

Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine

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Various detection methods of the specific product of reaction of superoxide ($O_2^{\bullet-}$) with hydroethidine (HE), namely 2-hydroxyethidium (2-OH- E^+), and with its mitochondria-targeted analog are described. The detailed protocol for quantification of 2-OH- E^+ , the unique product of HE/ $O_2^{\bullet-}$ in cellular systems, is presented. The procedure includes cell lysis, protein precipitation using acidified methanol and HPLC analysis of the lysate. Using this protocol, we determined the intracellular levels of 2-OH- E^+ and E^+ in the range of 10 and 100 pmol per mg protein in unstimulated macrophage-like RAW 264.7 cells. In addition to HE, 2-OH- E^+ and E^+ , we detected several dimeric products of HE oxidation in cell lysates. As several oxidation products of HE are formed, the superoxide-specific product, 2-OH- E^+ needs to be separated from other HE-derived products for unequivocal quantification.

INTRODUCTION

Superoxide radical anion ($O_2^{\bullet-}$), the one-electron reduction product of molecular oxygen, has been implicated in various physiological and pathological intracellular signaling processes^{1,2}. Increased production of $O_2^{\bullet-}$, its dismutation to hydrogen peroxide and molecular oxygen, as well as its rapid reaction with nitric oxide ($\bullet NO$) cause cellular oxidative stress resulting in the modification of lipids, proteins and DNA. However, because of its short lifetime, low fluxes and rapid reaction with cellular components including superoxide dismutase (SOD) or NO, the detection and quantification of $O_2^{\bullet-}$ in cellular systems has remained a challenging task³. In most chemical and biochemical systems, $O_2^{\bullet-}$ can be detected and quantified by way of its reaction with probes that produce easily detectable and relatively stable compounds (e.g., reduction of ferricytochrome c, oxidation of epinephrine to adrenochrome, spin trapping with cyclic nitrones). The specificity of each of these approaches can be validated by SOD, that is, an SOD-inhibitable signal would indicate an $O_2^{\bullet-}$ -dependent reaction of the probe. In cellular systems, however, the fluorogenic probe hydroethidine (HE, also known as dihydroethidium, DHE, Fig. 1) remains the probe of choice³. Recently, the derivative of HE bearing triphenylphosphonium moiety, called MitoSOX Red or Mito-HE (Fig. 1), has been synthesized. This probe was reported to accumulate in mitochondria and trap mitochondria-derived $O_2^{\bullet-}$ (ref. 4).

Recent studies categorically proved that the product of the reaction of HE with $O_2^{\bullet-}$ is 2-hydroxyethidium (2-OH- E^+), and that no ethidium (E^+) is produced from this reaction (Figs. 1,2)^{5,6}. Reports also show that no other biologically relevant oxidant reacts with HE to form 2-OH- E^+ . Thus, 2-OH- E^+ is a specific marker for $O_2^{\bullet-}$ (refs. 3–6). Rather conveniently, it has proven possible to prepare the authentic standard 2-OH- E^+ by an independent chemical synthetic route (Fig. 3)⁷. Reaction between HE and nitrosodisulfonate radical anion (Fremy's salt) yields 2-hydroxyethidium as the sole product⁷. Please note that an analogous reactivity also leads to the production of 2-OH-Mito- E^+ from Mito-HE see Box 1.

With regard to the mechanism of the reaction of $O_2^{\bullet-}$ with HE, it has been postulated that the radical formed in the first step of the reaction between HE and $O_2^{\bullet-}$ reacts quickly with another $O_2^{\bullet-}$ to give hydroperoxide, which, upon water elimination, would form the imino-quinone derivative of HE, which rearranges to form 2-OH- E^+ (Figs. 2,3)^{7,8}. A rate constant of $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ has been estimated for the first stage of this reaction, the formation of the HE-derived radical from HE and $O_2^{\bullet-}$ (ref. 8). The proposed mechanism of the reaction of HE with superoxide has the following implications for the intracellular detection and quantification of $O_2^{\bullet-}$ using HE as the detection probe:

1. The HE-radical intermediate formed in the assay does not react with oxygen (aromatic aminyl radicals are not reactive toward molecular oxygen). Therefore, unlike assays with

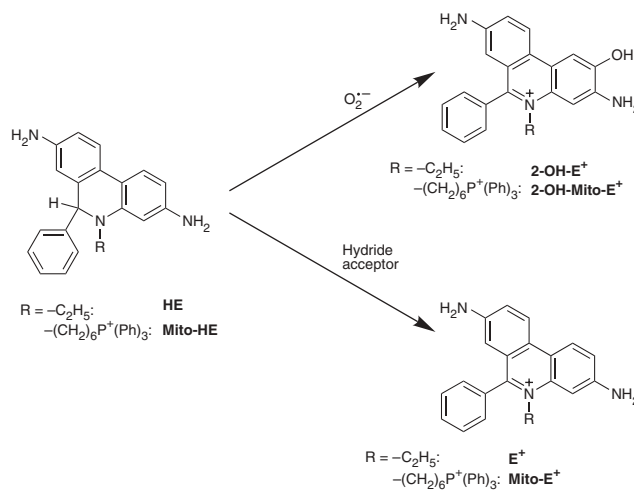


Figure 1 | Structures of hydroethidine (HE), Mito-HE and their oxidation products. 2-OH- E^+ , 2-hydroxyethidium.

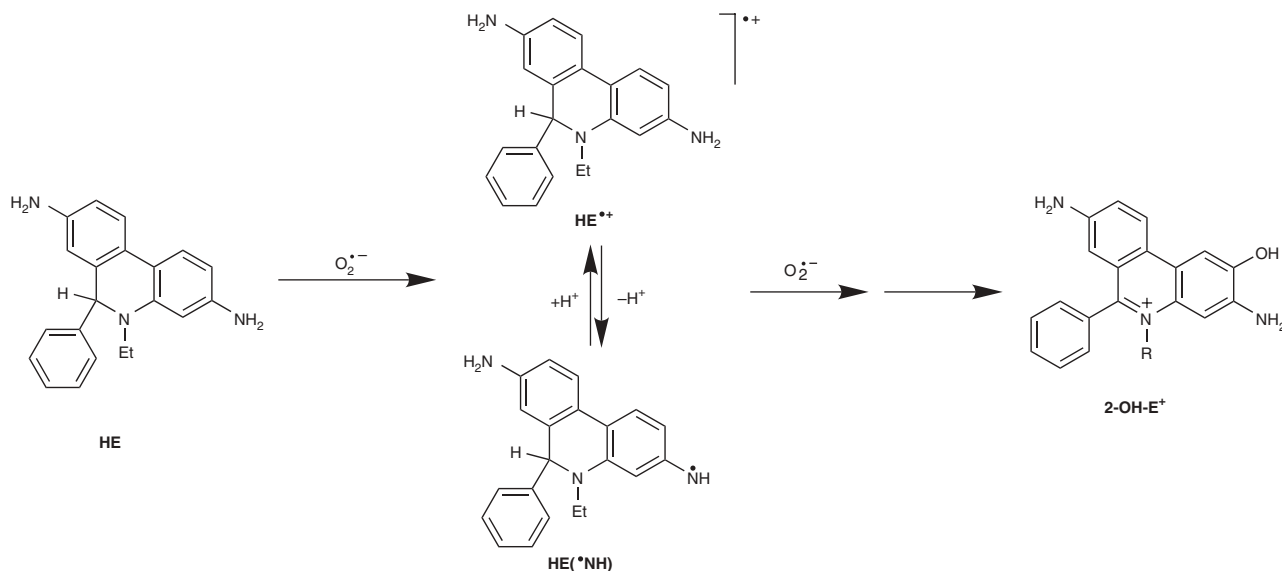


Figure 2 | Proposed mechanism of 2-hydroxyethidium (2-OH-E⁺) formation from the reaction between hydroethidine (HE) and O₂^{•-}.

other probes (lucigenin, 2',7'-dichlorofluorescein, luminol)⁹, the use of HE should not lead to the artifactual formation of superoxide.

- As the rate constant of the reaction of O₂^{•-} with HE is 1,000 times lower than that of the reaction of O₂^{•-} with SOD, the relative concentrations of HE and SOD are expected to affect substantially the yield of 2-OH-E⁺.
- With a constant flux of O₂^{•-}, the yield of 2-OH-E⁺ may be increased by the presence of enzymes or other oxidizing species capable of oxidizing HE to its radical cation (HE^{•+}) or the neutral radical (HE(•NH)). This radical

intermediate may, in fact, subsequently react with O₂^{•-} to form the product, 2-OH-E⁺. This reactive pathway would, however, result in an overall stoichiometric ratio of 1:1 between O₂^{•-} and 2-OH-E⁺ formed, as opposed to the expected 2:1.

- In oxidizing environments, other products of HE-derived radicals reactions (e.g., dimerization and/or disproportionation) will be formed which, in turn, will affect the effective HE concentration for superoxide trapping.

Although the measured amount of 2-OH-E⁺ is a good index of superoxide formation it cannot be directly equated to the value of intracellular flux of superoxide radical anion because of the following reasons:

- Owing to the competition with SOD and/or other intracellular superoxide scavengers, only a fraction of superoxide is scavenged by HE.
- Despite the theoretical stoichiometry of two superoxide molecules per molecule of 2-OH-E⁺ formed, it has been postulated that, similar to SOD, HE could also catalyze the dismutation of superoxide, potentially leading to an underestimation of superoxide levels⁹.
- Intracellular availability of HE may vary with time, and the fraction of superoxide scavenged by HE may, therefore, not be constant throughout the incubation period.

