl group attachment at C-9 position [77] or at C-2 position [73]. We favor the structure with the hydroxyl group attached at C-2 position (as shown in Figure 4) [73].

HE toxicity

HE was shown to be cytotoxic to protoplast at relatively higher concentrations (45 μ M), and the toxicity was increased in cells exposed to the UV light [78]. An inverse correlation between HE uptake and cell survival was observed in intact cells [45]. Intravenous injection of HE, however, protected against the injury caused by transient focal cerebral ischemia, which was attributed to scavenging of superoxide by HE [79].

Reaction between HE and oxidants

From reviewing the previous literature, it is evident that despite its extensive use in biological systems, the basic chemical reactivity of HE remains poorly understood. Thus, we feel it is necessary to present a short overview of the state-of-the-art of HE oxidation/reduction chemistry.

Two-electron oxidation to E⁺

HE can be readily converted to the ethidium cation by transferring the hydride anion (one proton and two electrons) to a suitable acceptor. In this regard, HE resembles the reduced form of the nicotinamide adenine dinucleotide (NADH). In fact, both HE and NADH can be converted to their oxidized forms (E⁺ and NAD⁺) by reacting with chloranil (tetrachloro-p-benzoquinone) [80]. Treatment of the cells with chloranil converted HE extensively into E⁺; however, it is not clear whether the conversion is solely non-enzymatic, or whether any cellular enzyme is involved in this conversion [81]. In addition, the one-electron oxidation of HE also leads to the formation of the ethidium cation (E⁺) along with other (dimeric) products under these conditions (*see below*) [73]. The proposed mechanism involves a disproportionation reaction of the radical intermediate (HE^{•+} or HE(NH[•])), along with the formation of the 2-electron oxidation product (E⁺).

Superoxide radical anion

The reactivity of HE with superoxide was initially deduced based on the inhibitory effect of SOD in potassium superoxide (KO₂) –induced oxidation of HE [46,53,82] and on the increased fluorescence intensity derived from HE incorporated into mitochondria in the presence of xanthine (X) and xanthine oxidase (XO) [47]. Many reports have since confirmed this finding [53,69,83-94]. The decrease in the fluorescence intensity observed in the presence of higher concentrations of KO₂ was attributed to the reaction between E⁺ and O₂^{•-} [53]. However, as neither E⁺ nor 2-OH-E⁺ reacts with O₂^{•-} [71], the loss of fluorescence may be due to the reaction with the species other than O₂^{•-}. Based on the fluorescence kinetics changes (monitored after mixing of HE with KO₂), formation of a transient fluorescent intermediate was also proposed [95].

In order to quantify the amount of superoxide trapped based on the yield of the HE-superoxide reaction product, the stoichiometry of this reaction needs to be determined. By analogy with the HE/Fremy's salt reaction, the stoichiometry for HE/O₂^{•-} reaction was postulated to be 1:2, i.e. two molecules of superoxide are consumed per one molecule of hydroethidine to form the fluorescent product [18]. However, this did not turn out to be the case using the X/XO system. It was suggested that HE may cause the dismutation of superoxide anion, thus lowering the yield of the oxidation product detected in the X/XO system [83]. The amount of 2-OH-E⁺ formed during oxidation of HE in X/XO system was nearly 3.6-fold lower as compared to superoxide generation [71]. In other studies, a 1:1 stoichiometry for HE reaction with O₂^{•-} was suggested at lower fluxes of superoxide [84,96,97]. The reported stoichiometry of 1:60 for HE/O₂^{•-} reaction in dimethyl sulfoxide (DMSO) [98] is also puzzling. Clearly, we still do not exactly know the mechanism and stoichiometry of 2-OH-E⁺ formation and additional investigation is necessary for establishing the exact mechanism and accurate stoichiometry of the HE/O₂^{•-} reaction.

Synthesis of 2-OH-E⁺: Reaction between nitrosodisulfonate radical dianion (Fremy's salt) and HE

We showed that HE reacts with nitrosodisulfonate radical dianion (NDS, a hydrolysis product of Fremy's salt) to form the 2-hydroxyethidium [18]. This reaction pathway is a facile route to synthesizing the authentic standard of 2-OH-E⁺. The stoichiometry of the reaction (leading to 2-OH-E⁺ formation) is 1:2 (HE:NDS). Based on the determined kinetic isotope effect (k_H/k_D) of 1.9, we proposed the hydrogen (deuterium) atom abstraction from one of the aromatic amine groups by NDS as the first step of the reaction. This is analogous to the initial hydrogen abstraction reaction between HE and O₂^{•-} forming a transient aromatic aminyl radical of HE, followed by the formation of the hydroperoxide and quinone imine forms of HE (Figure 5) [18]. Although the HE-derived radical was implicated in the reaction of HE with NDS, no effect of oxygen on the product yield was observed. Thus, it has been concluded that the radical formed is unreactive towards oxygen, in contrast to the radical species formed from other widely used fluorogenic probes, including dichlorodihydrofluorescein and dihydrorhodamine.

Reaction between manganese porphyrin complexes and HE

It was reported that Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride could quench the "DHE fluorescence" [81]. This suggests that the manganese-porphyrin complex can react with HE in the ground and/or the excited state. It was also reported that HE can react with another manganese-porphyrin complex, namely the manganese(III) tetrakis(4-benzoic acid)porphyrin (MnTBAP) [76]. The HPLC analysis of the reaction product(s) revealed the formation of E⁺. Mn(III)TBAP treatment was also shown to cause the depletion of HE in cells [76]. Thus, the decrease in the amount of superoxide-specific product may be due to lower intracellular availability of the probe. This is particularly worrisome as some porphyrin complexes are used as SOD mimetics to verify the intermediacy of intracellular superoxide using the HE probe.

Oxidation of HE by ferricyanide anion

It has been reported that HE reacts with ferricyanide anion ([Fe(CN)₆]³⁻) leading to the formation of product(s) with fluorescence properties similar to those of ethidium, although the fluorescence intensity was significantly lower [87]. This report also suggests that the stoichiometry of the reaction is 1:1, irrespective of the molar ratio between the reagents, and that a single product is formed. That observation led to the conclusion that a dimer was formed via the recombination of radicals formed from the one-electron oxidation of HE [87]. The HPLC analysis of the reaction products, however, indicated that several products are formed including E⁺ and the dimeric products of different redox state (HE-HE, HE-E⁺,

E^+E^+ , Figure 6) [73]. Ferricyanide-mediated oxidation of Mito-HE resulted in the formation of Mito- E^+ and the corresponding dimeric products. The reaction of HE (or Mito-HE) with ferricyanide has been proposed as a facile route for synthesis of the diethidium (E^+E^+ or Mito- E^+ -Mito- E^+) standard [99]. Measurement of dimeric products of HE can be used as a diagnostic quantitative marker for oxidant formation in cells.

Reaction between HE and H_2O_2 and organic peroxides?

Most previous reports indicate that HE does not react readily with hydrogen peroxide (H_2O_2) in cell-free solution [46,50,53,54,74,82,83,93]. Yet it has been reported that organic peroxide, *tert*-butyl hydroperoxide was able to oxidize HE with the formation of a fluorescent product [50]. However, this reaction appears to be highly unlikely in the absence of redox active metal ions or other catalyst. The presence of low-molecular weight complexes of iron and/or heme proteins (e.g. cytochromes) or in the cellular systems, may facilitate the reaction of HE with peroxides, as discussed below.

