Antioxidants and Redox Signaling

DOI: 10.1089/ars.2018.7535

News & Views

Are hydroethidine-based probes reliable for ROS detection?

Yi Xiao[1, 2] and David Meierhofer[1]*

Author Affiliations

[1]Max Planck Institute for Molecular Genetics, Ihnestraße 63-73, 14195 Berlin, Germany

[2]Freie Universität Berlin, Fachbereich Biologie, Chemie, Pharmazie, Takustraße 3, 14195 Berlin, Germany

Running head: Targeted LC-MS/MS method for ROS determination

*Author to whom correspondence should be addressed: meierhof@molgen.mpg.de

Phone: +49-30-8413-1567; Fax: +49-30-8413-1960

Word count (excl. ref., figure legends and notes): 1,912

Number of references: 9

Number of color figures: 3

Number of greyscale figures: 2

Keywords: Reactive oxygen species, ROS; superoxide, $O^{\cdot}{}_2^-$; mass spectrometry; multiple reaction monitoring, MRM; hydroethidine, HE; mito-hydroethidine, mitoSOX red or mito-HE; hydropropidine, HPr^+.

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

Abstract

Detection and quantification of the highly reactive and short lived superoxide ($O^{\bullet}_2{}^-$) can be challenging. Here, we present a new mass spectrometry (MS)-based method to detect and quantify $O^{\bullet}_2{}^-$ using three fluorogenic hydroethidine probes: HE, mito-HE, and HPr^+, which measure cytosolic, mitochondrial and extracellular $O^{\bullet}_2{}^-$, respectively. The probes and their oxidation products were simultaneously quantified by applying multiple reaction monitoring (MRM) with mass spectrometry that allowed the specific measurement of ROS distribution within the cell. The advantage of this LC-MS/MS method is that co-eluting compounds can be precisely distinguished using specific precursor and fragment masses. This method overcomes limitations from spectral overlap of $O^{\bullet}_2{}^-$ specific and non-specific products in fluorescence spectra or the low specificity associated with chromatography-based approaches. However, our experiments showed that these hydroethidine probes can be prone to autoxidation during incubation at 37 °C in Hank's solution. Cell treatments with strong oxidants did not significantly increase levels of the $O^{\bullet}_2{}^-$ radical. Thus, subtle changes in ROS levels in cell culture experiments might not be quantifiable. Our findings raise the question of whether hydroethidine-based probes can be used for the reliable detection of $O^{\bullet}_2{}^-$ radicals in cell culture.

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

Introduction

Reactive oxygen species (ROS) are chemically reactive oxygen-containing molecules that include peroxides, superoxide ($O^{\cdot}_2^-$), and hydroxyl radicals. ROS can be either natural byproducts of normal endogenous oxygen metabolism or generated by exogenous sources, such as UV and radiation. ROS were long thought to be potentially damaging byproducts of cellular metabolism that can affect DNA, lipids, and proteins. However, more recent studies highlight the important role of ROS in cell signaling, homeostasis, cancer and apoptosis (4). Thus, the ability to assess ROS status is valuable and many different methods have been established that allow the direct or indirect measurement of redox states; the advantages and disadvantages of these methods were reviewed recently (1).

The most commonly used fluorogenic probes for $O^{\cdot}_2^-$ detection, and the current "gold standard", are hydroethidine (HE; or dihydroethidium) and mitochondrial-targeted hydroethidine (mito-HE, mitoSOX red) for intracellular and mitochondrial $O^{\cdot}_2^-$ detection, respectively (3,8). Moreover, membrane-impermeable hydropropidine (HPr^+) that can detect extracellular $O^{\cdot}_2^-$ was recently developed (6). These three structurally analogous fluorogenic probes react with $O^{\cdot}_2^-$ in a very similar way, wherein $O^{\cdot}_2^-$-specific hydroxylated products (2-OH-E^+, 2-OH-mito-E^{2+}, and 2-OH-Pr^{2+}) are formed as the main products (Figure 1). However, many other oxidants are present in cells, including redox metal ions, heme proteins, and one- or two-electron oxidants, which can give rise to fluorescent ethidium and analogs (E^+, mito-E^{2+}, and Pr^{2+}) as well as weakly or non-fluorescent dimeric products (di-E^+, di-mito-E^{2+}, and di-Pr^{2+}; Figure 1) (3). The use of fluorescence microscopy or other fluorescence-based methods with these probes should be avoided, considering that the fluorescent characteristics of $O^{\cdot}_2^-$-derived marker products and other non-specific oxidized fluorescent products that form from these probes can have spectral overlap (3).