Despite these limitations, one can compare the amount of 2-OH-E⁺ produced in different conditions and obtain semi-quantitative data regarding the effect of various treatments or pathophysiological conditions on intracellular steady-state levels (or flux) of superoxide.

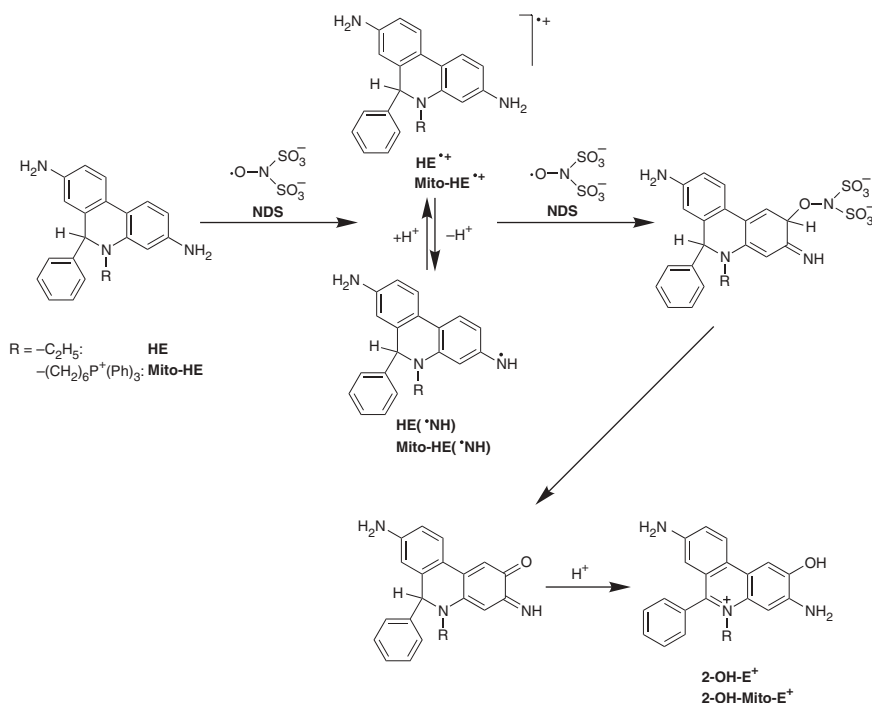


Figure 3 | Mechanism of the reaction of hydroethidine (HE) with nitrosodisulfonate radical dianion (NDS) adapted from ref. 7. 2-OH-E⁺, 2-hydroxyethidium.

BOX 1 | PREPARATION OF STOCKS AND STANDARDS OF Mito-HE AND ITS OXIDATION PRODUCTS 2-OH-Mito-E⁺ AND Mito-E⁺

Preparation of a 5 mM Mito-HE stock solution ● TIMING 15 min

1. Add 13.16 μl of DMSO to a vial containing 50 μg Mito-HE (MitoSOX Red), vortex for 5 s and spin down (1 min \times 1,000g at room temperature) to collect the solution at the bottom of the vial. The preparation should be carried out under subdued light.
2. (Optional) When DMSO should not be used (see Step 1), add 131.6 μl of 0.05 M H_3PO_4 aqueous solution to a vial containing 50 μg of Mito-HE to obtain 0.5 mM solution of Mito-HE. Vortex the resulting mixture for 10 min at 4 °C and centrifuge it (1 min \times 1,000g at room temperature) to collect the desired solution of Mito-HE in a 0.05 M aqueous solution of phosphoric acid (H_3PO_4) at the bottom of the vial.

▲ **CRITICAL STEP** Perchloric acid (HClO_4) should not be used at this stage because of the low solubility of Mito-HE in 0.1 M HClO_4 .

Synthesis of 2-OH-Mito-E⁺ ● TIMING 3 h

3. The standard, 2-OH-Mito-E⁺, may be prepared the same way as reported for 2-hydroxyethidium (2-OH-E⁺) (see Steps 20–26), however, that procedure can be scaled down for economic reasons. After synthesis, analyze the mixture obtained by HPLC to determine the retention time of 2-OH-Mito-E⁺ (major peak).
4. Because of the stronger binding of 2-OH-Mito-E⁺ to the C_{18} cartridge than in the case of 2-OH-E⁺, the elution profiles from the cartridge are not reproducible. Therefore, to improve purification efficiency, extract 2-OH-Mito-E⁺ along with Mito-E⁺ from the Mito-HE/NDS reaction mixture with *n*-BuOH (2 volumes of *n*-BuOH per one volume of the Mito-HE/NDS reaction mixture).
5. Repeat the extraction described in Step 4 and combine the *n*-BuOH extracts. Please note that the aqueous phase after extraction should be colorless.
6. Evaporate the solvent (*n*-BuOH) at 40 °C using rotary evaporator and redissolve the dry residue in a small amount (≤ 0.5 ml) of MeOH.
7. Transfer the MeOH solution into an HPLC vial. Add 1.5 ml water to the vial and mix the solution using a glass Pasteur pipette.
8. Inject the mixture into a semi-preparative C_{18} column (250 \times 10 mm²) and separate the products by increasing the concentration of MeCN in the mobile phase from 40 to 55% in water containing 0.1% TFA, over a period of 30 min at the flow rate of 2.4 ml min⁻¹. Monitor the elution of the compounds by UV absorption setting the detector at a wavelength of 290 nm.
9. Collect all the fractions corresponding to the peaks observed and analyze them by HPLC.
10. Lyophilize the fraction containing pure 2-OH-Mito-E⁺ and reconstitute it in 0.1 M HCl as described for 2-OH-E⁺ (see Step 26C(x)).

Synthesis of Mito-E⁺ ● TIMING 5 h

11. Formation of Mito-E⁺ is achieved through the reaction of Mito-HE with chloranil (tetrachloro-*p*-benzoquinone). Therefore, add 10 ml of MeOH to 4.92 mg of chloranil. Vortex the resulting mixture until the compound is completely dissolved to give a 2 mM solution. Place the solution on ice and keep it shielded from light.
12. Prepare 131.6 μl of a 5 mM solution Mito-HE in DMSO by combining the content of 10 vials, each containing 50 μg of Mito-HE in 13.16 μl DMSO, prepared in Step 1 above.
13. To a ≥ 20 ml amber glass vial, add 10 ml of water, 1.3 ml of 0.5 M aqueous solution of phosphate buffer pH 7.4, 1.3 ml of 1.0 mM diethylenetriaminepentaacetic acid and 32.9 μl of the solution of chloranil (2 mM) in MeOH.
14. Add 131.6 μl of 5 mM solution of Mito-HE in DMSO from Step 12, mix, and incubate the mixture for 4 h at room temperature. The reaction progress can be monitored by UV-visible absorption changes (the formation and growth of an absorption band with maximum at 488 nm will be noted). After incubation, analyze the obtained mixture by HPLC to determine the retention time of Mito-E⁺ (major peak). Mito-E⁺ can be extracted with *n*-BuOH from the reaction mixture and purified by HPLC as described above for 2-OH-Mito-E⁺.

Spurious parameters influencing the detection of 2-OH-E⁺

It is important to be aware that a number of external (nonbiological) factors may influence the measurement of intracellular 2-OH-E⁺:

Light. It has been shown that irradiation of HE solutions with light can cause HE photooxidation with the formation of both 2-OH-E⁺ and E⁺ (ref. 10). Thus, exposure of samples (during incubation or processing) to light can cause an increase in the levels of 2-OH-E⁺. Moreover, while direct efficient photooxidation of HE requires wavelengths close to the UV range, the presence of 2-OH-E⁺ sensitizes HE to photooxidation by visible light ($\lambda > 400$ nm). This can cause an apparent increase in E⁺ in systems generating superoxide.

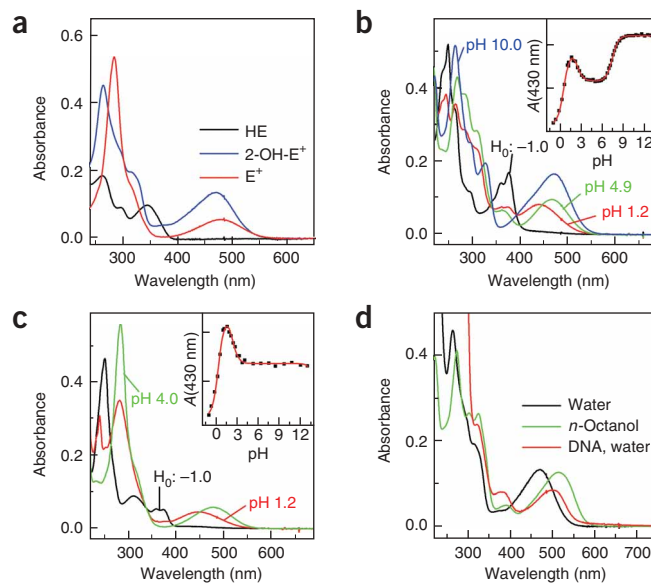
Test oxidants. As HE can also be oxidized by mild oxidants, proper controls should be run to check the reactivity of the test compounds (drugs) used in the experiments with HE. If the drug depletes HE (in the medium or intracellularly), the amount of

measured 2-OH-E⁺ may artifactually decrease owing to the decreasing levels of HE available for $\text{O}_2^{\bullet-}$ scavenging. This will lead to an incorrect interpretation of the data (i.e., decrease the intracellular level of superoxide). For instance, we have shown that treatment of the endothelial cells with Mn(III)TBAP decreases intracellular concentration of HE as well as 2-OH-E⁺, which leads to questionable interpretation of data¹⁰.