Cytochromes

Reports indicate that ferricytochrome *c* (cyt c^{3+}) can react with HE to form the ferrocycytochrome *c* (cyt c^{2+}) [83]. As one molecule of HE could cause the reduction of up to 4 molecules of cyt c^{3+} it was suggested that in addition to E^+ , the dimeric products are most likely formed [83]. Moreover, the kinetics of this reaction is biphasic, indicating a complex reaction mechanism [73,83,87]. Depending on the molar ratio between HE and cyt c^{3+} , different products were formed as monitored by the UV-Vis absorption and fluorescence techniques [87]. Also, no oxygen consumption during the reaction between cyt c^{3+} and HE was observed [87]. Thus, one can conclude that the reaction intermediate(s) and products formed during this reaction are inert towards molecular oxygen. HPLC analysis of the reaction mixtures containing HE and cyt c^{3+} revealed the formation of several products including E^+ , E^+E^+ (diethidium) and two additional products assigned to the dimers, HE-HE and HE- E^+ (Figure 6) [73]. Addition of cyt c^{3+} also catalyzed the reaction between HE and H_2O_2 giving rise to the same products. The reaction of Mito-HE with cyt c^{3+} results in the formation of Mito- E^+ as well as the dimeric products [73]. The rate constant for the reaction of HE and Mito-HE with cyt c^{3+} was estimated as $10^4 - 10^5 M^{-1}s^{-1}$, indicating the likelihood of a rapid consumption of these probes in the presence of excess cyt c^{3+} [73]. This type of reactivity of HE is not limited to cytochrome *c*, and other cytochromes present in mitochondria could cause the oxidation of HE as well [87]. Incubation of HE and Mito-HE with mitochondria yielded the same products (albeit in different ratios) as detected in the case of cyt c^{3+} [73]. Depletion of mitochondrial cytochrome *c* caused an increase of the probes' stability, suggesting that cytochrome *c* is largely responsible for the oxidation of HE or Mito-HE in the mitochondria.

HE oxidation by heme proteins

HE can be oxidized by horseradish peroxidase (HRP) and H_2O_2 with the formation of E^+ [46,74,86] along with non-fluorescent products [46]. Other studies showed that the product(s) of HE oxidation had absorption spectral characteristics similar to those of E^+ [88]. The stoichiometry of the reaction was determined to be 1:1 (i.e., 1 mol of HE oxidized per 1 mol of H_2O_2) [88].

Both hemoglobin and myoglobin react with HE to form the fluorescent products [87]. In both cases, a mixture of oxidation products was detected, similar to those formed during the reaction between HE and cytochrome *c*. In contrast to cytochrome *c*, methemoglobin and metmyoglobin-dependent oxidation of HE required the presence of oxygen [87].

Fenton's reagent

Several reports showed that HE can be oxidized by H_2O_2 in the presence of iron or copper [50,86] *via* the hydroxyl radical ($\cdot\text{OH}$) or higher oxidants of iron [50]. However, in most of these studies the stock solution of HE was prepared in DMSO or in dimethylacetamide. Thus, the actual oxidants could be the solvent-derived radicals, as both DMSO and dimethylacetamide could scavenge $\cdot\text{OH}$ radicals. Moreover the HPLC analysis of the products of the incubation of HE with $\text{Fe}^{2+}/\text{EDTA}/\text{H}_2\text{O}_2$ revealed the formation of both 2-OH- E^+ and E^+ [74]. Recently, it has been reported that the formation of 2-OH- E^+ during oxidation of HE by Fenton's reagent is SOD-inhibitable, and so the formation of 2-OH- E^+ in this system was attributed to superoxide formation [80]. The results show that the metal chelator-derived radical formed from the oxidizing species generated in the Fenton reaction could form the superoxide anion. The addition of ethanol or formate anion to the Fenton's system also increased the yield of 2-OH- E^+ *via* superoxide formation [80]. Thus, the notion that hydroxyl radical or other oxidants derived from the Fenton system can form 2-OH- E^+ from HE without the intermediacy of superoxide has not been substantiated.

Hydroxyl radical

Previous reports showed that $\cdot\text{OH}$ radical does not oxidize HE to a fluorescent product [53]. However, other studies showed the formation of a fluorescent product during irradiation of HE solutions, which has been attributed to the product of reaction of HE with hydroxyl radical [93,96]. It was suggested, based on the fluorescence spectral characteristics, that 2-OH- E^+ was one of the products of the reaction of HE with hydroxyl radical [100]. However, without the HPLC analysis of the products formed, these conclusions remain speculative. Direct monitoring of the reaction of HE with hydroxyl radical by pulse radiolysis indicated the formation of HE radical cation (*see below*), and the rate constant value of $7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ was determined for this reaction [80].

Nitrogen oxides

Published reports consistently showed that $\cdot\text{NO}$ does not react with HE [53,86,94]. However, when HE was incubated with $\cdot\text{NO}$ in the presence of oxygen, a fluorescent product was detected [86]. In addition, it was also shown that the nitrogen dioxide radical ($\cdot\text{NO}_2$) could oxidize HE to a fluorescent product [86]. Structures of the products derived from HE were not, however, determined.

Peroxynitrite

Early on, it was reported that $\cdot\text{NO}$ or ONOO^- was unreactive towards HE. This conclusion was based on the lack of increase of the ethidium fluorescence [53]. It was then postulated that $\cdot\text{NO}$ can compete with HE for $\text{O}_2\cdot^-$ thus inhibiting oxidation of HE [53,69,91,101]. However, it was also shown that incubation of SIN-1 (that slowly releases ONOO^- in the presence of oxygen) or a bolus amount of ONOO^- with HE led to the formation of a fluorescent product [50,86,91], and that reaction can be inhibited by the addition of glutathione [91]. Product(s) formed from the reaction between HE and peroxynitrite were totally different from 2-OH- E^+ [69]. In another study it was postulated that, whereas peroxynitrite alone does not oxidize HE to 2-OH- E^+ nor E^+ , the presence of CO_2 stimulated oxidation of HE by ONOO^- [74]. Moreover, the authors reported that, while no fluorescent product was formed during the reaction of HE with ONOO^- , a product absorbing at 430 nm was detected [74]. These studies clearly indicate that in the presence of ONOO^- , HE is oxidized to the products different from 2-OH- E^+ .

Hypochlorous acid

It has been reported that HE undergoes oxidation by HOCl to form a product with fluorescence characteristics similar to that of the ethidium cation [86,96]. Another study indicated that several products of similar spectral (UV-Vis absorption and fluorescence) characteristics were formed during HOCl-mediated oxidation of HE [88].

Photooxidation

In a cell-free system, HE was relatively stable, when exposed to an excitation light corresponding to that of ethidium (at 510 nm) [53]. On the other hand, when irradiated with UV light, HE undergoes oxidation yielding fluorescent product(s) [53,102]. The HPLC analysis of the products of HE photolysis revealed the formation of 2-OH-E⁺ and, with continued irradiation with the visible light, E⁺ was also formed [76]. The photooxidation of HE was shown to be oxygen-dependent, as after deoxygenation of the solution the process was inhibited [76]. It was also reported that the exposure of acetonitrile cell extracts to ambient light caused an increase in the levels of 2-OH-E⁺ [74].

We have previously shown that in the presence of 2-OH-E⁺, HE can undergo oxidation under the exposure to visible light, with the formation of E⁺ as a final product [76]. The extent of HE oxidation was proportional to the concentration of 2-OH-E⁺, and the photosensitizing effect of 2-OH-E⁺ was abrogated by deoxygenation of the reaction mixture, suggesting the involvement of molecular oxygen in the reaction. However, azide, a well-known scavenger of singlet oxygen, did not inhibit HE photooxidation, thus excluding the involvement of the singlet oxygen in the reaction [76].

Singlet oxygen

Although previous reports indicate that HE does not react with singlet oxygen (¹O₂) [53,96], a more recent report suggests that HE scavenges ¹O₂, with a rate constant of $1.1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ in acetonitrile [103]. The product analysis of the reaction between HE and singlet oxygen has not been, however, reported.