The aim of this study was to develop a robust, rapid and quantitative LC-MS/MS-based method to measure $O^{\cdot}_2^-$ levels in cells. Furthermore, the use of extracellular, intracellular, and mitochondria-targeted fluorogenic probes can allow spatial resolution of the ROS source. Thus, mass fingerprints for HE, mito-HE, and HPr^+ and their oxidation products were generated to uniquely identify and quantify the compounds. We established and

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

optimized a single short LC-MS/MS method that involves applying multiple reaction monitoring (MRM) to simultaneously monitor all educts and their oxidation products in a cell system. The separation and determination of these probes and corresponding oxidation products was previously achieved by high performance liquid chromatography (HPLC)-based analyses (1,3). Our LC-MS/MS method is superior to methods that rely solely on HPLC, as the identity of every peak can be verified in the mass spectrum, and co-eluting peaks can be easily distinguished by mass differences in the unique fragment masses as part of a multiplexed single short LC-MS/MS run. DNA intercalations will not interfere with our method, as only metabolites and not DNA was extracted (3). By applying our LC-MS/MS method, we found that in Hank's solution all three probes: HE, mito-HE, and HPr^+, were prone to autoxidation, which resulted in an increase in the levels of $O^{\cdot}{}_2^-$-specific products. Thus, results obtained using HE, mito-HE, and HPr^+ as probes for $O^{\cdot}{}_2^-$ detection should be interpreted with care due to the high autoxidation rate of these molecules. The observed autoxidation in Hank's solution could compromise detection of endogenous $O^{\cdot}{}_2^-$ production, and could confound the actual $O^{\cdot}{}_2^-$ state in many experiments.

Results

Establishment of a MRM-based method to monitor HE, mito-HE, HPr^+, and their oxidation products.

To identify and quantify relative differences in the three fluorogenic probes and their oxidation products in cell culture systems, we developed a targeted LC-MS/MS approach, based on multiple reaction monitoring (MRM). The mass spectrometry parameters for specific transitions of all monitored compounds were individually identified, optimized and the three best transitions per metabolite were selected for the final method. The identity of fragments was verified by high resolution mass spectrometry (Figure 2). To increase the robustness of the LC-MS/MS method, we monitored the correct retention time of all peaks, the peak shapes, and the ion ratios. The ion ratios were calculated by dividing all transitions by the largest peak area per compound and the values must match the ratios calculated for the pure probes (2). All selected transitions, including internal standards, were thus combined in a single LC-MS/MS method operating in the positive ionization

mode (Figure 3A, Table 1). Transitions for the three fluorogenic compounds and their $O^{\cdot}_2^-$ specific products were additionally displayed individually (Figure 3B-G).

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

Fluorogenic probe stability is a significant concern

A mass spectrometry approach overcomes spectral overlap issues in fluorescence-based methods, as it relies on the exact masses of specific transitions. Given the natural tendency of the probes to undergo autoxidation, we first monitored probe stability. Several time series using different light and temperature conditions were performed to monitor the stability of educts and oxidation products in a cell free environment. Regardless of the conditions, rapid autoxidation rates were observed in all experiments, which translated to an increase in the levels of $O^{\cdot}_2^-$ -specific products (Figure 4). A 150% increase within 60 minutes was observed for the $O^{\cdot}_2^-$-specific products from all three probes under the "25 °C without light" condition at this time point (Figure 4A-C). Under standard laboratory light conditions, levels of the $O^{\cdot}_2^-$-specific products increased by over 200% and after 2 hours to more than 300% above initial values (Figure 4D-F). Light conditions had a larger impact on $O^{\cdot}_2^-$-specific products relative to that seen for temperature increases to 37 °C (Figure 4G-I). In contrast, the internal standard showed a standard deviation of 5.5 % within all experiments, indicating normal variations of the LC-MS/MS runs and in turn robust measurement conditions.

Effects of strong ROS stimulations in cells were smaller than those for autoxidation

HepG2 cells were treated with strong ROS inducers, such as rotenone and H_2O_2, as well as the ROS quencher N-acetyl-cysteine (NAC) (5,9), to identify maximum effects in a cell system. We asked whether or not these chemical treatments show stronger ROS signals than the autoxidation rates of the probes themselves.

Upon treatment with NAC, levels of all oxidation products did not change significantly, except for Pr^{2+}. Indeed, the fold-changes of these oxidation products were slightly reduced for HE and HPr^+ and unchanged for mito-HE (Figure 5A). After incubating the cells with H_2O_2, levels of $O^{\cdot}_2^-$-specific products were unchanged for mito-HE compared to controls and even reduced for HE and HPr^+ (Figure 5B). Rotenone treatment was associated with insignificant reductions in $O^{\cdot}_2^-$ specific products for HE and HPr^+, and only 2-OH-mito-E^{2+} showed a significant increase of 50%. This result was perhaps predictable, given that

mitochondria are sites of electron leakage (Figure 5C). All harsh treatments did not induce significant changes in the amounts of $O^{\cdot}{}_2^-$ specific products, most likely because the cell culture medium already induced a high oxidation rate of the three probes, which then diminished cell-specific changes.

Together these results indicate that the autoxidation effects of the three probes were high upon incubation at 25 °C or 37 °C, especially under standard room light conditions. This autoxidation can compete with experimentally-induced ROS changes to diminish or perhaps obscure the true ROS status in the presence of harsh treatments with H_2O_2, rotenone, or NAC.