Sonication. Sonication is a widely used technique to achieve cell lysis. However, ultrasound treatment is known to cause the formation of superoxide radical anion in the presence of oxygen, and, indeed, we observed the formation of 2-OH-E⁺ after sonication of an aqueous solution of HE¹⁰. Recently, it has been reported that sonication of cells treated with HE does not cause an increase in 2-OH-E⁺ (ref. 11). This may be attributed to the protective effect of the cells (i.e., scavenging of superoxide by cellular components). However, treatment of cells with test compounds may diminish their ability to scavenge superoxide, making the experiment more susceptible to sonication-induced overestimation of superoxide.



Figure 4 | UV-visible absorption spectra. (a) Hydroethidine (HE), 2-hydroxyethidium (2-OH-E⁺) and E⁺ (10 μM each) in 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA. (b,c) The dependence of UV-visible absorption spectra of 2-OH-E⁺ (10 μM, panel b) and E⁺ (10 μM, panel c) on pH of the solution. Insets: pH titration curve of the absorbance detected at 430 nm (for E⁺ and 2-OH-E⁺). Red solid line shows the fitted curve based on the determined pK_a values. (d) 10 μM 2-OH-E⁺ in the absence (black line) and presence (red line) of DNA (1 mg ml⁻¹) in 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA. The spectrum of the compound in *n*-octanol saturated with PBS is shown as a green line.



Inhibitor-derived oxidants. One should take into account the possibility of formation of inhibitor-derived radicals that can react with HE, causing a decrease in 2-OH-E⁺ levels.

Binding of the compounds to the vial walls. While preparing and handling the solutions of HE, Mito-HE and their oxidation products, one should consider the ability of these compounds to bind to the vial walls and pipette tips. As we attribute this phenomenon mostly to hydrophobic interactions, we recommend the use of pure organic solvents [DMSO, methanol (MeOH), ethanol (EtOH)] or mixtures of acidic solutions with organic solvents (0.1 M aqueous phosphate buffer pH 2.6, 25% MeOH) for the storage and transfer of solutions containing these compounds and for the preparation of solutions of standards for HPLC analysis.

Instability of HE solutions. HE can undergo oxidation during prolonged storage at room temperature (20–25 °C). The auto-oxidation process can be slowed down by adding diethylenetriaminepentaacetic acid (DTPA, 100 μM) and/or by storing the solution at lower temperatures (on ice or at 4 °C in the refrigerator). In any event, to minimize risks, we recommend preparing fresh solutions of HE for each experiment.

Intracellular level of HE. Inside cells, HE competes for superoxide with SOD and other targets. Therefore, as mentioned above, the amount of 2-OH-E⁺ formed is also a function of intracellular HE concentration. Compounds that can affect cell membrane permeability to HE or the rate of HE consumption via superoxide-independent mechanisms (e.g., peroxidase-catalyzed HE oxidation¹²) will alter 2-OH-E⁺ yields irrespective of the intracellular steady-state level of superoxide. Monitoring the intracellular level of HE is, therefore, critical for proper interpretation of the data. Note that the yield of 2-OH-E⁺ is not a linear function of HE concentration and thus a simple division of 2-OH-E⁺ amount by intracellular HE concentration may not be adequate to account for changes in HE level¹³.

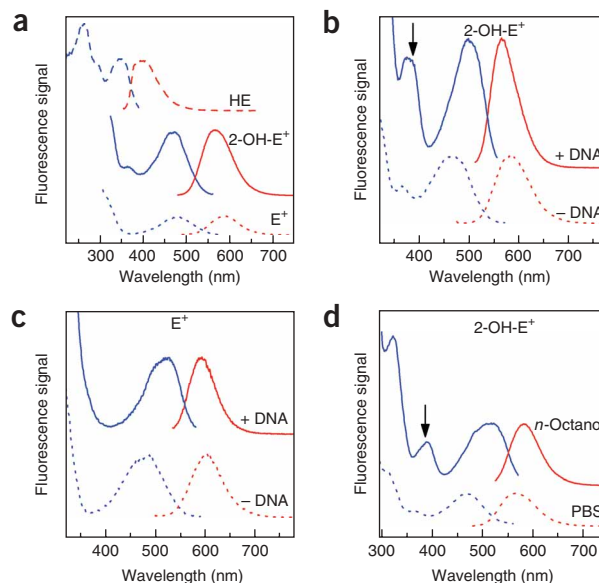
Methods of 2-hydroxyethidium quantification

UV-visible absorption spectroscopy. A major advantage of the spectrophotometric detection of 2-OH-E⁺ (and 2-OH-Mito-E⁺) is the ready availability of spectrophotometers in most laboratories, short time for analysis and the possibility of real-time monitoring of 2-OH-E⁺ formation in chemical and biochemical systems. As shown in **Figure 4**, 2-OH-E⁺ spectrum exhibits absorption maxima at 266 and 470 nm at pH 7.4. As the spectral overlap with other solutes and with HE in the UV range is prominent, the absorption maximum at 470 nm is preferred for 2-OH-E⁺ quantification. However, even at this wavelength the quantification may be

ambiguous owing to interference from other oxidation products of HE absorbing at this wavelength (e.g., E⁺, **Fig. 4a**). Therefore, the spectrophotometric detection should be accompanied by HPLC analysis of the products. In some cases, 2-OH-E⁺ can be separated from other solutes absorbing light at 470 nm by extracting 2-OH-E⁺ with *n*-BuOH. After the subsequent evaporation of *n*-BuOH, the dry residue should be redissolved in the appropriate solvent¹⁴. It should be noted that it is much easier to dissolve the dry residue containing 2-OH-E⁺ in acidic solution [0.1 M hydrochloric acid (HCl)] than in pure water owing to protonation of the compound (see below), which leads to higher solubility in the aqueous solution. In this case, however, the analysis needs to be adjusted to the experimental conditions. As shown in **Figure 4b,c**, the UV-visible spectral properties of 2-OH-E⁺ and E⁺ change with the pH of the solution. Comparison of the pK_a values of E⁺ (pK_as of 0.4 and 2.1, see inset in **Fig. 4c**) and those of 2-OH-E⁺ (pK_as of 0.5, 2.2 and 7.3, see inset in **Fig. 4b**) clearly indicates the involvement of the aromatic hydroxyl group in the acid–base equilibria at neutral pH in the case of 2-OH-E⁺. As the extinction coefficient of 2-OH-E⁺ is sensitive to changes in pH, a rigorous control of pH is required for the spectrophotometric assay of 2-OH-E⁺. Please note that, as Mito-E⁺ and 2-OH-Mito-E⁺ have almost the same acid–base properties as E⁺ and 2-OH-E⁺ (pK_a = 0.2 and 2.0 for Mito-E⁺ and 0.4, 1.9 and 7.2 for mito-2-OH-E⁺), the pH should be rigorously controlled also when working with the mitochondria-targeted analog. The absorption spectrum of 2-OH-E⁺ is also affected by environmental factors (binding to DNA or polarity of the solvent) as shown in **Figure 4d**. Therefore, a calibration curve should be prepared and the extinction coefficient of 2-OH-E⁺ determined under the same experimental conditions.

Fluorescence spectroscopy. Fluorescence spectroscopy, while still allowing the ‘real time’ monitoring of oxidation/hydroxylation of HE to 2-OH-E⁺, is more selective than spectrophotometry. The former approach, in fact, enables quantitative analysis in the presence of multiple components absorbing at 470 nm. Fluorescence-based techniques (including fluorescence microscopy and flow cytometry) have been used for the HE-based detection of

Figure 5 | Fluorescence properties of hydroethidine (HE) and its oxidation products. In all panels the blue lines represent the excitation spectra and red lines the emission spectra. **(a)** Fluorescence spectra of HE (1 μM , dashed lines), 2-hydroxyethidium (2-OH-E⁺) (10 μM , solid lines) and E⁺ (10 μM , dotted lines) in 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA. **(b,c)** The fluorescence spectra of 2-OH-E⁺ (panel **b**) and E⁺ (panel **c**) in the absence (dotted lines, compounds' concentration: 10 μM) and the presence (solid lines, compounds' concentration: 1 μM) of DNA (1 mg ml⁻¹) in 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA. **(d)** Fluorescence spectra of 2-OH-E⁺ (10 μM) in Dulbecco's PBS (DPBS) (dotted lines) and in *n*-octanol saturated with DPBS (solid lines). The arrows indicate an additional excitation band present in the case of 2-OH-E⁺.