Ultrasound

An increase in HE-derived fluorescence intensity was observed after a shock wave treatment of cell suspension or a cell-free system containing HE [104,105]. It was shown that the increase in HE-derived fluorescence after treating cells with shock waves correlates directly with the cell membrane damage and increased membrane permeability, thus confounding the interpretation of the results of the flow cytometry measurements [106]. The sonication of HE aqueous solution causes a rapid consumption of HE along with the formation of 2-OH-E⁺ and probably other products [76]. The formation of 2-OH-E⁺ has been interpreted in terms of superoxide formation in the solution during sonication, as the addition of SOD inhibited the formation of 2-OH-E⁺ [76]. It was, however, reported that the sonication of the cell extract in acetonitrile did not cause formation of E⁺ or 2-OH-E⁺ [74]. These findings can be rationalized by the protective effects of the cell components present in the extract and/or the different sonochemistry of acetonitrile as compared to aqueous solution. Nevertheless, care should be taken when exposing HE to ultrasound [107-113].

Other reactions

The six-membered nitroxide, Tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), was shown to increase the HE-derived fluorescence in cells, when both compounds were co-incubated [114]. This indicates the possibility of a direct reaction between the two compounds. The acidity of the solution may affect HE stability. Reports indicate an increase

in HE stability due to protonation or enhanced oxidation of HE, as has been reported for trichloroacetic acid [18, 76,87].

Intermediates formed during oxidation of HE

The intermediate radical formed during the one-electron oxidation of HE has been characterized spectroscopically and kinetically using the pulse radiolysis technique [80]. One electron oxidation of HE forms a radical cation ($\text{HE}^{\bullet+}$) with the absorption spectral maxima at 455 nm and 700 nm. It has been shown that the hydroxyl radical can quantitatively oxidize HE to the radical cation, $\text{HE}^{\bullet+}$. During the reaction between HE and superoxide, $\text{HE}^{\bullet+}$ is presumably formed which reacts rapidly with another superoxide molecule ($k \approx 2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$). This reaction is responsible for the specific formation of 2-OH- E^+ during HE reaction with superoxide. During the reaction between HE and nitrosodisulfonate radical dianion, another intermediate product, with the absorption maximum at 460 nm and 670 nm, has been detected and assigned tentatively to the corresponding iminoquinone form of oxidized hydroethidine [18].

Physico-chemical analyses of HE-derived products

Acid-base properties

At neutral pH, the ethidium cation exists as a single, positively charged species. However, in acidic solution, it undergoes double protonation with the pK_a values of 0.4-0.8 and 2.0-2.1 for the protonation of aromatic amine groups at positions 3 and 8, respectively [115,116]. In the case of 2-OH- E^+ , an additional acid-base equilibrium is expected – with the involvement of the aromatic hydroxyl group [18,116]. In fact, the pK_a value of 7.3, next to 0.5 and 2.2, has been reported for this cation [116]. This indicates that at pH 7.4, both cationic (2-OH- E^+) and zwitter-ionic (2-O⁻- E^+) forms of the molecule should be present in almost equal amounts. Similar pH equilibria have been observed for mitochondria-targeted compounds, with pK_a values of 0.2 and 2.0 for Mito- E^+ and 0.4, 1.9 and 7.2 for 2-OH-Mito- E^+ [116].

Binding to DNA

The binding of ethidium cation to polyanions, including DNA, is well known [117,118]. Ethidium has been used as a fluorescent stain for DNA and RNA, as the fluorescence intensity of DNA-bound ethidium is *ca.* 40 times higher than of unbound E^+ [119,120]. 2-OH- E^+ also binds to DNA with a concomitant increase in the quantum yield of fluorescence [69,72,116]. No DNA binding capabilities have been reported for the dimeric (HE-HE and HE- E^+) oxidation products of HE; however, the dimer E^+-E^+ binds to DNA, as reflected by the red shift of its UV-Vis absorption spectrum [73]. The oxidation products of Mito-HE show enhancement in the fluorescence intensity upon binding to DNA [77]. The binding to DNA causes changes in the spectroscopic as well as in the physico-chemical properties of the cations [117,121]. For example, the pK_a value of the hydroxyl group of 2-OH-Mito- E^+ in aqueous solutions containing DNA is 10.4 [77], as compared to the value of 7.2 in the absence of DNA [73].

Binding to the cation-exchange column

The cationic species, E^+ and 2-OH- E^+ , can also bind to the cation exchange columns, and this property has been utilized for purification of HE stock solutions from contaminating E^+ [122]. This property can be also used for isolation of E^+ and 2-OH- E^+ from cell and tissue extracts [84,98].

Redox reactions

Ethidium is reduced to HE via a hydride anion transfer (Figure 2). This reaction has been used to synthesize HE which is accomplished by a two-electron reduction of E^+ to HE using sodium borohydride [17]. It was reported previously that E^+ can be reduced enzymatically to HE by glucose oxidase [83]. Although the possibilities of an enzymatic reduction of E^+ to HE in cellular systems have not yet been explored, a recent report suggests that 2-OH- E^+ is formed from E^+ in endothelial cells *via* reduction of E^+ to HE [71]. The electrochemical reduction of E^+ has also been studied and HE was reported as the final product [123]. Two reduction peaks (at -0.232 V and -556 V vs. Ag/AgCl) have been identified during E^+ reduction. Moreover, the product formed by the reduction of E^+ at the potentials between -0.3 V and -0.45 V, which was assigned to a carbon-centered ethidium radical (E^\bullet), was highly fluorescent ($\lambda_{exc} = 260$ nm, $\lambda_{emi} = 450$ nm) [123]. The radical intermediate formed from the one-electron reduction of ethidium cation has been characterized by pulse radiolysis and based on the differences in electronic absorption spectra, it was concluded that the intermediate formed is structurally different from the species formed upon one-electron oxidation of HE [80]. 2-OH- E^+ was shown to be unreactive towards the biologically relevant reductants, involving ascorbic acid and glutathione [71].

In the presence of strong oxidants (e.g. dibromine radical anion, azidyl radical), ethidium could be oxidized to form the radical dication E^{*2+} that exists in equilibrium with its deprotonated form, ethidium radical cation, $E(\bullet NH)^+$ ($pK_a = 8.2$) [121]. However, a recent study showed that while 2-OH- E^+ can be oxidized by horseradish peroxidase enzyme (HRP) and hydrogen peroxide, E^+ was unreactive under those conditions. This feature enabled quantification of 2-OH- E^+ and E^+ by measuring the fluorescence intensity of the mixture before and after incubation with HRP and H_2O_2 [84,98].

Other considerations affecting HE-derived fluorescence

The ethidium cation, a two-electron oxidation product of HE, was shown to interact with antimycin, a mitochondrial complex III inhibitor, thereby causing an increase in the fluorescence intensity [124]. Mn(III)TBAP has been reported to react with E^+ and 2-OH- E^+ , which can potentially complicate the interpretation of results of its use as an SOD-mimetic agent [76]. Moreover, the cationic porphyrins reportedly quench the fluorescence of ethidium bound to DNA, but not that of ethidium in DNA-free solution [125]. Thus, in addition to HE consumption, the metal porphyrin complexes may also decrease the fluorescence intensity observed by physical and/or chemical interaction with the fluorescent products. The ethidium cation is known to undergo self-association [126-128], but the possibility of self- or hetero-association processes of 2-OH- E^+ is not known.

Methods for detecting HE and its oxidation products in cells and tissues

As ethidium has long been assumed to be the sole product of HE oxidation, the quantification of HE oxidation was based on the changes in the fluorescence intensity. With increased understanding of the oxidative chemistry of HE, it became apparent that even for qualitative analysis, the selective detection of the specific products is critical. The fluorescence settings with greater sensitivity for 2-OH- E^+ than for E^+ have been established to achieve more selective detection of superoxide-specific products. However, to obtain the whole profile of HE oxidation products, the methods based on separation of HE and its products, including HPLC techniques, have been established. The ability to detect the extent of HE consumption and monitor the formation of the oxidation product(s) is a critical factor in HE-based assay for superoxide. Each detection method described below has its own advantages as well as limitations that should be taken into consideration.