Discussion

At present, fluorescence- and HPLC-based approaches are frequently used with hydroethidine-based probes such as HE, mito-HE or HPr^+ to assess the ROS status in cells (3,7,8), However, such probes have several drawbacks in fluorescence or HPLC applications, particularly because of spectral overlap in fluorescence assays and low specificity in HPLC methods. In this study, we developed the first LC-MS/MS method that can simultaneously detect and quantify these three fluorogenic probes and their corresponding oxidation products. This method resulted in a rapid, sensitive and specific LC-MS/MS method to elucidate the ROS status in a cell system with spatial resolution. Three specific transitions for each of the educts and oxidized products as well as the ion ratios between the transitions and the correct retention time were monitored to ensure high selectivity. This method eliminates problematic fluorescence spectral overlap that is typical of fluorescence-based techniques and the low specificity associated with the use of only HPLC-based methods. However, we found that these hydroethidine-based probes were intrinsically prone to oxidation. Incubation at 25 °C or 37 °C in our stability test led to rapid probe autoxidation, which could complicate the results obtained when these probes are used for ROS detection. Accordingly, the harsh conditions used for ROS induction and inhibition treatments generated results for the oxidized fluorophores that lacked significance and reproducibility. Hence, subtle changes in ROS levels that can occur in

biological systems and that can be used to achieve new insights into redox homeostasis may not be not be detectable.

In addition to the instability of hydroethidine-based probes, other inherent characteristics can impede ROS detection, including complex chemical reactions, intercalation with DNA, and interference with heme-containing enzymes (1). Heme proteins, such as hemoglobin and myoglobin, can react with HE and form fluorescent products. These chemical reactions can produce many other oxidation products that can compete with radical specific products and may influence the amount of detectable $O^{\bullet}_2{}^{-}$-specific products. In addition, formation of $O^{\bullet}_2{}^{-}$-specific products could be influenced by peroxidase reactions that can interfere with $O^{\bullet}_2{}^{-}$ quantification (1). Meanwhile, mito-HE can be translocated to other intracellular organelles that have a higher negative membrane potential (3).

Our targeted LC-MS/MS method allows the selective identification and quantification of the fluorogenic compounds HE, mito-HE, and HPr and their respective $O^{\bullet}_2{}^{-}$-specific products. However, we also detected a high autoxidation rate for the probes alone in Hank's solution. These findings suggest that, despite the many recent methodological improvements in ROS quantification, direct measurements of ROS levels based on hydroethidine probes may be in inaccurate due to artifactual effects of autoxidation. Experiments performed using these probes should thus be interpreted with caution, and autoxidation rates should be monitored.

Innovation

Current methods to determine ROS levels within cells have limitations such as spectral overlap and low specificity in fluorescence- and HPLC-based approaches, respectively. In this study we showed that three hydroethidine-based probes and their oxidized products can be measured precisely, quantitatively and simultaneously using specific precursor and fragment masses in a 10-min targeted LC-MS/MS method. However, our results also indicated that these fluorophores are prone to autoxidation during sample treatment. As such, results generated using these probes must include stability checks. Overall, our findings indicate that ROS determination using hydroethidine-based probes should be

done with care and take into consideration how autoxidation could affect measured ROS values.

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

Notes

Chemicals

Hydroethidine (HE, D1168) and mito-hydroethidine (mito-HE, M36008) were purchased from Invitrogen (Carlsbad, CA). Hydropropidine (HPr^+) was a kind gift from Prof. Jacek Zielonka, Medical College of Wisconsin. Potassium nitrosodisulfonate (NDS, 220930), chloranil (45374), potassium ferricyanide (455946), diethylenetriaminepentaacetic acid (DTPA, D6518), bromodeoxyuridine (B9285), uridine (U3003), N-acetyl-cysteine (NAC) (A9165), hydrogen peroxide (H1009), rotenone (R8805) and Triton X-100 (T9284) were all purchased from Sigma Aldrich (St. Louis, MO).

Synthesis of oxidation products derived from HE, mito-HE, and HPr^+

The synthesis of oxidation products was done as previously described (8). In brief, 2-OH-E^+ and 2-hydroxy-mito-ethidium (2-OH-mito-E^{2+}) were synthesized by oxidizing HE and mito-HE with potassium nitrosodisulfonate (NDS). E^+ and mito-ethidium (mito-E^{2+}) were synthesized by reacting HE and mito-HE with chloranil, diethidium (di-E^+) and di-mito-ethidium (di-mito-E^{2+}) and reacting HE and mito-HE with potassium ferricyanide. 2-hydroxypropidium (2-OH-Pr^{2+}), propidium (Pr^{2+}), and dipropidium (di-Pr^{2+}) were derived from HPr^+ and synthesized using a procedure similar to that used to synthesize corresponding products from HE (6).

Generation of MRM methods for fluorogenic probes and derived oxidation products and LC-MS/MS conditions

Educts and oxidation products of the three probes were tuned individually to identify and optimize specific transitions for the QTrap 6500 mass spectrometer. About 100 ng/µL of all compounds were dissolved in methanol and a constant flow of 7 µL/min was used. The automatic optimization procedure in the analyst software (v.1.6.2) was used. The ten most intense fragment masses per compound were optimized for collision energy, declustering potential, and the collision cell exit potential (Table 1).

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

Three different LC columns and several different buffer conditions were used to identify the highest peak areas, optimal peak shapes, and retention times, as described previously (2). Finally, a Reprosil-PUR C18-AQ (1.9 µm, 120 Å, 150 x 2 mm ID; Dr. Maisch; Ammerbuch, Germany) with the following buffer and run conditions was selected for metabolite separation: A1: LC-MS grade water; 0.1% formic acid; B1: LC-MS grade acetonitrile; 0.1% formic acid. Gradients and flow conditions were as follows: the compounds were separated by a linear increase of B1 from 20% to 95% in 8 min and maintained at 95% B1 for 1 min. The concentration of B1 was then reduced to 20% in 1 min, and this level was maintained until minute 10. All optimized MRM transitions and retention times are shown in Table 1.