superoxide radical anion in a variety of biological systems for over a decade^{15,16}. The fluorescence spectra of HE, E⁺ and 2-OH-E⁺ are shown in **Figure 5**. Whereas HE will not interfere with the fluorescence detection of 2-OH-E⁺, there is significant spectral overlap between 2-OH-E⁺ and E⁺. Therefore, the detection and quantification of 2-OH-E⁺ by fluorescence spectroscopy also requires HPLC analysis to ensure that 2-OH-E⁺ is the only fluorescent product formed following HE oxidation. Note that, in virtually all the biological systems investigated, we detected both 2-OH-E⁺ and E⁺ in different ratios and hence the use of the fluorescent microscopy methods alone can be misleading. The fluorescence intensity of 2-OH-E⁺ and E⁺ (as well as their mitochondria-targeted analogs) increases upon binding of these compounds to DNA (**Fig. 5b,c**). The fluorescence intensities of both E⁺ and 2-OH-E⁺ increase when excitation light in the range of 450–500 nm is used. 2-OH-E⁺ displays an additional excitation band with a maximum between 350 and 400 nm (as indicated by an arrow in **Fig. 5b,d**). This particular spectral feature has been used in the selective detection of 2-OH-E⁺ (and 2-OH-Mito-E⁺)⁴. However, E⁺ also is fluorescent when excited at those wavelengths. Thus, depending on its concentration, E⁺ can significantly contribute to the total fluorescence intensity measured, thereby confounding the analysis. Moreover, that new excitation band between 350 and 400 nm increases in intensity when *n*-octanol is used as a solvent (**Fig. 5d**) or following decrease of the pH of the solution. We attribute this excitation band to the protonated form of 2-OH-E⁺, and conclude that, depending on the intracellular distribution of 2-hydroxyethidium, different fluorescence intensity can be detected. These results further strengthen the case for using HPLC analysis to validate the formation of 2-OH-E⁺ and 2-OH-Mito-E⁺.

Separation of the oxidation products. As UV-visible absorption and fluorescence spectroscopy alone cannot be used to accurately quantify 2-OH-E⁺ in the presence of ethidium, the separation of these products is essential^{5,6}. HPLC enables the analysis of complex mixtures and the quantification of both HE and its oxidation

products. Several methods of extraction of these products from cells and tissues have been reported^{6,11,17–19}, and, depending on either the cell type or the tissue type, different extraction protocols may be required to obtain satisfactory extraction efficiency.

As a consequence of the fluorescent properties of 2-OH-E⁺ and E⁺, HPLC has been used in conjunction with fluorescence detection^{4–8,11,17,20,21}. Recently, we have shown that electrochemical detection is approximately ten times more sensitive than fluorescence detection^{8,10,13}. The selective detection of 2-OH-E⁺ in pure solution and in extracts from cells (in the presence of E⁺) has been also achieved by HPLC-mass spectrometry (HPLC-MS)^{5,7,19}. This technique has the advantage that the compounds can be resolved based not only on their different retention time but also on their different *m/z* values (314 and 330 for E⁺ and 2-OH-E⁺, respectively), thereby increasing selectivity compared with regular HPLC.

Other methods for the selective detection and quantification of 2-OH-E⁺ extracted from biological systems include micellar electrokinetic chromatography coupled with laser-induced fluorescence detection^{22,23} and purification of the cell (or tissue) extract by cation-exchange and hydrophobic micro-column chromatography followed by detection of the fluorescence intensity of the mixture of 2-OH-E⁺ and E⁺ in the presence of DNA before and after consumption of 2-OH-E⁺ by an HRP/H₂O₂ system¹⁸. It is noteworthy that the micellar electrokinetic chromatography coupled with laser-induced fluorescence detection is the most sensitive method available for detection of 2-OH-E⁺ reported to date (limit of detection: 0.15 amol)²³.

MATERIALS

REAGENTS

- Acetonitrile, CH₃CN, MeCN, gradient grade, for HPLC (Sigma-Aldrich, cat. no. 34851) **! CAUTION** Highly flammable and harmful.
- BSA (Sigma, cat. no. A8806)
- Argon gas (4.8 grade; Praxair)
- Bradford reagent (Sigma, cat. no. B6916) **! CAUTION** Corrosive.
- 1-Butanol, *n*-BuOH (Sigma-Aldrich, cat. no. 34867) **! CAUTION** Harmful.
- DTPA (Fluka, cat. no. 32319) **! CAUTION** Irritant, dangerous for the environment.
- DMSO, ACS spectrophotometric grade (Sigma-Aldrich, cat. no. 15,493-8)

- Dulbecco's PBS (DPBS; Sigma, cat. no. D8537)
- EtOH (200 proof; Aldrich, cat. no. E7023) **! CAUTION** Highly flammable.
- Ethidium bromide, E⁺Br⁻—5-ethyl-6-phenyl-3,8-diaminophenanthridinium bromide (Fluka, cat. no. 46065) **! CAUTION** Very toxic.
- Formic acid (HCOOH, 98.0–100%; Riedel-de Haën, cat. no. 27001) **! CAUTION** Corrosive.
- HCl (fuming, ≥ 37%; Fluka, cat. no. 84415) **! CAUTION** Corrosive.
- HE or DHE—5-ethyl-5,6-dihydro-6-phenyl-3,8-diaminophenanthridine (Invitrogen, cat. no. D-1186 or Fluka, cat. no. 37291). **! CAUTION** Potentially harmful—undergoes oxidation to a very toxic compound, ethidium.

- MeOH (for HPLC; Sigma-Aldrich, cat. no. 34860) **! CAUTION** Highly flammable and toxic.
 - Mito-HE—5-(triphenylphosphonium)hexyl-5,6-dihydro-6-phenyl-3,8-diaminophenanthridine, MitoSOX Red (Invitrogen, cat. no. M36008) **! CAUTION** Potentially harmful—undergoes oxidation to an analog of ethidium.
 - Perchloric acid (HClO₄, 70%; Fluka, cat. no. 77227) **! CAUTION** Corrosive and oxidizing.
 - Phosphoric acid (H₃PO₄, ≥85 weight % in H₂O; Sigma-Aldrich, cat. no. 215104) **! CAUTION** Corrosive.
 - Potassium nitrosodisulfonate, NDS, Fremy's salt (Aldrich, cat. no. 220930) **! CAUTION** Harmful.
 - Potassium phosphate dibasic (K₂HPO₄; Fluka, cat. no. 60356)
 - Potassium phosphate monobasic (KH₂PO₄; Sigma-Aldrich, cat. no. P0662)
 - Tetrachloro-*p*-benzoquinone, chloranil (optional, see **Box 1**; Fluka, cat. no. 23280) **! CAUTION** Irritant, dangerous for the environment.
 - Trifluoroacetic acid (TFA, 100%; Pierce, cat. no. 28903) **! CAUTION** Corrosive.
 - Triton X-100 (Sigma-Aldrich, cat. no. T9284) **! CAUTION** Harmful, dangerous for the environment.
 - Water for the solution preparation and for HPLC-EC mobile phase should be purified by Milli-Q system (Millipore) and passed through a Preveil C18 SPE cartridge (Alltech) to remove traces of organic contaminants.
- EQUIPMENT**
- HPLC system with electrochemical detection system (optional, see Step 53, ESA Biosciences Inc.) consisting of Model 582 Pump, with RS-232 Control (ESA Biosciences Inc., cat. no. 70-4050); Gradient Upgrade for CoulArray System (ESA Biosciences Inc., cat. no. 70-4051); Model 5600A CoulArray Detector with computer, 8 Channel (ESA Biosciences Inc., cat. no. 70-4325); CoulArray Organizer with a Temperature Control (ESA Biosciences Inc., cat. no. 70-4340T); Model 542 Autosampler with Sample Tray Cooling, Biocompatible Fluid Path and Standard vial tray (84 positions; ESA Biosciences Inc., cat. no. 70-4152); Model 542 Autosampler Start-up Kit for CoulArray and Coulchem Systems (ESA Biosciences Inc., cat. no. 70-4139)
 - HPLC system with absorption and fluorescence detectors and gradient mixer (optional, see Step 53, Agilent 1100 system; Agilent)
 - HPLC system with MS detector (optional, see Step 53, LC-MSD SL system based on a HPLC Agilent 1100; Agilent)
 - CO₂ incubator (Fisher)
 - Refrigerated centrifuge (Eppendorf, model 5417R)
 - Anaerobic chamber (model 855-AC, Plas-Labs Controlled Atmosphere (Anaerobic) Chamber)
 - Safelight lamp (Kodak 5.5" round adjustable safelight lamp, model B; Kodak, cat. no. 141 2212, equipped with Kodak OC safelight filter; Kodak, cat. no. 152 1483)
 - Rotary evaporator (Büchi Rotavapor R-205 equipped with heating bath B-490, vacuum controller V-800 and vacuum pump Vac V-500, Büchi)
 - Low temperature (−80 °C) freezer (model 8581, Thermo Forma Ultra Low Temperature Upright Freezer; Thermo Forma)
 - C₁₈ column, optional—for methods A and B (see Step 53), Kromasil C₁₈ HPLC column, 250 mm × 4.6 mm, 5 μm, 100 Å (Alltech, cat. no. 62000); guard cartridge (Kromasil C₁₈ guard cartridges; Alltech, cat. no. 96163); cartridge holder (All-Guard cartridge holder; Alltech, cat. no. 80101)
 - Ether-linked phenyl column, optional—for method C (see Step 53), Synergi Polar RP HPLC column, 250 mm × 4.6 mm, 4 μm, 80 Å (Phenomenex, cat. no. 00G-4336-E0); guard cartridge (Synergi Polar RP Securityguard cartridges; Phenomenex, cat. no. AJ0-6076); guard cartridge holder (Phenomenex, cat. no. KJ0-4282)
 - Ether-linked phenyl column, optional—for method D (see Step 53), Synergi Polar RP HPLC column, 250 mm × 2.0 mm, 4 μm, 80 Å (Phenomenex, cat. no. 00G-4336-B0); guard cartridge (Synergi Polar RP Securityguard cartridges; Phenomenex, cat. no. AJ0-6075); guard cartridge holder (Phenomenex, cat. no. KJ0-4282)
 - Semi-preparative HPLC column (Beckman Ultrasphere ODS, 250 mm × 10 mm; Beckman, cat. no. 235328)
 - SPE cartridges—Extract Clean SPE Preveil C18 1000 (Alltech, cat. no. 605430)
 - Adjustable-volume pipettes 2.5 μl, 10 μl, 20 μl, 100 μl, 200 μl, 1 ml + tips (adjustable-volume pipettes; Eppendorf Research)
 - HPLC amber vials (wide opening screw top amber vials with write-on spot; Agilent, cat. no. 5182-0716)
 - Inserts for HPLC vials (100-μl deactivated glass inserts with polymer feet and mandrel interior; Agilent, cat. no. 5181-8872)
 - Screw caps with septa for HPLC vials (polypropylene screw caps with septa for wide opening screw top vials, PTFE/red silicone rubber septa; Agilent, cat. no. 5182-0717)
 - 1.5-ml microcentrifuge clear tubes (VWR, cat. no. 89000-028)
 - 1.5-ml microcentrifuge amber tubes (VWR, cat. no. 89000-030)
 - 15-ml centrifuge tubes (ISC BioExpress, cat. no. C-3394-1)
 - Insulin syringes for cell lysis (Becton Dickinson, cat. no. 329461)
 - 0.45-μm membrane filter (Versapor —450 membrane; PALL, cat. no. 66408)
- REAGENT SETUP**
- 0.5 M phosphate buffer pH 7.4** Mix KH₂PO₄ (final concentration: 0.11 M) with K₂HPO₄ (final concentration: 0.39 M) and dissolve these solids in an amount of water for HPLC-EC that is up to 90% of the desired final volume of the solution. To check the pH of the buffer, take an aliquot, dilute it with nine volumes of water and measure the pH of this 1:10-diluted buffer solution. If the pH value is not in the range 7.3–7.5, adjust the pH of the original buffer by addition of concentrated HCl or NaOH and check the buffer's pH again as above. After obtaining the desired pH of the buffer solution, add water to obtain the final solution volume as planned. Finally, filter the buffer solution by passing it through 0.45-μm membrane filter and store in the 4 °C refrigerator for up to 1 month.
- 1 M phosphate buffer pH 2.6** Prepare as described above, but by mixing H₃PO₄ (final concentration: 0.3 M) with KH₂PO₄ (final concentration: 0.7 M). The final pH of the buffer, after 1:10 dilution with water should be in the range 2.5–2.7.
- 0.2 M HClO₄ in MeOH** Add 85.5 μl of 70% HClO₄ per 4.915 ml of ice-cold MeOH. Keep the resulting solution in the 4 °C refrigerator.