Fluorescence-based techniques

The red fluorescence (excitation \approx 500 nm, emission \approx 600 nm, Figure 3) formed from HE has been used in the detection of intracellular superoxide for the last two decades. As HE is also fluorescent (excitation at 350 nm, emission at 400 nm, Figure 3B), the blue fluorescence intensity due to HE has been used to monitor its intracellular uptake. The major advantages of fluorescence detection are the possibility of “real-time” monitoring of the probe oxidation and the lack of cumbersome sample processing. Moreover, using the plate reader, large number of samples can be analyzed simultaneously. The fluorescence microscopy enables the determination of the intracellular/tissue localization of the fluorescent products of HE oxidation and HE distribution. The use of fluorescence detection methods for superoxide measurements was deemed to be inappropriate [87,99,116,129] due to several limitations, including the confounding effects of the non-specific oxidation product(s) of HE contributing to the total fluorescence intensity and light-sensitivity of HE. Selective detection of 2-OH-E⁺ using the excitation light at 396 nm was recently suggested [77,130]. The excitation band between 350 nm and 400 nm was shown to be present in the case of 2-OH-E⁺ (and 2-OH-Mito-E⁺) but not E⁺ (or Mito-E⁺) (Figure 3C). However, it should be emphasized that even at that wavelength, E⁺ will still absorb the excitation light and emit the fluorescence, although to a much lower extent than 2-OH-E⁺. Given that the levels of E⁺ detected in the biological systems are nearly ten-fold higher than that of 2-OH-E⁺, E⁺ could significantly contribute to the fluorescence intensity [116]. As additional oxidative products of HE in cells continue to be discovered, the interpretation of the changes in the fluorescence intensity should be made with caution.

The determination of the intracellular levels of HE using the fluorescence assay remains problematic. Although HE can be detected by fluorescence with a high sensitivity in cell-free systems, using the excitation around 350 nm and emission above 400 nm, the interfering autofluorescence due to the reduced forms of NAD(P) coenzymes in cells makes it difficult to determine intracellular HE levels. The changes in the cellular levels of NAD(P)H can complicate data interpretation. For example, it has been shown that rotenone treatment causes an increase in NAD(P)H autofluorescence [131], which could be mistaken for increased intracellular HE levels.

The fluorescent properties (excitation/emission maxima, quantum yields of fluorescence) of HE oxidation products can be affected by their intracellular localization. Besides binding to polyanionic macromolecules such as DNA, the hydrophobic environment can also affect the fluorescence of E⁺ and 2-OH-E⁺. For example, the partitioning of 2-OH-E⁺ into n-octanol phase, or binding to DNA is accompanied by a shift in the maxima of excitation and emission of fluorescence, resulting in the appearance of an additional excitation band between 350 nm and 400 nm [116].

Another drawback of the fluorescence-based detection is the photosensitivity of HE (*see above*), which can affect the formation of the fluorescent products, especially during constant monitoring of intracellular HE. Moreover, the excitation of the products of HE oxidation may stimulate further HE oxidation, as has been shown in the case of 2-OH-E⁺ [76].

HPLC separation of HE oxidation products

Clearly, there are many drawbacks with the fluorescence approaches for detection of superoxide in intact cells or tissue. The HPLC-based methods allow for detection of both HE and the whole range of the oxidation products. In addition to the analytes' resolution based on the different retention times, the detectors coupled with HPLC systems typically offer the additional selectivity options via different detector settings, including different absorption

wavelengths (UV-Vis absorption detectors), excitation/emission wavelengths (fluorescence detectors), different electric potentials (electrochemical detectors) or different mass/charge values (MS detectors). The major drawbacks of the HPLC methods include the lengthy analysis time (typically 30-75 min per sample) and the need to optimize the sample extraction procedures. The development of new HPLC technology (ultra-performance liquid chromatography, UPLC) aimed at shortening the analysis time by decreasing the column length and particle size (while retaining the resolution characteristic for longer columns) may pave the way for faster analysis of HE and its oxidation products. Different extraction/sample preparation procedures from cells/tissues have been described, but no systematic studies have been reported on the actual efficiency of the extraction together with the stability of HE and its products during sample preparation. Although the HPLC-based separation and detection methods have been most popular, other interesting approaches include the low-pressure chromatography and electrophoresis-based separation.

HPLC with UV-Vis absorption and/or fluorescence detection—The HPLC method coupled with fluorescence detection has been employed to selectively detect and quantify 2-OH-E⁺ and E⁺ both in cell-free and cellular systems [18,69,71-74,80,81,99,116,132-149]. The absorption detection at 290 nm and 500 nm has also been used for monitoring the HE oxidation products [80,99,116], and detection at 245 and/or 350-370 nm for monitoring HE levels [71,74,99,116,148]. Recently, the fluorescence detection of HE during HPLC analysis has been shown to be highly sensitive, and the method of programming of the fluorescence detector to detect both HE and its oxidation products during a single HPLC run has been proposed [99].

With Mito-HE, the HPLC methods with absorption/fluorescence detection have been described [73,77,99,116]; however no data on HPLC-based assay for Mito-HE and its oxidation products extracted from cell cultures or tissue have been published to date.

HPLC with electrochemical detection—To increase the sensitivity of the assay, the HPLC system equipped with electrochemical detection has been used to quantify HE and its oxidation products [76]. The electrochemical coulometric detection method for 2-OH-E⁺ is at least an order of magnitude more sensitive than the fluorescence detection [76]. This may, however, be dependent on the instrumental setup [99]. The reported HPLC method with amperometric detection of HE and 2-OH-E⁺ requires shorter time for sample analysis [150]. The electrochemical detection methods have already been applied to investigate HE oxidation in cell-free systems [73,76,80,99,151], cell culture systems [99,116,150-154] and in tissues from *in vivo* experiments [155]. The analysis of Mito-HE and its oxidation products by HPLC with electrochemical detection has been also described [73,99,116].

HPLC with mass spectrometric detection—As 2-OH-E⁺, E⁺ and E⁺-E⁺ are ionic species, their detection is easily achieved using mass spectral analysis [99]. Other analytes (HE, HE-HE and HE-E⁺) can be also monitored using mass spectrometric detectors, but in the case of HE and HE-HE, only the protonated forms can be detected [116]. To date, there are several reports utilizing HPLC-MS method for qualitative and/or quantitative analysis of HE oxidation products in cell-free and cellular systems [18,69,84,99,116,156]. Despite the selectivity of the MS detection due to different masses of the analytes, it needs to be emphasized that resolution based only on m/z differences may be not adequate for quantitative measurements. This is due to the overlap of the signals obtained from different protonation/isotopic forms of the analytes. For example, at m/z value of 316, in addition to the singly-protonated HE form, the isotopic peak of E⁺ is also present; while at m/z value of 314 (used for E⁺ quantification), the protonated form of HE-E⁺ can also be detected [116]. Despite the inherent sensitivity afforded by the additional positive charge localized on the

triphenylphosphonium moiety, no reports on the HPLC-MS detection of Mito-HE and its oxidation products have been published so far.

Electrophoresis-based separation—The separation of the charged oxidation products of HE has also been obtained using the micellar electrokinetic capillary chromatography technique, and the fluorescent products have been quantified using the laser-induced fluorescence detection system [75,157]. The method has also been used to detect 2-OH-Mito-E⁺ and Mito-E⁺ [158]. This is the most sensitive method that is currently available (detection limit of 0.15 amol for 2-OH-E⁺) [75]. An additional advantage of this method is a short analysis time, as it takes less than three minutes to analyze a sample. The major drawback of the published method is that only the fluorescent cations (2-OH-E⁺ and E⁺, and their mitochondria-targeted analogs formed from Mito-HE) have been detected. Thus the probe (HE or Mito-HE) uptake into cells remains unknown, complicating the interpretation of the results.