Fragmentation patterns of the probes and $O^{\cdot}_2{}^{-}$-specific products were recorded by a Q Exactive HF (Thermo Fisher Scientific, Waltham, MA) mass spectrometer with a high resolution of 60,000. The fragment mass identity was verified using ACD Spectrus Processor 2017.2.1 software. Assigned fragment masses differed by less than 0.001 Da from theoretically calculated masses and assignments are shown in Figure 2.

Sample preparation for fluorogenic probe stability tests and metabolite extraction

The stability of all three fluorogenic probes was tested in Hank's solution (Thermo Fisher Scientific, Waltham, MA, 14025) to evaluate autoxidation rates under cell-free conditions. Each probe (2 µM) was incubated in 50 µL Hank's solution for 0, 10, 30, 60, and 150 min under the following three conditions in triplicate: 25°C with light, 25°C without light, and 37°C without light. 200 µL acetonitrile and 20 µL of 1 mM internal standard (chloramphenicol) were added, vortexed (800 rpm) for 5 min at room temperature and centrifuged at 8,000 g for 5 min at 4°C to extract the probes. 20 µL of each supernatant was analyzed by LC-MS/MS to evaluate the autoxidation rates.

Metabolite extraction and determination of ROS status in a cell line

For proof of principle, ROS signatures were induced or scavenged by chemical treatments to detect compartment-specific signals for HE, mito-HE, and HPr^+ using the established LC-MS/MS method. HepG2 cells were treated with two chemical ROS stimuli, H_2O_2 (100 µM) and rotenone (1 µM) for 2 hours, as well as with the ROS scavenger N-acetyl-cysteine

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

(NAC, 1 mM) for 20 hours. HepG2 cells were cultivated in DMEM (Dulbecco Modified Eagle Medium, Life Technologies, New York NY) containing 4.5 g/L glucose, supplemented with 10% fetal bovine serum (Silantes, Munich, Germany), 1% penicillin-streptomycin-neomycin (Invitrogen, Carlsbad, CA), at 37 °C in a humidified atmosphere of 5% CO_2. Cells were split into 6-well plates (9 cm^2) and grown to full confluency. After treatment, cells were incubated with either 2 µM HE, mito-HE, or HPr^+ for 30 min at 37 °C in biological triplicates.

Cells were washed with ice-cold phosphate-buffered saline twice and immediately lysed in 200 µL ice-cold phosphate-buffered saline containing 0.1% Triton X-100. The cell suspensions were transferred into 1.5-ml black tubes to avoid light exposure and placed on ice for further disruption by shear forces induced by 10 repeated injections through a 26G cannula. The homogenates were centrifuged for 5 min at 600 g at 4°C. Supernatants (50 µL) were mixed with 200 µL acetonitrile and 20 µL 1 mM internal standard (chloramphenicol) and placed on a shaker (800 rpm) to allow protein precipitation for 5 min at 4°C. The protein precipitate was pelleted by centrifugation at 20,000 g for 10 min at 4 °C, and the resulting supernatant was used for LC-MS/MS analysis.

LC-MS/MS instrument settings

Supernatant (20 µL) was injected and compounds were separated on a LC instrument (1290 series UHPLC; Agilent, Santa Clara, CA), coupled online to a triple quadrupole hybrid ion trap mass spectrometer QTrap 6500 (Sciex, Foster City, CA). All transitions and compound-specific settings are shown in Table 1. Data acquisition was performed with an ion spray voltage of 5.5 kV in the positive mode for the electro spray ionization source, N^2 as the collision gas was set to medium, the curtain gas was at 30 psi, the ion source gas 1 and 2 was at 50 and 70 psi, respectively, and the interface heater temperature was set to 350 °C. Mass spectrometry data have been deposited into the publicly accessible repository PeptideAtlas under the identifier PASS01157.

Data evaluation and statistics

Metabolite identification was based on three levels: (i) the correct retention time, (ii) three transitions, (iii) and matching MRM ion ratios of tuned pure metabolites as described

previously (2). Peak integration was performed using MultiQuant™ software v.2.1.1 (Sciex, Foster City, CA). All peaks were reviewed manually and adjusted if necessary. The peak area of the first transition per metabolite was used for subsequent calculations. An internal standard was used to normalize all LC-MS/MS runs for instrumental variations. HE, mito-HE, and HPr^+ were used to normalize respective oxidized derivatives for the total input in cell culture experiments. Two tailed unpaired t-tests were performed.

Acknowledgments

Hydropropidine was a kind gift from Prof. Jacek Zielonka, Medical College of Wisconsin. Our work was supported by the Max Planck Society and the China Scholarship Council to Y.X.

Authors Disclosure Statement

No competing financial interests exist.

Author Contribution

Y.X performed the experiments, analyzed the data and prepared the figures. D.M. conceived the study and wrote the paper.

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

References

1. Dikalov SI, Harrison DG. Methods for detection of mitochondrial and cellular reactive oxygen species. *Antioxid Redox Signal* 20: 372-82, 2014.

2. Gielisch I, Meierhofer D. Metabolome and proteome profiling of complex I deficiency induced by rotenone. *J Proteome Res* 14: 224-35, 2015.