PROCEDURE

Preparation of 20 mM HE stock solution in a darkroom (under amber light) ● TIMING 3 h

- 1| Place in an anaerobic chamber 50 plastic 1.5-ml amber or black Eppendorf tubes, 100-μl and 1-ml pipettes, appropriate pipette tips, ~5 ml DMSO in a 15-ml tube. Please note that, alternatively, instead of DMSO the solvent used at this point may be an aqueous solution of H₃PO₄ (0.05 M) or HClO₄ (0.1 M). For most experiments it is most convenient to use the stock solution of HE in DMSO. However, when DMSO could interfere with the assay (e.g., in the case of reaction of HE with Fenton's reagent or for spectroscopic measurements <300 nm) DMSO should be avoided. To prepare a 5 mM HE solution in 0.05 M H₃PO₄ (or in 0.1 M HClO₄), add 634 μl of 0.05 M aqueous solution of H₃PO₄ (or of 0.1 M HClO₄) per 1 mg of HE in amber glass vial, vortex for 1 min and keep on ice. Check then the concentration of HE by the procedure analogous to the one described below for HE stock solution in DMSO (Steps 11–19).
- 2| Purge the chamber with argon gas.
- 3| Remove oxygen from DMSO by bubbling with argon inside the anaerobic chamber for 30 min.
- 4| Turn off the room light. Use darkroom amber light.

PROTOCOL

- 5| Place the HE vial in the anaerobic chamber.
- 6| Weigh ~5–6 mg of HE in 1.5-ml Eppendorf tube. Write down the exact mass and calculate the volume of DMSO needed to prepare a 20 mM solution according to the equation:

$$V[\text{ml}] = 1,000 \times \frac{m[\text{g}]}{315.42[\text{g/mol}] \times 0.02[\text{mol/dm}^3]},$$

where m —is the mass of HE (in g), 315.42 g mol⁻¹—molecular weight of HE, 0.02 mol dm⁻³—concentration of HE in the stock solution (for 0.00631 g HE, one should add 1.0 ml DMSO)

- 7| Add the calculated volume of deoxygenated DMSO and shake the tube to make sure all HE has been dissolved.
- 8| Take 20 μl aliquots of the HE stock solution and transfer into the prepared amber (or black) Eppendorf tubes. Take the tubes out of the anaerobic chamber.
- 9| Place the tubes containing HE stock solution in a –80 °C freezer.
- **PAUSE POINT** The stock solution of HE in DMSO thus prepared is stable at –80 °C for at least 6 months.
- 10| Before each experiment (see below), thaw the HE stock solution at room temperature for ~5 min.
- 11| Determine the concentration of the HE stock solution by spectrophotometry: place 0.998 ml of aqueous solution of 50 mM phosphate buffer pH 7.4 containing 100 μM DTPA in a quartz cuvette.
- 12| Add 2.5 μl 20 mM HE stock solution in DMSO.
- 13| Mix the solution quickly and collect the UV-visible spectrum in the range 200–800 nm.
- 14| Scan the appropriate blank by the same procedure but using pure DMSO instead of HE stock solution.
- 15| Subtract the spectrum of the blank solution from the spectrum of HE solution.
- 16| Read the absorbance values at 265 and 345 nm.
- 17| Calculate the concentration of HE in the cuvette using the absorbance values at 265 and 345 nm using the equation:
- $$c_{\text{cuvette}}[\text{mol/dm}^3] = \frac{A}{\epsilon[\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}] \times l[\text{cm}]}$$
- 18| Plug the relevant extinction coefficients listed in **Table 1** into the equation in Step 17.
- 19| Calculate the concentration of HE in stock solution (c_{stock}) using the average value of the concentration determined at 265 and 345 nm (c_{cuvette}).

$$c_{\text{stock}}[\text{mol/dm}^3] = 400 \times c_{\text{cuvette}}[\text{mol/dm}^3]$$

Synthesis of 2-hydroxyethidium ● TIMING 3 h

20| Add 10 ml of 50 mM aqueous solution of phosphate buffer pH 7.4 containing 100 μM DTPA to ~3.6 mg NDS (Fremy's salt). Note that the commercially available NDS powder contains ~75% of the compound (based on spectrophotometric analysis). It is normal that dissolution of the yellow powder of Fremy's salt in aqueous solution colors the solution blue. This is due to the

TABLE 1 | The extinction coefficient values for the standards of HE and Mito-HE and their oxidation products at pH 7.4.

Standard	Wavelength λ_{max} (nm)	ϵ (λ_{max}) ($\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$)	Reference
HE	265	1.8×10^4	7
	345	9.75×10^3	7
E ⁺	480	5.8×10^3	7
2-OH-E ⁺	470	1.2×10^4	10
Mito-HE	267	1.6×10^4	This work
	355	7.5×10^3	This work
Mito-E ⁺	488	5.8×10^3	4
2-OH-Mito-E ⁺	478	9.4×10^3	4

2-OH-E⁺, 2-hydroxyethidium; HE, hydroethidine.

dissociation of the (yellow) dimeric nitrosodisulfonate (Fremy's salt powder) into the (blue) monomeric form of nitroxide radical.

▲ **CRITICAL STEP** Owing to its instability, especially in acidic solution, the NDS solution should be prepared fresh, stored at 4 °C and used within a couple of hours of preparation.

21 Record the UV-visible absorption spectrum of the NDS solution prepared in Step 20 after adjusting the baseline reading with 50 mM aqueous solution of phosphate buffer pH 7.4 containing 100 μM DTPA (blank solution).

22 Record the absorbance values at 248 and 545 nm.

23 Calculate the concentration of NDS in the cuvette using the absorbance values (A) at 248 and 545 nm and the optical path length ($l = 1.0$ cm) according to the equation:

$$c_{\text{cuvette}}[\text{mol}/\text{dm}^3] = \frac{A}{\varepsilon[\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}] \times l[\text{cm}]}$$

24 Use the extinction coefficient values of 1.69×10^3 and $20.8 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ at 248 and 545 nm, respectively, to calculate an average NDS concentration.