Microcolumn/HRP assay—The separation of HE and its oxidation products has been also achieved using the sequential cation exchange and hydrophobic microcolumn low pressure chromatography [84,98]. After separation, a mixture of 2-OH-E⁺ and E⁺ was obtained and after adding DNA, the fluorescence intensity of the mixture was measured. Subsequently 2-OH-E⁺ was converted into a non-fluorescent product by HRP/H₂O₂ system, and the fluorescence intensity was measured again. From the difference in the fluorescence intensities, the amount of 2-OH-E⁺ was calculated [84,97,98]. The major advantage of this assay is the possibility of being able to perform parallel processing of a number of samples, thus decreasing the total analysis time. However, this method is not any more sensitive than the typical HPLC method with fluorescent or electrochemical detection [76,84]. The major drawback of the assay is that 2-OH-E⁺ is not quantified directly, but its amount is calculated from the difference in the fluorescence intensities measured before and after incubation with HRP/H₂O₂ [98]. Another disadvantage is the lack of detection of additional (e.g., dimeric) oxidation products of HE.

Factors affecting the yield of 2-hydroxyethidium in cells and tissues

The question is: is HE the ideal intracellular probe for measuring superoxide? Intracellularly, HE has to compete with SOD ($k(\text{SOD} + \text{O}_2^{\bullet-}) = 2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$), which is present at a micromolar concentration in the cytosol. To outcompete SOD, HE levels should reach millimolar concentrations in cells, which may be cytotoxic. Thus, although 2-hydroxyethidium, a specific marker for superoxide radical anion, has been used to monitor changes in superoxide levels, the amount of 2-OH-E⁺ produced is determined not only by intracellular superoxide generation, but also by other variables, e.g., HE uptake, HE consumption rate, and its availability and efficiency in the competition for superoxide with SOD, and intracellular peroxidatic activities [116]. The intracellular uptake of HE by cells is a function of its concentration and stability in the medium and the efficiency of transport from the extracellular medium into the cells.

Stability of HE in the tissue culture media

In a typical cell culture medium (e.g., Dulbecco's Modified Eagle Medium, DMEM), lacking HE-reactive additives (for example Mn(III)TBAP [76]), HE is relatively stable and virtually constant steady-state concentration of extracellular HE can be achieved. As a part of the experimental procedure, the stability of HE in the specific media has to be tested. HE has been used in the quantification of superoxide production by cellular organelles isolated from animal tissues or cultured cells, including microsomes [159,160], mitochondria

[161-163] or cell membranes [74]. In all these instances, no data concerning the stability of HE in the incubation medium were provided.

While maintaining a constant HE level in the extracellular medium to assure the continuous availability of the probe is a relatively easy task in cell culture experiments, the situation is much more complicated in *ex vivo* tissue culture experiments, where the probe has to be transported to the site of superoxide production through multiple cellular compartments. Examples of the use of HE in *ex vivo* experiments include the detection of *in situ* production of superoxide in brain tissue [53,164-173], spinal cord sections [174-176], blood vessels [74,91,138,139,141,145,177-270], diaphragms [52,85,271-273], various components of kidney [94,274-280], heart [201,281-291], islets of Langerhans [292], penis sections [293], liver sections [294,295], lungs [296,297], retinal tissue sections [298], eye sections [299-301] and prostate tissue [302]. HE has been also used to detect superoxide in tissue homogenates [207,279,303-306] as well as in whole blood [307-311]. Although in some cases HE was constantly infused during the experiment, the availability of the probe for the cellular uptake remains unknown. Even more challenging is the task of delivering and maintaining a constant supply of HE to the specific organ in the *in vivo* experiments. The transport of HE from the site of injection to the organ of interest *via* the circulatory system is limited by the high reactivity of the probe with the heme proteins to form the ethidium cation (*see above*). Nonetheless the application of HE to detect *in vivo* production of superoxide has been reported [84,97,107-113,155,164,312-339]. It has been postulated that after an intravenous injection, HE is translocated to the brain [108-111,312,316-318,321-325,328,329,331], kidney [327] and eyes [315,319]. When HE was administered subcutaneously, 2-OH-E⁺ has been detected of in the heart, brain, kidney, liver, lungs and spleen [84]. After the intraperitoneal injection of HE solution, the red fluorescence has been detected in animals' brains [97,112,113,314,320] and hearts [334]. With a few exceptions ([97,155]), the estimation of transport efficiency of HE to the targeted organ in most of those reports was based on the fluorescence intensity of the oxidation products (E⁺ and 2-OH-E⁺), which does not provide any information concerning the availability of HE for reaction with superoxide. Moreover, as no reports have been reported on the distribution of E⁺ (or 2-OH-E⁺) in *in vivo* experiments, it remains to be established whether the fluorescence signals observed are due to oxidation of HE or transport of the oxidized product(s) formed elsewhere to the target organ.

Transport efficiency through cell membranes

The transport of HE from the extracellular environment into the cytosol is thought to occur by passive diffusion, although it remains to be established how different incubation conditions affect the efficiency of the cellular uptake of HE. Under various conditions (including oxidative stress), the permeability of the cell membranes changes [340-342]. It is yet to be established whether this would cause changes in the uptake of HE. With increased intracellular uptake of HE, one would expect an increased level of HE oxidation products, including 2-OH-E⁺, even in the absence of increased steady-state level of superoxide (*see below*).

Intracellular stability of HE

Intracellularly, HE is consumed *via* both superoxide-dependent and- independent pathways. Although the oxidation products that are not specific for superoxide (E⁺, dimers) are usually formed in higher yields than the superoxide-specific product, 2-OH-E⁺ in biological systems, the identity of the superoxide-independent pathways still remains to be established. It has been postulated that HE undergoes NADP⁺-dependent enzymatic dehydrogenation to produce E⁺ [20,22,45]; however other pathways, including oxidation by heme proteins [73] may be also involved. While the intracellular HE concentration is clearly an important factor

controlling the amount of 2-OH-E⁺ produced, its actual concentration inside the cells is usually not measured. Based on the published data for intracellular HE levels in endothelial cells (*ca.* 0.2 nmol/mg protein after treatment of the cells with 10 μM HE [99]) and the endothelial cell volume reported (1 – 7 μl/mg protein, depending on the cellular environment [343]), one can estimate the average concentration of intracellular HE in the low micromolar to sub-millimolar range. In view of the high reactivity of SOD towards superoxide and its intracellular concentration within the micromolar range, the majority of superoxide is probably scavenged by SOD. Thus, the amount of superoxide trapped by HE does not reflect its total intracellular level, and the fraction of superoxide scavenged by HE should be a function of the ratio of HE to SOD levels. Instead of using the absolute fluorescence intensity of the oxidized product, the ratio of the fluorescence intensities of the oxidized product to unreacted HE has been used as a marker of superoxide (or ROS) production [227,260,275,277,344-348]. In some reports, the amount of 2-OH-E⁺ and/or E⁺ has been “normalized” to the amount of HE consumed, thus giving the fraction of HE converted to the specific product [74,134]. Also the ratio of the amount of 2-OH-E⁺ to the intracellular level of E⁺ formed has been used to “normalize” the amount of 2-OH-E⁺. This is based on the assumption that the concentration of E⁺ is not influenced by the treatments used, and thus reflects mainly the intracellular uptake of HE [135]. To normalize the data to the intracellular concentration of HE, however, one has to measure the HE levels during the experiment. Assuming that the intracellular steady-state concentration of HE is reached quickly, as compared to the duration of the assay, and is maintained until the end of incubation at a consistent level, one can analyze the concentration of HE in cell lysate after the incubation. This value may be used to “normalize” the amount of 2-OH-E⁺ to intracellular HE availability and to determine its efficiency in competition with SOD for superoxide. While the straightforward way to “normalize” to HE level is to divide the intracellular amount of 2-OH-E⁺ by the intracellular amount of HE [150], one has to keep in mind that even in a simple competition between scavengers A and B (two pseudo-first-order reactions, with the second-order rate constants k_A and k_B , respectively), the concentration of the specific product (c_A), is given by the equation:

$$c_A = \frac{c_0 \cdot \frac{k_A \cdot [A]}{k_B \cdot [B]}}{1 + \frac{k_A \cdot [A]}{k_B \cdot [B]}}$$

where c_0 is the concentration of the common reactant, for which the compounds A and B compete. This equation can be simplified to the linear dependence of concentration c_A on $[A]/[B]$ value:

$$C_A = c_0 \cdot \frac{k_A \cdot [A]}{k_B \cdot [B]}$$

only, when $k_A \cdot [A] \ll k_B \cdot [B]$. In case of the competition between HE and SOD for superoxide, this requirement would mean that the amount of superoxide scavenged by HE will be the linear function of HE only when the concentration (and activity) of SOD is constant, with SOD scavenging most of the intracellular pool of superoxide. Although it can be expected that in many cases these conditions are met, it is not clear whether the intracellular competition between HE and SOD can be adequately described by this simple model.