3. Kalyanaraman B, Dranka BP, Hardy M, Michalski R, Zielonka J. HPLC-based monitoring of products formed from hydroethidine-based fluorogenic probes--the ultimate approach for intra- and extracellular superoxide detection. *Biochim Biophys Acta* 1840: 739-44, 2014.

4. Kim J, Kim J, Bae JS. ROS homeostasis and metabolism: a critical liaison for cancer therapy. *Exp Mol Med* 48: e269, 2016.

5. Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, Robinson JP. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem* 278: 8516-25, 2003.

6. Michalski R, Zielonka J, Hardy M, Joseph J, Kalyanaraman B. Hydropropidine: a novel, cell-impermeant fluorogenic probe for detecting extracellular superoxide. *Free Radic Biol Med* 54: 135-47, 2013.

7. Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, Murphy MP, Beckman JS. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. *Proc Natl Acad Sci U S A* 103: 15038-43, 2006.

8. Zielonka J, Hardy M, Kalyanaraman B. HPLC study of oxidation products of hydroethidine in chemical and biological systems: ramifications in superoxide measurements. *Free Radic Biol Med* 46: 329-38, 2009.

9. Zmijewski JW, Banerjee S, Bae H, Friggeri A, Lazarowski ER, Abraham E. Exposure to hydrogen peroxide induces oxidation and activation of AMP-activated protein kinase. *J Biol Chem* 285: 33154-64, 2010.

Abbreviations

2-OH-E^+ = 2-hydroxyethidium

2-OH-mito-E^{2+} = 2-hydroxy-mito-ethidium

2-OH-Pr^{2+} = 2-hydroxypropidium

Di-E^+ = diethidium

Di-mito-E^{2+} = di-mito-ethidium

Di-Pr^{2+} = dipropidium

DMEM = Dulbecco Modified Eagle Medium

DTPA = diethylenetriaminepentaacetic acid

E^+ = ethidium

H_2O_2 = hydrogen peroxide

HE = hydroethidine

HepG2 = human liver cancer cell line

HPLC = high performance liquid chromatography

HPr^+ = hydropropidine

LC-MS/MS = Liquid chromatography-tandem mass spectrometry

Mito-E^{2+} = mito-ethidium

Mito-HE = mito-hydroethidine

mitoSOX red = mito-hydroethidine

MRM = multiple reaction monitoring

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

MS = mass spectrometry

NAC = N-acetyl-cysteine

NDS = nitrosodisulfonate

$O^{\cdot}{}_2^-$ = superoxide

Pr^{2+} = propidium

ROS = reactive oxygen species

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

Tables

Table 1. MRM transitions and MS parameters of the fluorogenic probes.

Compound	Q1 mass (Da)	Q3 mass (Da)	RT (min)	DP (V)	CE (V)	CXP (V)	MRM ion ratio
HE	316.2	210.1	6.75	76	43	24	1
HE	316.2	287.1	6.75	76	29	28	0.71
HE	316.2	271.1	6.75	76	41	14	0.16
2-OH-E^+	330.2	301.1	4.95	101	37	8	1
2-OH-E^+	330.2	300.1	4.95	101	55	28	0.93
2-OH-E^+	330.2	255.1	4.95	101	69	12	0.84
E^+	314.1	284.1	5.15	1	51	24	1
E^+	314.1	286.1	5.15	1	39	16	0.80
E^+	314.1	285.1	5.15	1	37	18	0.69
di-E^+	313.2	285.1	5.22	141	35	26	1
di-E^+	313.2	284.1	5.22	141	41	26	0.77
di-E^+	313.2	299.1	5.22	141	31	10	0.56
mito-HE	316.7	278.1	7.49	1	19	32	1
mito-HE	316.7	209.1	7.49	1	43	22	0.28
mito-HE	316.7	183.1	7.49	1	77	16	0.14
2-OH-mito-E^{2+}	323.7	300.1	5.53	111	41	14	1

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

2-OH-mito-E^{2+}	323.7	262.1	5.53	111	33	24	0.72
2-OH-mito-E^{2+}	323.7	289.1	5.53	111	37	8	0.54
mito-E^{2+}	316.1	285.1	5.64	96	31	18	1
mito-E^{2+}	316.1	262.1	5.64	96	31	18	0.80
mito-E^{2+}	316.1	289.1	5.64	96	37	14	0.61
di-mito-E^{2+}	316.1	285.1	5.63	111	31	26	1
di-mito-E^{2+}	316.1	262.1	5.63	111	33	22	0.84
di-mito-E^{2+}	316.1	345.1	5.63	111	31	26	0.72
HPr^{+}	415.3	272.1	4.53	60	49	16	1
HPr^{+}	415.3	328.1	4.53	60	25	10	0.25
HPr^{+}	415.3	300.1	4.53	60	31	20	0.80
2-OH-Pr^{2+}	215.1	266.1	4.42	60	25	24	1.00
2-OH-Pr^{2+}	215.1	300.1	4.42	60	23	26	0.75
2-OH-Pr^{2+}	215.1	72.0	4.42	60	25	10	0.63
Pr^{2+}	207.2	72.0	4.37	60	23	8	1
Pr^{2+}	207.2	250.0	4.37	60	25	24	0.64
Pr^{2+}	207.2	163.5	4.37	60	21	16	0.40
di-Pr^{2+}	413.1	326.1	4.35	111	21	26	1
di-Pr^{2+}	413.1	331.1	4.35	111	21	18	0.02
di-Pr^{2+}	413.1	213.1	4.35	111	37	20	0.00
IS	323.1	275.1	5.00	50	20	14	1.00