25 To 24 ml of water in a 100-ml amber glass bottle, add 4 ml of 0.5 M aqueous phosphate buffer pH 7.4, 4 ml of 1 mM aqueous solution of DTPA and 200 μl of 20 mM solution of HE in DMSO (see above). After mixing, add slowly 8.0 ml of 1.0 mM NDS while slowly stirring the solution. If the determined concentration of NDS (calculated in Step 24) is different from 1 mM, the volume should be adjusted accordingly. Leave the solution at room temperature for 2 h. The solution should turn yellow after mixing. After incubation analyze the obtained mixture by HPLC to determine the retention time of 2-OH-E⁺ (major peak).

▲ **CRITICAL STEP** Excess of NDS should be avoided as it will react with the product, 2-OH-E⁺, to form undefined product(s) which will lower the yield of 2-OH-E⁺.

26 Along with the reduction products of NDS, the final reaction mixture may contain small amounts (<10%) of E⁺. The 2-OH-E⁺ thus synthesized can be purified by three alternative approaches: method A, HPLC-based purification; method B, purification on silica gel column⁷; and method C, purification on Alltech Prevail SPE C₁₈ cartridge¹⁰. Method A is recommended for small quantities (<1 mg) of 2-OH-E⁺. It requires the semi-preparative HPLC column and yields the compound as a trifluoroacetate salt. Method B is recommended for large quantities (>50 mg) of 2-OH-E⁺. Method C is recommended for intermediate amounts of 2-OH-E⁺ (0.5–50 mg).

(A) HPLC-based purification ● TIMING 75 min per injection

(i) Follow the method described in **Box 1** for the synthesis of 2-OH-Mito-E⁺, but use the gradient starting at 10% MeCN and change it to 70% MeCN over a time of 46 min.

(B) Purification on silica gel column ● TIMING 6 h

(i) Follow the procedure described elsewhere⁷.

(C) Purification on Alltech Prevail SPE C₁₈ cartridge ● TIMING 6 h

(i) Condition the cartridge by passing 6 ml of water, followed by 3 ml of water/MeOH (50/50) mixture, 3 ml of pure MeOH and again 6 ml of pure water.

(ii) Load the reaction mixture (obtained after Step 25) onto the cartridge.

▲ **CRITICAL STEP** All the fractions obtained in Steps 26C(ii)–(vii) should be collected and the volumes written down to allow subsequent estimation of the amount of 2-OH-E⁺ purified.

(iii) Wash the cartridge with 4 × 3 ml of water.

(iv) Wash the cartridge with 2 × 3 ml of water/MeOH (50/50) mixture.

(v) Wash the cartridge with 3 × 3 ml of water/MeOH (20/80) mixture. 2-OH-E⁺ (the orange band) should elute in this step. Use more water/MeOH (20/80) mixture if the eluate is still colorful, which indicates incomplete elution.

(vi) Wash the cartridge with 2 × 3 ml of pure MeOH.

(vii) Continue washing the cartridge until the second band (E⁺, the pink band) is eluted.

(viii) Analyze by HPLC 10 μl aliquots of each fraction after mixing with 90 μl of 0.1 M aqueous phosphate buffer pH 2.6 solution containing 25% MeOH.

(ix) Based on HPLC analysis take the fraction containing pure 2-OH-E⁺ and lyophilize it or evaporate the solvent under air flux. The other fractions containing 2-OH-E⁺ and E⁺ should be kept for repurification.

(x) Based on the volume of the 2-OH-E⁺ fraction collected and the concentration of 2-OH-E⁺ (the concentration in the eluate was 10× higher than in the solution analyzed by HPLC) calculate the amount of 2-OH-E⁺ and the volume of 0.1 M HCl needed to prepare a 10 mM 2-OH-E⁺ solution. Follow the directions reported below for final dilution and determination of the concentration of 2-OH-E⁺.

PROTOCOL

Preparation of the stock solutions of the standards for oxidation products of HE (and Mito-HE) ● TIMING 1 h

27| To prepare each solution, first add 0.1 M HCl to the solid and vortex until the compound is completely dissolved to form a ~10 mM solution and then dilute the solution 1:100 with water to obtain 0.1 mM solution of the standard in 1 mM HCl.

■ **PAUSE POINT** The solutions of the standards in 1 mM aqueous solution of HCl should be stable for at least 6 months when stored at 4 °C.

28| To determine the concentration of the standards by UV-visible spectrophotometry, place 100 µl of aqueous solution of 100 mM phosphate buffer pH 7.4 containing 200 µM DTPA in an amber glass vial.

29| Add 100 µl of the 100 µM standard stock solution prepared in Step 27.

30| Mix the solution quickly, transfer it into a microcuvette (designed for 100 µl of the solution) and collect the UV-visible spectrum in the range 200–800 nm.

31| Scan the appropriate blank by the same procedure but using 100 µl of 1 mM HCl instead of standard stock solution.

32| Subtract the spectrum of the blank solution from the spectrum of standard solution.

33| Read the absorbance values at the relevant λ_{\max} values for each standard solution (see **Table 1**).

34| Calculate the concentration of standard in the cuvette (c_{cuvette}) using the measured absorbance values (A) and the optical path length ($l = 1.0$ cm) according to the equation:

$$c_{\text{cuvette}}[\text{mol}/\text{dm}^3] = \frac{A}{\varepsilon[\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}] \times l[\text{cm}]}$$

35| Calculate the concentration of the standard in stock solution (c_{stock}) using the determined concentration c_{cuvette} .

$$c_{\text{stock}}[\text{mol}/\text{dm}^3] = 2 \times c_{\text{cuvette}}[\text{mol}/\text{dm}^3]$$

Detection of 2-OH-E⁺ in cells ● TIMING 7 h

36| Prepare three sets of 1.5-ml tubes: the first set (empty tubes) will be used to store aliquots of cell lysate for protein determination; the second set (filled with 100 µl of 0.2 M HClO₄ in MeOH per each tube) will be used to perform protein precipitation from the lysate; and the third set (filled with 100 µl of 1 M phosphate buffer pH 2.6) will be used to precipitate perchlorate anions and adjust pH of the cell extract. Place all three sets of tubes on ice.

▲ **CRITICAL STEP** Steps 37–53 should be carried out under subdued light and the samples should always be kept on ice during sample processing. The procedure described herein can be used for 60-mm diameter dishes. The reagents' volumes should be appropriately scaled, depending on the size of the cell dish.

37| Incubate the cells of choice with cell culture medium containing 10 µM HE (5 µl of 20 mM HE in DMSO per 10 ml of medium) for 30 min.

38| To stop the incubation, remove the medium and wash the cells with ice-cold DPBS buffer.

39| Scrape the cells immediately in 1 ml of ice-cold DPBS, transfer the cell suspension into a 1.5-ml Eppendorf tube and place the tube on ice.

40| Pellet the cells by centrifugation: 5 min × 1,000g at 4 °C.

41| Remove the supernatant carefully by aspiration.

■ **PAUSE POINT** Although it is recommended that the samples be analyzed immediately, the cell pellets can be frozen and stored at –80 °C, typically for up to 1 month, until the day of the HPLC analysis. Please note that all subsequent steps should be carried out on the day of the HPLC analysis.

42| To the cell pellets add 150 µl of ice-cold DPBS containing 0.1% (by vol) of Triton X-100 (lysis buffer).

43| Lyse the cells by drawing the mixture in and out of the insulin syringe. Repeat ten times.

44| Spin down the unlysed cells by centrifugation: 5 min × 1,000g at 4 °C.

45| Transfer 100 µl of the lysate supernatant into the tube containing 100 µl of 0.2 M HClO₄ in MeOH (see Step 36), vortex the tube for 10 s and place it back on ice to allow protein precipitation. Leave it on ice for 1–2 h.

BOX 2 | ELUTION CONDITIONS FOR THE ANALYSIS OF Mito-HE, 2-OH-Mito-E⁺ AND Mito-E⁺

In **Tables 2–4** are reported the elution conditions for HPLC analyses of Mito-HE and its oxidation products (HPLC-FL, method A; HPLC-EC using a Kromasil C₁₈ column, method B; and HPLC-EC using an ether-linked phenyl column, method C). Please note that the HPLC-MS approach is not covered here as we have not carried out this procedure for the analysis of Mito-HE, 2-OH-Mito-E⁺ and Mito-E⁺ in our laboratory.

Each method covered involves re-equilibration of the column for 15 min under the conditions described at time: 0 min. All other conditions are the same as described in Step 53 for HPLC analyses of HE and its oxidation products.

Method A (analogous to method described in Step 53A) ● **TIMING** 65 min per injection see **Table 2** for details.

Method B (analogous to method described in Step 53B) ● **TIMING** 60 min per injection see **Table 3** for details.

Method C (analogous to method described in Step 53C) ● **TIMING** 70 min per injection see **Table 4** for details.

46| Transfer 2 µl of the lysate supernatant into an empty 1.5-ml tube placed on ice.

■ **PAUSE POINT** The cell lysate mixed with acidified MeOH may be stored on ice for 2 h. During that time one can analyze the protein levels in the lysates as well as take a break.