Intracellular levels of superoxide radical anion

It is now established that 2-OH-E⁺ is the sole product of the reaction of superoxide with HE in well-defined chemical and enzymatic systems. However, it remained to be established whether the product is also formed in cellular systems and can be used as a diagnostic marker for intracellular superoxide. Several investigators have shown that modulation of intracellular superoxide level causes changes in the amount of 2-OH-E⁺ formed. As the steady-state concentration of superoxide is dependent on both the rates of its formation and decay, stimulation of superoxide production or its scavenging has been used to validate the utility of the assay. Incubation of cells with menadione, a well known redox-cycling agent, has been shown to increase the level of 2-OH-E⁺ formed in isolated mitochondria [75], endothelial cells [71,72,84], spermatozoa [81], ovary cells [150], intact aortic segments [71] and in various mouse organs [84], and this effect was inhibited by treatment of cells with PEG-SOD [71], MnTBAP [72] or Tempol [84], potential scavengers of superoxide.

Results from these studies suggest that 2-OH-E⁺ can be used as the diagnostic marker product of superoxide radical. It has been shown that other redox active agents (e.g., paraquat [137] and mitoquinone [146]), cause an increase of the amount of intracellular 2-OH-E⁺. Angiotensin II, a physiologically-relevant stimulus, increased the amount of 2-OH-E⁺, presumably by activating the NADPH oxidase. The separation and quantitation of the HE oxidation products revealed the increased formation of 2-OH-E⁺ or both 2-OH-E⁺ and E⁺ following angiotensin II treatment [71,134,136,138,145]. The bacterial endotoxin, lipopolysaccharide (LPS), has been reported to increase the formation of intracellular 2-OH-E⁺ [74], which was attributed to activation of NADPH oxidase by LPS. Other physiological stimuli that can potentially stimulate superoxide production and their signaling pathway, including ceramide [72] and arachidonic acid [156], increase the intracellular level of 2-OH-E⁺ as well. The drug phenylephrine, α_1 -adrenergic receptor agonist, has also been reported to increase the amount of 2-OH-E⁺, as measured by HPLC [139]. 1-Methyl-4-phenylpyridinium cation (MPP⁺), a neurotoxin used in the cellular models of Parkinson disease, stimulated the formation of 2-OH-E⁺ in neuroblastoma cells [142].

The manipulation of the cellular antioxidant system has also been shown to affect the level of 2-OH-E⁺ detected. For example, depletion of intracellular GSH levels by incubating cells with buthionine sulfoximine has been shown to increase the intracellular concentration of 2-OH-E⁺ [71]. On the other hand, overexpression of intracellular SOD1 has been shown to inhibit angiotensin II-stimulated 2-OH-E⁺ formation [74]. The transfection studies should, however, be done with appropriate care as it has been shown that an empty vector can affect intracellular 2-OH-E⁺ and E⁺ levels [74]. Similarly, while incubating cells with PEG-SOD causes a decrease in 2-OH-E⁺ level, PEG alone and PEG-catalase have also been shown to exhibit the inhibitory effects [74]. These effects have been attributed to PEG-induced changes in the cell membrane fluidity, which may cause changes in the transmembrane transport of HE and/or its oxidation products.

The steady-state level of HE-derived radical

According to the proposed mechanism of the reaction of HE with superoxide (Figure 5), HE undergoes oxidation to the HE radical form (HE^{•+} or HE(•NH)) during the first step of the reaction. HE-derived radical reacts with another molecular of superoxide leading to the formation of 2-OH-E⁺. The first step – one electron oxidation of HE may be achieved by a variety of oxidants [18,73,80,87,88], and thus one would predict that superoxide-independent oxidation of HE to its radical can increase the yield of 2-OH-E⁺ at a constant flux of superoxide. This hypothesis, however, needs to be verified experimentally. With HE-based cell-free assay for superoxide, in the absence of other superoxide scavengers, one-electron oxidants could change the stoichiometry of the reaction from 1:2 (HE:O₂^{•-}) to 1:1,

thus doubling the amount of 2-OH-E⁺. In the presence of other scavengers (*e.g.* SOD in cells), the efficiency of trapping superoxide by HE can be increased even more, as HE-derived radical reacts with superoxide much faster than HE (estimated rate constant of HE-derived radical with O₂^{•-}, $k \sim 2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ [80]). For example, the oxidation of HE by cytochrome *c* may account the increased red fluorescence intensity observed during the release of cytochrome *c* from mitochondria into cytosol during apoptosis [349]. The occurrence of one-electron oxidation of HE in cells can be verified by monitoring the levels of HE-derived dimeric products using HPLC-based assays. The presence of the compounds capable of reacting with HE-derived radical may cause a decrease in the amount of 2-OH-E⁺. Reduction of the radical back to the parent molecule (HE) is another complicating factor. The reactivity of HE-derived radical with biologically relevant reductants remains to be determined.

Stability and intracellular retention of 2-OH-E⁺

The use 2-OH-E⁺ for the (semi)quantitative analysis of superoxide formation inside the cells is based on the assumption that 2-OH-E⁺ formed intracellularly is retained inside the cells and does not undergo metabolic conversion to other species during the course of the experiment. Thus, the intracellular amount of 2-OH-E⁺ should linearly increase with incubation time and superoxide flux. Due to the destructive nature of the HPLC-based methods, each sample can be analyzed only for a single timepoint. In most reports, the analysis of the samples has been done using a single incubation time, without investigating the time-course of the 2-OH-E⁺ accumulation. As 2-OH-E⁺ binds to DNA, it is generally assumed that its transport out of the cells is negligible. It has been reported that after loading the endothelial cells with 2-OH-E⁺, its intracellular levels monitored for an hour remained unchanged [71]. In addition, incubation of 2-OH-E⁺ with the cell homogenate did not induce metabolic changes to the compound [71]. These results indicate the general stability of 2-OH-E⁺ in endothelial cells. However, the possibility of its metabolism or transport in other types of cells remains to be explored. Reports indicate that in some biological samples, peroxidase-catalyzed oxidation of 2-OH-E⁺ may decrease its levels, and hence the use of a non-specific peroxidase inhibitor, potassium cyanide, was recommended to overcome such problems [98].

HE-based localization of the sources of superoxide

The HE-based fluorescence assay has been used to identify the localization of superoxide source in tissues and cells. Those experiments are based on several assumptions:

1. The effectiveness of scavenging of superoxide by HE is similar in different cellular compartments, *i.e.* HE is distributed uniformly over the whole cell or tissue.
2. The fluorescent compound formed from HE oxidation remains stationary at the site of its formation, *i.e.* no intracellular or intercellular transport of the fluorescent product occurs during the experiment.
3. The fluorescent product formed is due to the reaction of HE with superoxide, *i.e.* the fluorescence intensity originates mostly from 2-OH-E⁺.
4. The differences in the fluorescence quantum yields of 2-OH-E⁺ in different environments cannot account for the observed differences in the fluorescence intensity.

These aspects are critically important for proper interpretation of results. HE distribution (and its kinetic parameters) in the whole organ or even in intact tissues has not been established. Even if it were to distribute evenly, the fluorescent products derived from HE are likely to translocate inside the cells. Due to the extreme affinity of E⁺ to DNA, the

prevailing view is that the fluorescent oxidation product of HE relocates to the nucleus and binds to DNA. It has been shown that HE-derived red fluorescence colocalizes with the blue fluorescence of the nucleus-specific fluorescent dye – 4,6-diamino-2-phenylindole (DAPI) [252]. With regard to mitochondria, binding to mitochondrial nucleic acids has been assumed. It has been reported that HE and Mito-HE oxidation products can bind to the isolated mitochondria [73,350,351]. Moreover, E^+ has been widely used to delete mitochondrial DNA in intact cells [352]. Thus, it is likely that the fluorescent products of HE oxidation may bind to mitochondrial DNA after translocation from the cytosol.