IS	323.1	165.0	5.00	50	37	11		0.52
IS	323.1	83.0	5.00	60	95	9		0.23

***HE**, hydroethidine; **2-OH-E^{+}**, 2-hydroxyethidium; **E^{+}**, ethidium; **di-E^{+}**, diethidium; **mito-HE**, mito-hydroethidine; **2-OH-mito-E^{2+}**, 2-hydroxy-mito-ethidium; **mito-E^{2+}**, mito-ethidium; **di-mito-E^{2+}**, di-mito-ethidium; **HPr^{+}**, hydropropidine; **2-OH-Pr^{2+}**, 2-hydroxypropidium; **Pr^{2+}**, propidium; **di-Pr^{2+}**, dipropidium; **IS**, internal standard chloramphenicol; **RT**, retention time; **DP**, declustering potential; **CE**, collision energy; **CXP**, collision cell exit potential; MRM ion ratio, peak areas of all transitions were divided by the highest peak area per compound.*

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Figure Legends

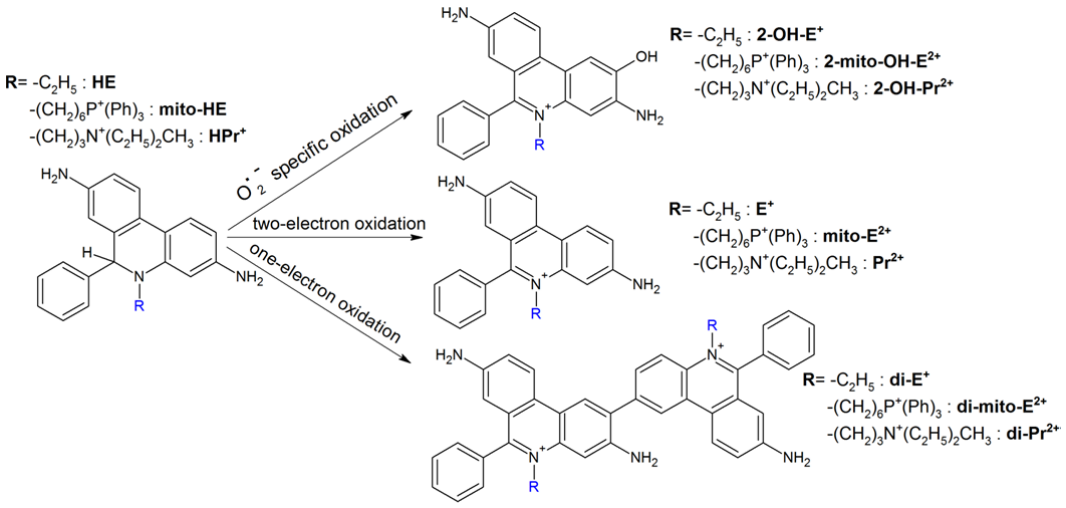

Figure 1. Products formed from HE, mitoHE, and HPr. Adapted from Kalyanaraman et al., Arch Biochem Biophys, 2014.