47| Determine the protein concentration in the lysates using the Bradford reagent as follows: Prepare the blank solution (no BSA) and BSA standard solutions (0.5, 1, 1.5, 2, 3, 4 and 5 mg ml⁻¹) in DPBS containing 0.1% Triton X-100. Transfer 2 µl aliquots of the BSA standard solutions into empty 1.5-ml tubes. Add 998 µl (per sample) of Bradford reagent to the vials containing the BSA standard solutions and to those containing 2 µl each of the cell lysates from Step 46. Vortex the tubes and place them in the area shielded from light. Incubate the mixtures for 20 min at room temperature. Before absorption measurement vortex each tube once more (2 s) and transfer the mixture into a disposable plastic cuvette. Place the cuvette in the spectrophotometer. Measure immediately the absorbance at 595 nm. Based on a calibration curve obtained using the BSA standard solutions, calculate the protein concentration in the cell lysates.

48| Pellet the protein precipitate (obtained in Step 45) by centrifugation: 30 min × 20,000g at 4 °C.

49| Transfer 100 µl of the supernatant to the tube containing 100 µl of 1 M phosphate buffer pH 2.6, vortex for 5 s.

50| Pellet the excess buffer and KClO₄ precipitate by centrifugation: 15 min × 20,000g at 4 °C.

51| Transfer 150 µl of the supernatant into the HPLC vial equipped with 200-µl conical glass insert, seal the vial and place it on ice.

52| When all samples are in HPLC vials, place them in the autosampler with the tray cooled down to 4 °C.

53| Analyze the standards (HE, 2-OH-E⁺ and E⁺) and the samples from the cell lysis experiment by HPLC. For compound detection, one can use fluorescence and UV-visible absorption (HPLC-FL)⁷, method A; electrochemistry (HPLC-EC)^{8,10}, methods B and C; or MS^{5,7,19} (HPLC-MS), method D. To make an informed decision as to which analytical approach to implement, please note that the HPLC-electrochemistry (HPLC-EC) system affords greater sensitivity than HPLC-FL in the analysis of HE and its oxidation products. Moreover, the electrochemical properties of HE, 2-OH-E⁺ and E⁺ are distinctly different to one another^{8,10}, thus the analytes can be resolved not only based on their different retention times but also based on their different oxidation potentials. HPLC-MS is the most selective approach for the detection of the analytes, but the cost of such a system is much higher than either of the other systems mentioned above. Please note that, although when applying HPLC-FL using a C₁₈ column (with the mobile phase containing TFA) we obtain good separation between the peaks of 2-OH-E⁺ and E⁺, in the case of HPLC-EC, when acidic phosphate buffer is used instead of TFA, the separation is not always satisfactory. Therefore, the additional option (method C) is described, in which the C₁₈ column is replaced by an ether-linked phenyl column, which affords a much better peak separation. The same type of column is also used for HPLC-MS, for which the mobile phase contains HCOOH instead of TFA. Also in case of unsatisfactory resolution on C₁₈ column in the case of TFA-modified mobile phase (HPLC-FL) the ether-linked phenyl column can be used. Please note that the schematic HPLC conditions that we recommend for the analogous analyses of Mito-HE, 2-OH-Mito-E⁺ and Mito-E⁺ are reported in **Box 2** and **Tables 2–4**.

(A) HPLC with fluorescence and UV-visible absorption detection ● **TIMING** 75 min per sample

- (i) To separate HE, E⁺ and 2-OH-E⁺, inject 50 µl of sample (the actual sample to be tested or the 2-OH-E⁺ standard prepared in Steps 21–28 above) into the HPLC system with a Kromasil C₁₈ column equilibrated with 10% CH₃CN in water containing 0.1% (vol/vol) TFA.

TABLE 2 | Elution conditions for the analysis of Mito-HE and its oxidation products by HPLC with fluorescence detection (**Box 2**, method A).

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
10	55	45
30	45	55
40	0	100
46	0	100
50	80	20

TABLE 3 | Elution conditions for the analysis of Mito-HE and its oxidation products by HPLC with electrochemical detection (Box 2, method B).

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	50	50
25	10	90
30	0	100
40	0	100
45	50	50

TABLE 4 | Elution conditions for the analysis of Mito-HE and its oxidation products by HPLC with electrochemical detection (Box 2, method C).

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	35	65
30	0	100
50	0	100
55	35	65

- (ii) Use a gradient elution method with two mobile phases: A—water containing 0.1% (vol/vol) TFA and B—99.9% acetonitrile, 0.1% (vol/vol) TFA. Increase linearly the concentration of B phase from 10 to 70% in 46 min at a flow rate of 0.5 ml min⁻¹.
- (iii) During the next 1 min (46–47 min after injection) increase the concentration of B to 100% and keep this concentration fixed for the next 8 min (55 min after injection).
- (iv) Over the next 5 min (from 55 to 60 min after injection) gradually decrease B concentration to the initial value of 10% then re-equilibrate the column for 15 min.
- (v) Use fluorescence detection at 356 and 510 nm (excitation) and 595 nm (emission) as well as the absorbance at 220, 250, 290, 370 and 500 nm to monitor the reaction products in the test sample.
- (vi) Measure the areas of the peak of 2-OH-E⁺ obtained following HPLC of the 2-OH-E⁺ standards prepared in Steps 20–26, and use this values to infer a correlation between those areas and the known concentrations of the standards. Identify the peak due to 2-OH-E⁺ in the test sample through the fluorescence trace recorded using an excitation at 510 nm and emission at 595 nm, and measure the area of the peak. Use the area/concentration correlation obtained through the 2-OH-E⁺ standards to calculate the concentration in the test sample.

(B) HPLC with electrochemical detection (HPLC-EC) using a Kromasil C₁₈ column ● TIMING 60 min per sample

- (i) Use a gradient elution method with two mobile phases: A—50 mM phosphate buffer (pH 2.6), 10% acetonitrile, 90% water; and B—50 mM phosphate buffer (pH 2.6), 60% acetonitrile, 40% water. Deoxygenate the mobile phase by bubbling with argon gas. Start mobile phase deoxygenation at least 30 min before beginning HPLC analysis and continue bubbling argon through the mobile phase during analysis of the samples.
- (ii) Separate HE, 2-OH-E⁺ and E⁺ on a Kromasil C₁₈ column using a gradient elution with ratios of A and B mobile phases changing from 7:3 to 3:7 over a period of 20 min (ref. 10).
- (iii) Over the next 5 min (20–25 min after injection) increase the fraction of the mobile phase B to 100% and maintain this setting for the next 15 min (25–40 min after injection).
- (iv) During the next 5 min (40–45 min after injection) decrease the concentration of CH₃CN gradually in the mobile phase to the initial values, then re-equilibrate the column for 15 min.

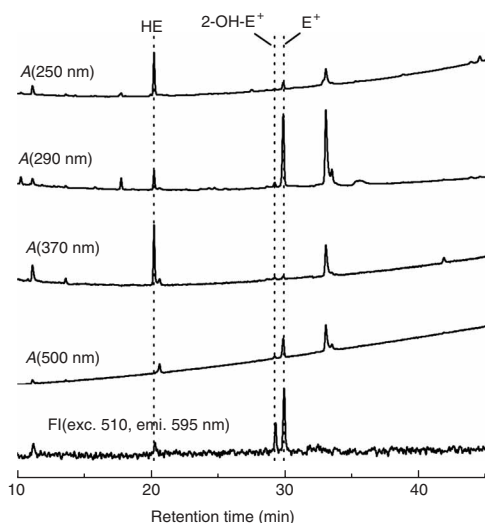


Figure 6 | HPLC of the extract from RAW 264.7 cells. Cells were treated with hydroethidine (HE; 10 μM) in DMEM containing 2% FBS for 20 min at 37 °C and processed as described in the PROCEDURE. An HPLC system with absorption and fluorescence detectors equipped with a Kromasil C₁₈ column was used (method A). 2-OH-E⁺, 2-hydroxyethidium.

- (v) Throughout the experiment, set the detector channels to the potentials 0, 200, 280, 365, 400, 450, 500 and 600 mV versus the palladium reference electrode.
- (vi) The quantification of 2-OH-E⁺ is performed by adding the areas of the peaks observed at 200, 280 and 365 mV. While some 2-OH-E⁺ will undergo oxidation at 200 mV, most of the compound should undergo oxidation at 280 mV and the remaining compound will be oxidized at 365 mV. Other potentials can be used for quantification of HE (0 and 200 mV), E⁺ (365–500 mV) and other products.
- (vii) Quantify the amount of 2-OH-E⁺ in the test sample in an analogous way as described in Step 53A(vi).

(C) HPLC with electrochemical detection (HPLC-EC) using an ether-linked phenyl column ● TIMING 60 min per sample

- (i) To obtain a better separation between 2-OH-E⁺ and E⁺ peaks than in method B, use an ether-linked phenyl column (250 mm × 4.6 mm) instead of a C₁₈ column⁸.
- (ii) The elution method uses the same two mobile phases as described in method B above. Separate HE, 2-OH-E⁺ and E⁺ using a gradient elution with ratios of A and B mobile phases changing from 3:2 to pure B phase over a period of 30 min using a flow rate 0.5 ml min⁻¹.