However, reports also suggest that E^+ formed in mitochondria can exit out of the mitochondria after the addition of an uncoupling agent, thus causing a change in the fluorescence intensity [353-355]. Similar findings were reported using an antimicrobial peptide histatin 5 in yeast. Here the increase in HE-derived fluorescence intensity has been attributed to the release of the ethidium cation from the mitochondrial matrix rather than to oxidation of HE [356]. The most overlooked aspect of HE-derived fluorescence is the relative contribution from 2-OH- E^+ , the fluorescent product of HE and superoxide, to the total fluorescence intensity. In the absence of information regarding the identity of the product formed, it is virtually impossible to deduce the nature of the actual oxidant and its site of production.

Summary

Currently, HE is the most commonly used ROS-specific probe, especially for detecting intracellular superoxide. There is an ever increasing number of reports on the detection of superoxide with HE and Mito-HE, its mitochondria-targeted analog. These reports often lack any information with respect to superoxide reaction with the probes [129]. It has been nearly six years after publication of the initial report suggesting that HE/superoxide reaction forms 2-hydroxyethidium but not ethidium as a major product. However, many publications still claim that ethidium is the major product of HE and superoxide [357]. This review emphasizes the various factors that can affect the yield of 2-hydroxyethidium, the diagnostic marker of superoxide and HE reaction. The major conclusion is that the knowledge of the whole profile of HE oxidation products together with the intracellular levels of HE is required to make any conclusion regarding the amount of intracellular superoxide and/or the effect of the specific inhibitors. The use of a HPLC-based methodology is currently the only way of fulfilling this requirement. HE-based fluorescence microscopy alone will not be able to provide trustworthy information on intracellular superoxide formation. That said, both HE and Mito-HE (Mito-SOX) could, however, serve as ideal probes for measuring intracellular oxidant formation. As radicals derived from HE and Mito-HE do not react with oxygen to form additional superoxide and H_2O_2 , disproportionation and radical recombination products derived from HE (ethidium, 2-hydroxyethidium, and dimers) and Mito-HE (Mito-ethidium, 2-hydroxy-mito-ethidium, and the corresponding dimers) yield a quantitative measurement of oxidants formed in the cytosolic and mitochondrial regions.

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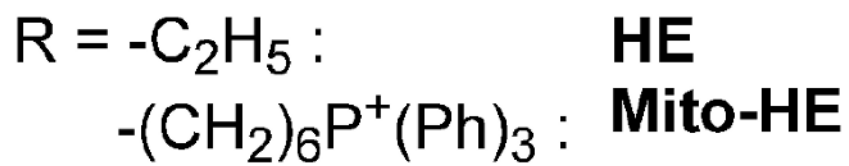
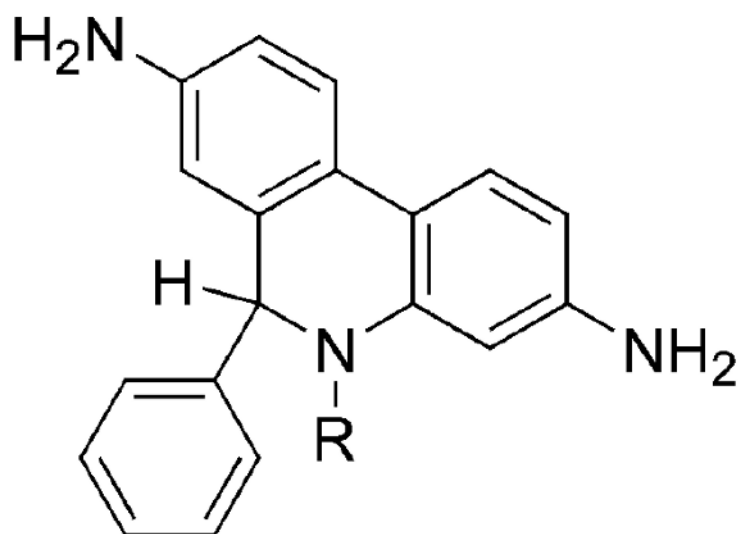


Figure 1.
Structure of HE and Mito-HE.

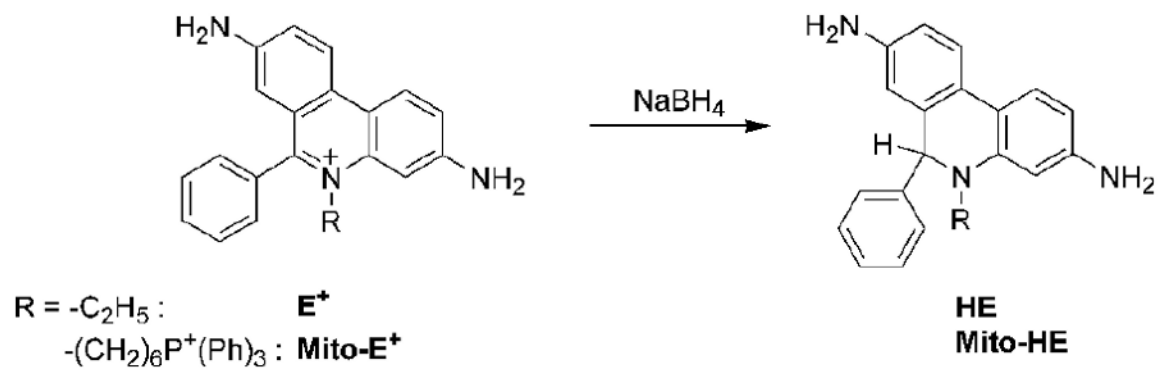


Figure 2.
Reduction of \mathbf{E}^+ and $\mathbf{Mito-E}^+$ by NaBH_4 .

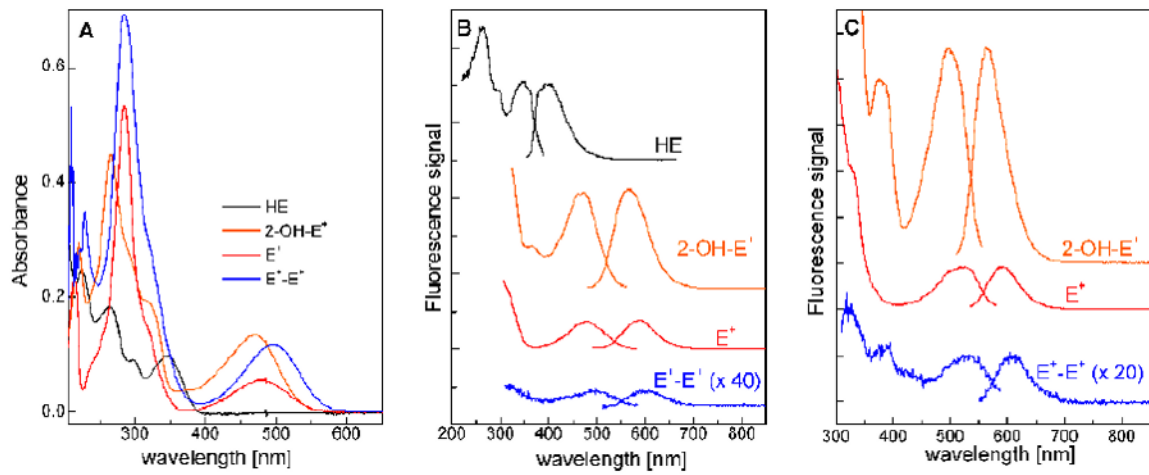


Figure 3. Spectroscopic properties of HE and its oxidation products. (A) UV-Vis absorption spectra; (B) Fluorescence spectra; (C) Fluorescence spectra in the presence of DNA.