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

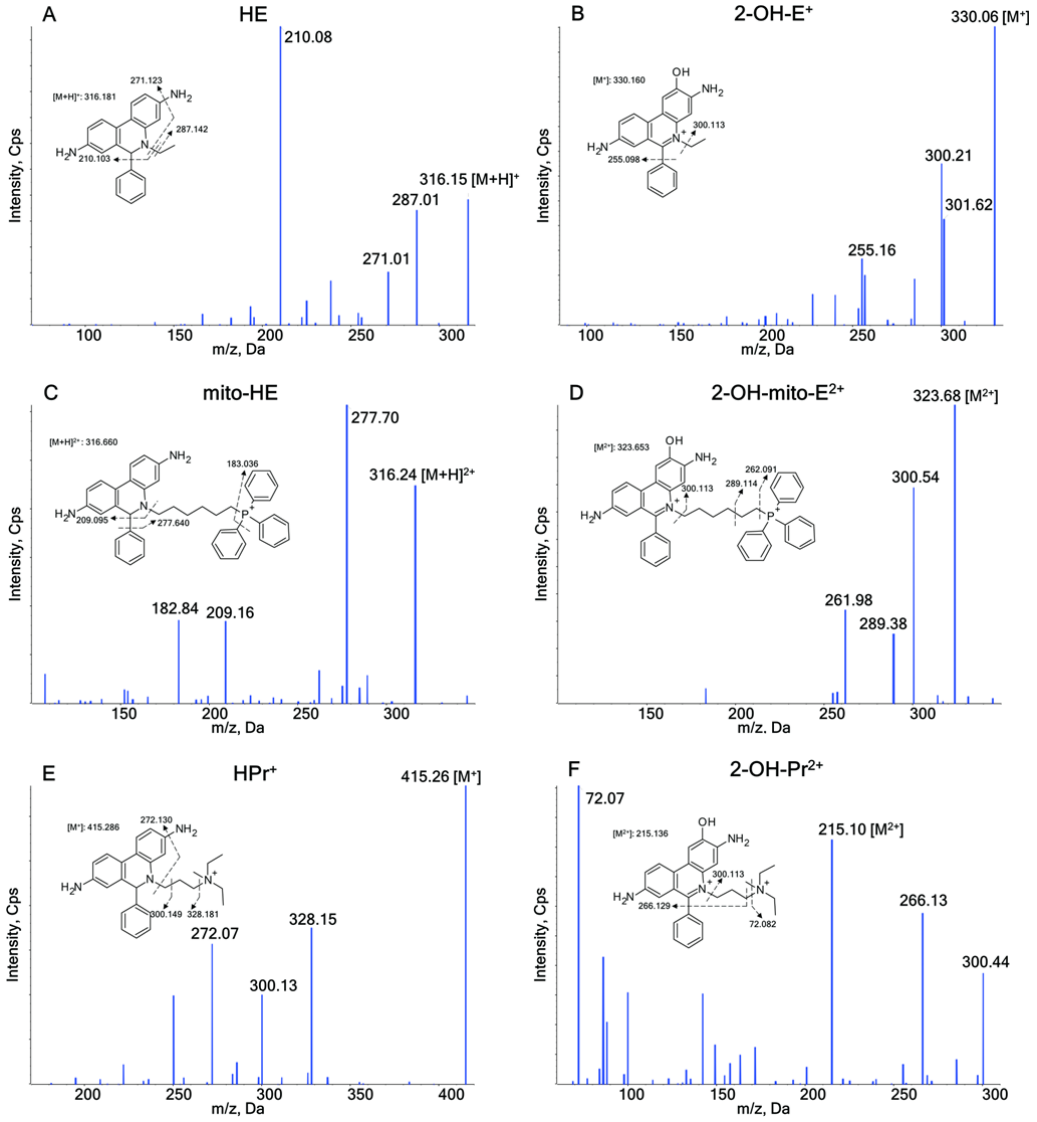

Figure 2. Specific fragment spectra (MS^2) obtained for (A) HE, (B) its $O^{\cdot}{}_2^-$ specific oxidized product 2-OH-E^+, (C) mito-HE and (D) 2-OH-mito-E^{2+}, (E) HPr^+ and (F) 2-OH-Pr^{2+}. Only the three indicated transitions per compound were included in the final MRM method that featured high intensities, unique transitions, and no matrix effects. Precursor ions are indicated by [M+H]. Structures and predicted fragments for all probes are shown. Cps, counts per second. **HE**, hydroethidine; **2-OH-E^+**, 2-hydroxyethidium; **mito-HE**, mito-hydroethidine; **2-OH-mito-E^{2+}**, 2-hydroxy-mito-ethidium; HPr^+, hydropropidine; **2-OH-Pr^{2+}**, 2-hydroxypropidium.

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

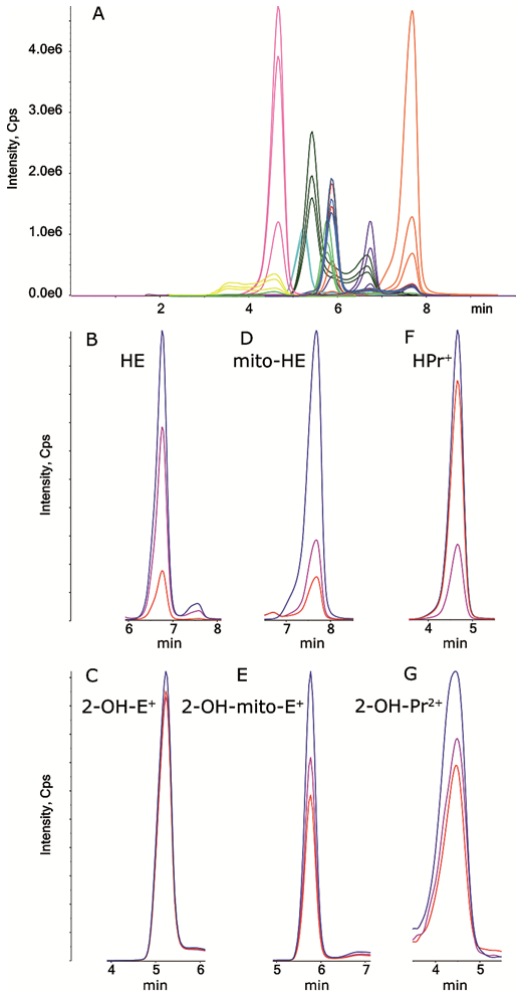

Figure 3. MRM transitions of three fluorogenic probes and their oxidation products, separated on a reverse phase C18 column. (A) All transitions were monitored in a single 10 minute LC-MS/MS run; transitions of the most important compounds are displayed individually as: (B) HE, (C) 2-OH-E^+, (D) mito-HE, (E) 2-OH-mito-E^{2+}, (F) HPr^+ and (G) 2-OH-HPr^{2+}. **HE**, hydroethidine; **2-OH-E^+**, 2-hydroxyethidium; **mito-HE**, mito-hydroethidine; **2-OH-mito-E^{2+}**, 2-hydroxy-mito-ethidium; HPr^+, hydropropidine; **2-OH-Pr^{2+}**, 2-hydroxypropidium.