- (iii) Pump the pure mobile phase B through the column for the next 10 min (30–40 min after injection).
- (iv) Over the next 5 min (40–45 min after injection) restore the initial composition of the mobile phase then re-equilibrate the column for 15 min.
- (v) Calculate the peak areas in the same way as described in Step 53B(vi).
- (vi) Quantify the amount of 2-OH-E⁺ in the test sample in an analogous way as described in Step 53A(vi).

(D) HPLC with MS detection ● TIMING 90 min per sample

- (i) For the separation and detection of HE, 2-OH-E⁺ and E⁺, use an HPLC system with an MS detector (LC-MSD SL, see EQUIPMENT), an electrospray ionization source and a single quadrupole mass analyzer. Inject 20 μl of sample into the HPLC system with an ether-linked phenyl column (250 mm × 2.0 mm) equilibrated with 20% CH₃CN in water containing 0.1% (vol/vol) HCOOH.

▲ CRITICAL STEP As MS detectors are typically not compatible with phosphate buffer, the HPLC-MS system should be equipped with a valve allowing online buffer removal. Alternatively, cell pellets (obtained in Step 41) should be extracted directly with organic solvent (EtOH) and the supernatant diluted 1:1 with water before injection.

- (ii) Use a gradient elution method with two mobile phases: A—water containing 0.1% (vol/vol) HCOOH and B—99.9% acetonitrile, 0.1% (vol/vol) HCOOH. Separate HE and its oxidation products through a linear increase in B phase concentration from 20 to 100% in 60 min at a flow rate of 0.1 ml min⁻¹.
- (iii) Over the next 10 min (60–70 min after injection), pump the B mobile phase through the column.
- (iv) Decrease the concentration of B back to 20% over the next 5 min (70–75 min after injection).
- (v) Re-equilibrate the column by pumping the mobile phase containing 20% B through the column over the next 15 min.
- (vi) For the 2-OH-E⁺ quantification use the peak area detected at *m/z* = 330. Quantify the amount of 2-OH-E⁺ in the test sample in an analogous way as described in Step 53A(vi).

? TROUBLESHOOTING

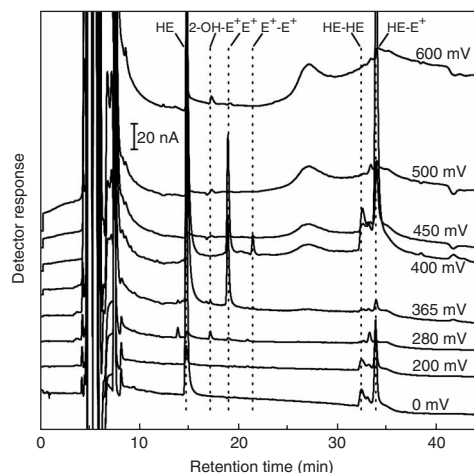


Figure 7 | HPLC-EC chromatogram of the extract from RAW 264.7 cells. Sample was prepared and processed as described in the **Figure 6** caption. The HPLC-EC system equipped with a Synergi Polar RP HPLC column was used (method C). 2-OH-E⁺, 2-hydroxyethidium; EC, electrochemistry; HE, hydroethidine.

● TIMING

Steps 1–9, preparation of the solutions of HE and standards of oxidation products: ~3 h; Steps 10–19: 30 min; Steps 20–25: 3 h; Step 26: ~6 h; Steps 27–35: 1 h; Step 36, detection of 2-OH-E⁺ in cells: 30 min; Steps 37–39, depending on the actual experiment, but typically <2 h; Steps 40 and 41: 15 min; Steps 42–44: 25 min; Steps 45 and 46: 15 min; Step 47: up to 2 h 35 min; Step 48: 45 min; Steps 49 and 50: 30 min; Steps 51 and 52: 15 min; Step 53, HPLC analysis: 75 min (method A), 60 min (methods B and C) or 90 min (method D) per sample. Include at least four more samples for running the blank and standards (HE, 2-OH-E⁺ and E⁺) samples.

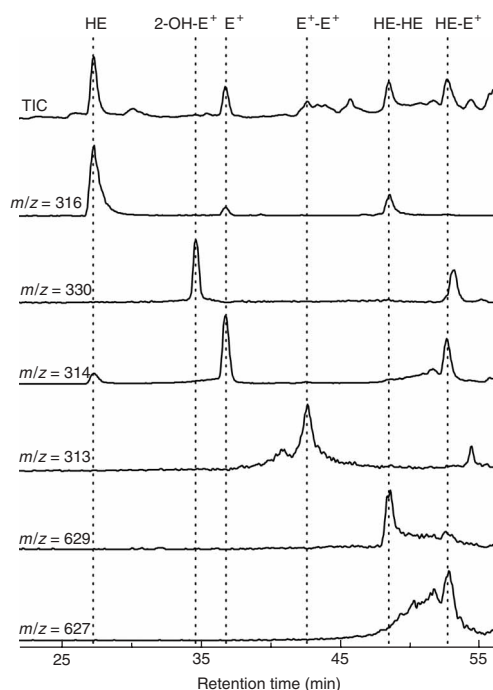


Figure 8 | HPLC-MS chromatogram of the cell extract from RAW 264.7 cells after incubation with HE as described in the **Figure 6** caption. The cell pellet was extracted with pure ethanol, centrifuged (15 min × 20,000*g*, 4 °C) and the supernatant mixed with water (1:1) before the injection on column. An HPLC-MS system equipped with a Synergi Polar RP HPLC column was used (method D). Total ionic current (TIC) represents the mass range *m/z* from 100 to 1,000. The calculated (and detected) *m/z* values are as follows: HE: M+H⁺: 316.2, M+2H⁺: 158.6; 2-OH-E⁺: M: 330.2; E⁺: M: 314.2; dimer E⁺-E⁺: M: 313.2; dimer HE-HE: M+H⁺: 629.3, M+2H⁺: 315.2, M+3H⁺: 210.5; dimer HE-E⁺: M: 627.3, M+H⁺: 314.2, M+2H⁺: 209.8 where M denotes the parent compound. 2-OH-E⁺, 2-hydroxyethidium; HE, hydroethidine; MS, mass spectrometry.

PROTOCOL

Note: As for the Step 53 the samples are placed in the refrigerated autosampler, there is no need for the person carrying out the analysis to be present during the whole time the HPLC analysis is being done. The HPLC sequence can be started at the beginning of the break after Step 46, so that at least one standard will be done before the end of cell samples preparation, giving the person a chance to control whether the HPLC system is working properly.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 5**.

TABLE 5 | Troubleshooting table.

Problem	Possible reason	Solution
The absorbance at < 300 nm is too high (> 2) when collecting the spectra of hydroethidine (HE) or standards	Plastic cuvettes used	Use quartz cuvettes with the 90% transmittance region starting at ≤ 200 nm
	The concentration of DMSO in the solution is too high	Use higher concentration of the compound in DMSO solution, so that a smaller volume can be added to the aqueous solution for the spectroscopic measurements
No HPLC peaks detected	No HE left in the stock solution (all compound underwent oxidation)	Check the stock solution of HE by HPLC (after dilution) and, if necessary, prepare a fresh stock solution
HE peak not detected	HE completely consumed during incubation	Use higher volume of medium and/or higher concentration of HE Shorten the time of incubation
	HE destroyed during sample processing	Minimize the exposure of the samples to light and make sure the samples' temperature is always ≤ 4 °C. Make sure that after lysis the samples are always in acidic conditions and contain at least 25% of organic solvent
Cells detach from plates' surfaces	HE is toxic to cells	Decrease HE concentration in the medium and/or shorten the time of incubation
Unknown peaks coeluted with HE or its oxidation products	The cell constituents or chemicals from the cell treatment elute at the same retention time as the compounds measured	Change the HPLC method (column type and/or gradient)
		Change the detection method (switch between fluorescence, electrochemical and mass spectrometry detection)
Results from different experiments carried out applying the same treatments to the same cell lines vary significantly	Different cell properties (number of passage, confluence)	Perform the HE incubation experiment using cells from the same batch grown to the same percentage of confluence
	Different time of samples' processing	Write down the time points for the samples processing individually for each sample. Make sure that the time of cells incubation and processing is the same for all samples

ANTICIPATED RESULTS

The typical chromatograms that are obtained by analysis of the extracts from unstimulated RAW 264.7 cells incubated for 20 min with HE (10 μ M in the medium) are shown in **Figure 6** (HPLC with absorption and fluorescence detection), **Figure 7** (HPLC with electrochemical detection) and **Figure 8** (HPLC with MS detection). Note that HPLC enables the detection of several products formed from HE reactions: HE alone, 2-OH-E⁺, E⁺ and other derivatives, some of them we attribute to HE radical dimerization products²⁴. As shown in **Figure 6**, at least under the chosen HPLC elution conditions, only 2-OH-E⁺ and E⁺ contribute significantly to the total fluorescence intensity. The calculation of the amount of 2-OH-E⁺ based on the HPLC peak area gives the value of 10 pmol of 2-OH-E⁺ per mg protein. From the same chromatogram the amount of E⁺ is calculated as

100 pmol of E⁺ per mg protein, clearly indicating that E⁺ should be considered as a significant contributor to the total fluorescence intensity observed in fluorescence microscopy and flow cytometry measurements in cells.

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