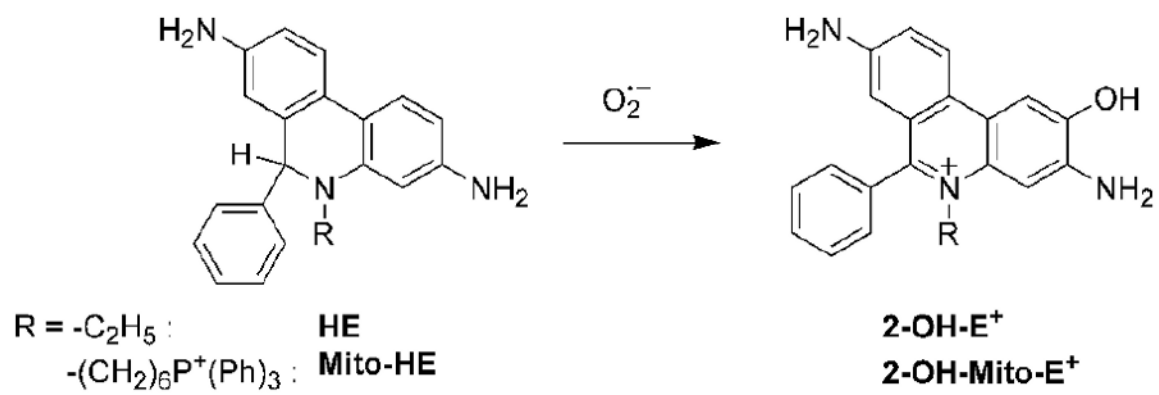


Figure 4.
Formation of 2-hydroxyethidium.

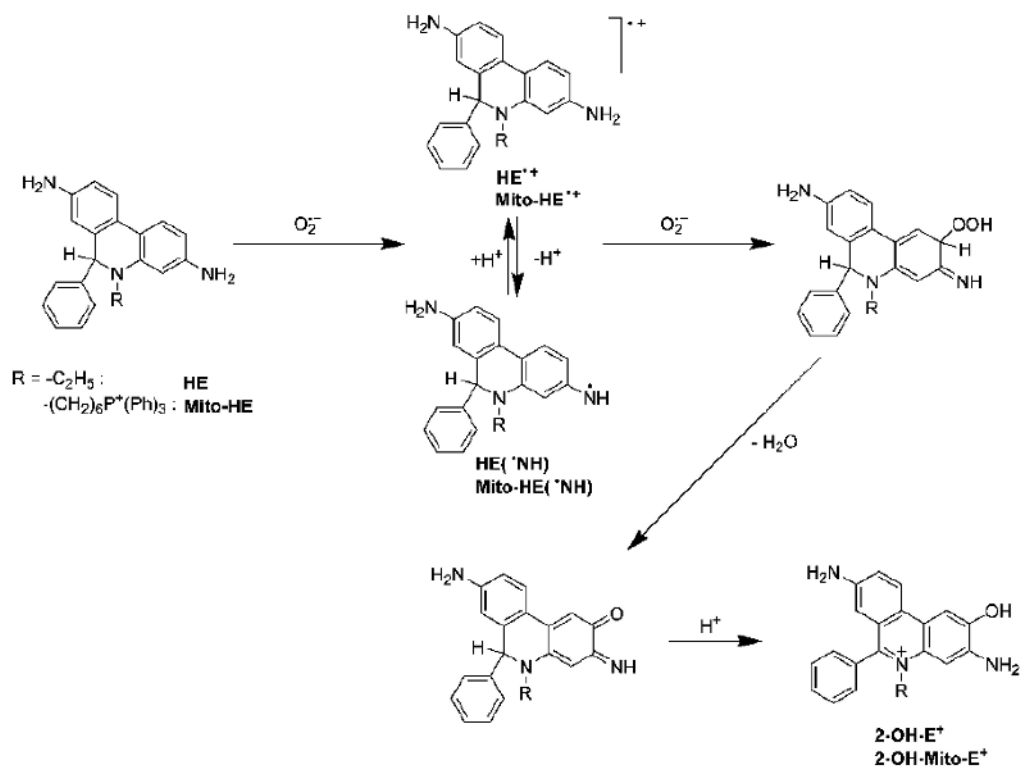


Figure 5.
Hypothetical mechanism of the reaction of HE with superoxide radical anion.

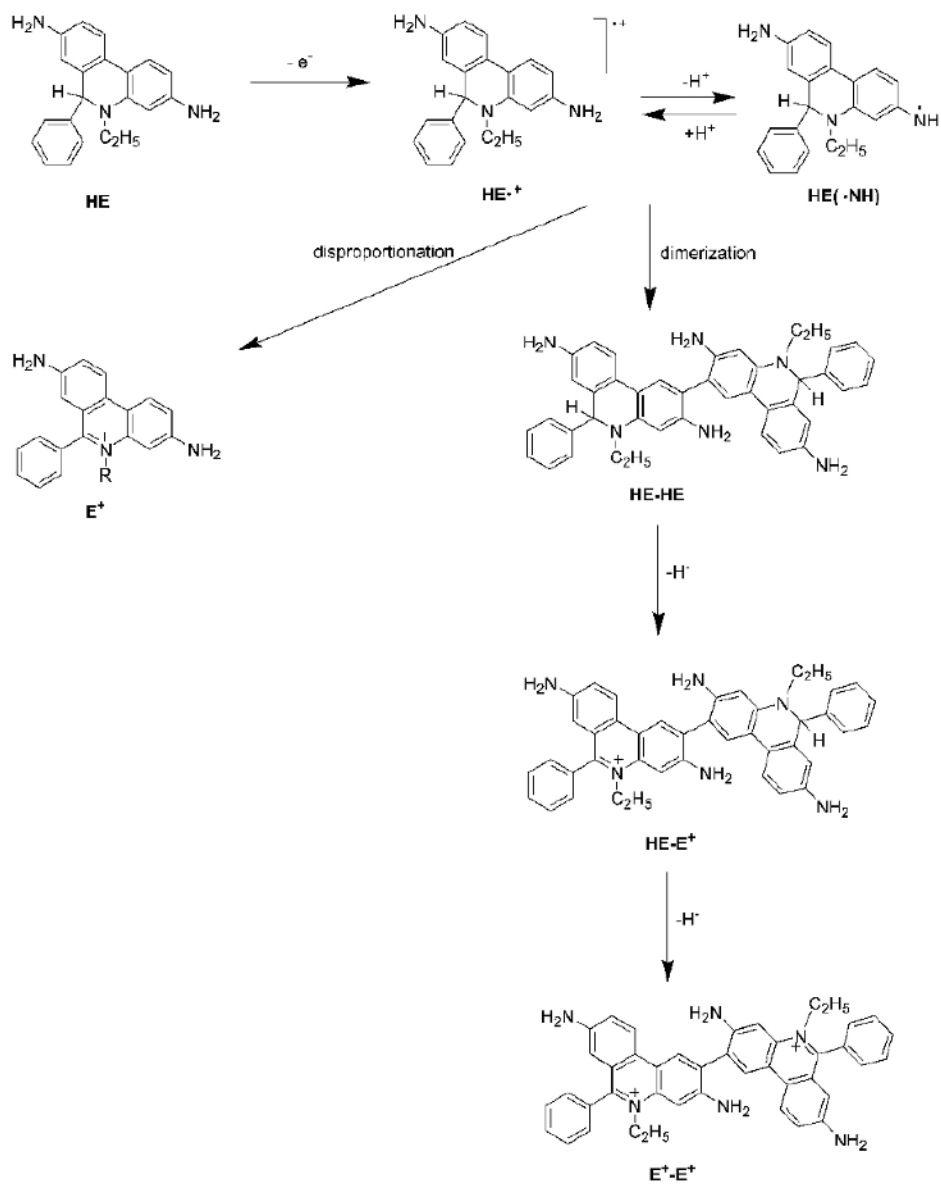


Figure 6.
Transformations of HE upon one-electron oxidation.