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)

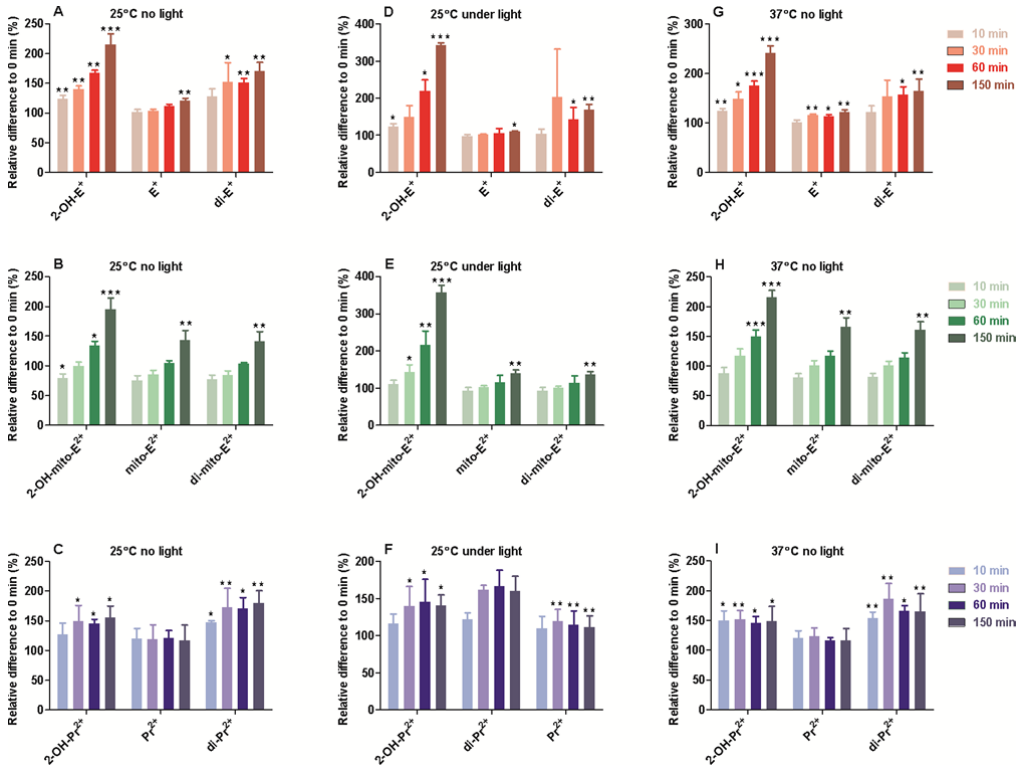

Figure 4. Stability tests for fluorogenic probes. Probes were incubated in Hank's solution under (**A, B, C**) 25 °C without light, (**D,E, F**) 25 °C with light, and (**G,H,I**) 37 °C without light. **2-OH-E^{+}**, 2-hydroxyethidium; **E^{+}**, ethidium; **di-E^{+}**, diethidium; **2-OH-mito-E^{2+}**, 2-hydroxy-mito-ethidium; **mito-E^{2+}**, mito-ethidium; **di-mito-E^{2+}**, di-mito-ethidium; **2-OH-Pr^{2+}**, 2-hydroxypropidium; **Pr^{2+}**, propidium; **di-Pr^{2+}**, dipropidium. Results are based on three biological experiments (*p < 0.05; **p < 0.01; ***p < 0.001; two tailed unpaired t-test with 0 min group). Error bars, mean ± S.D.

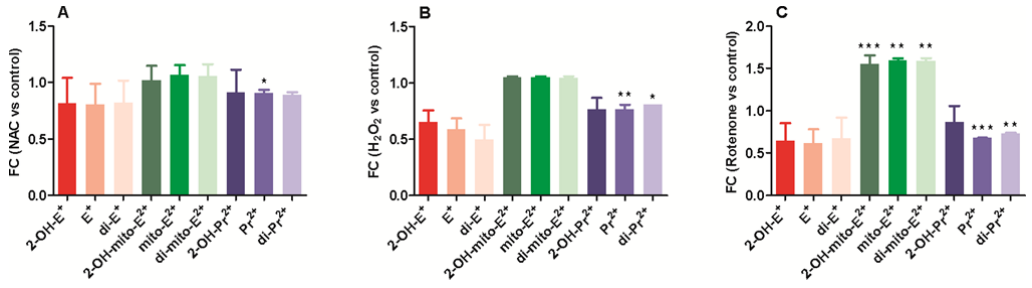

Figure 5. ROS determination of fluorogenic probes after chemical stimulation with (**A**) NAC, (**B**) H_2O_2, and (**C**) rotenone in HepG2 cells. **2-OH-E$^+$**, 2-hydroxyethidium; **E$^+$**, ethidium; **di-E$^+$**, diethidium; **2-OH-mito-E^{2+}**, 2-hydroxy-mito-ethidium; **mito-E^{2+}**, mito-ethidium; **di-mito-E^{2+}**, di-mito-ethidium; **2-OH-Pr^{2+}**, 2-hydroxypropidium; **Pr^{2+}**, propidium; **di-Pr^{2+}**, dipropidium; N-acetyl-cysteine (NAC); FC, fold change. Results are based on three biological experiments (*p < 0.05; **p < 0.01; ***p < 0.001; two tailed unpaired t-test with control group). Error bars, mean ± S.D.

Antioxidants and Redox Signaling
Are hydroethidine-based probes reliable for ROS detection? (DOI: 10.1089/ars.2018.7535)