Selective fluorescent imaging of superoxide in vivo using ethidium-based probes

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The putative oxidation of hydroethidine (HE) has become a widely used fluorescent assay for the detection of superoxide in cultured cells. By covalently joining HE to a hexyl triphenylphosphonium cation (Mito-HE), the HE moiety can be targeted to mitochondria. However, the specificity of HE and Mito-HE for superoxide in vivo is limited by autooxidation as well as by nonsuperoxide-dependent cellular processes that can oxidize HE probes to ethidium (Etd). Recently, superoxide was shown to react with HE to generate 2-hydroxyethidium [Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vasquez-Vivar, J. & Kalyanaraman, B. (2003) Free Radic. Biol. Med. 34, 1359–1368]. However, 2-hydroxyethidium is difficult to distinguish from Etd by conventional fluorescence techniques exciting at 510 nm. While investigating the oxidation of Mito-HE by superoxide, we found that the superoxide product of both HE and Mito-HE could be selectively excited at 396 nm with minimal interference from other nonspecific oxidation products. The oxidation of Mito-HE monitored at 396 nm by antimycin-stimulated mitochondria was 30% slower than at 510 nm, indicating that superoxide production may be overestimated at 510 nm by even a traditional superoxide-stimulating mitochondrial inhibitor. The rate-limiting step for oxidation of superoxide was $4 \times 10^6$ M$^{-1}$s$^{-1}$, which is proposed to involve the formation of a radical from Mito-HE. The rapid reaction with a second superoxide anion through radical–radical coupling may explain how Mito-HE and HE can compete for superoxide in vivo with intracellular superoxide dismutases. Monitoring oxidation at both 396 and 510 nm of excitation wavelengths can facilitate the more selective detection of superoxide in vivo.

Oxidative stress resulting from mitochondrial dysfunction has been implicated in neurodegeneration, aging, cancer, and diabetes (1, 2). Oxidative damage to mitochondrial electron transport complexes and DNA can lead to mitochondrial DNA mutations, aberrant electron transport, disruption of calcium homeostasis, and activation of apoptosis (3). Mitochondria consume ~85–95% of the oxygen inspired during respiration (1), most of which is reduced to water, but a small portion (estimates range from <0.1% to as high as 4%) of electrons leak from the respiratory chain to reduce oxygen to superoxide (O$_2^-$) (4, 5). Complexes I and III are major sites of O$_2^-$ formation (3, 4). Both membrane potential and reduction state of respiratory chain carriers affect O$_2^-$ production (3).

Assessing the generation of O$_2^-$ in mitochondria is confounded by the lack of a sensitive and specific assay (6). Some O$_2^-$ sensors actually generate O$_2^-$ by either uncoupling the respiratory chain (e.g., luminol) or reacting with oxygen (e.g., luminol and nitroblue tetrazolium; refs. 7 and 8). Many O$_2^-$ sensors react with intracellular oxidoreductases and can artificially generate a “superoxide” signal [e.g., hydroethidine (HE), cytochrome $c$, nitroblue tetrazolium, epinephrine, lucigenin, and luminol; ref. 8]. To circumvent these technical problems, we report a technique to detect O$_2^-$ using HE and the recently developed probe, MitoSOX red, a mitochondrial superoxide indicator (Invitrogen, Mito-HE). Mito-HE is comprised of HE, a commonly used probe for O$_2^-$, linked by a hexyl carbon chain to a triphenylphosphonium (TPP⁺) group. TPP⁺ cations target molecules to mitochondria, because the positive charge on the phosphonium is surrounded by three lipophilic phenyl groups, which facilitates movement across phospholipid bilayers and accumulation into the mitochondrial matrix in response to the negative membrane potential (9).

HE is the two-electron reduced form of ethidium (Etd⁺; 3,8-diamo-no-5-ethyl-6-phenylanthranilide). In 1984, Gallop et al. (10) reported that HE was readily taken up and internalized by live cells where HE can be oxidized to Etd⁺, which intercalates into nucleic acid, greatly enhancing its fluorescence when using 535-nm excitation and 610-nm emission wavelengths (11). Oxidation to Etd⁺ was originally attributed to the metabolic state of the cell and the cell’s ability to dehydrogenate HE (11). However, in 1990, Rothe and Valet (12) showed in vitro that HE was oxidized by potassium superoxide to a red fluorescent product. HE has since been widely used to detect reactive oxygen species during the phagocytic respiratory burst (12, 13) and for the detection of intracellular O$_2^-$ (6, 14). However, Rothe and Valet also showed that HE was oxidized not only by O$_2^-$ but also by H$_2$O$_2$ plus peroxidase (12). HE may also be oxidized by other intracellular processes, involving oxidases or cytochromes, to yield Etd⁺ (11, 15–18). Consequently, increased Etd⁺ fluorescence is not necessarily proof of O$_2^-$ production. Swannell et al. (19) proposed HE could be oxidized to more than one red fluorescent product. In 2003, Zhao et al. (20) reported that HE is oxidized by O$_2^-$ to yield a hydroxylated product (HO-Etd⁺). The initial oxidation of HE had been proposed to involve the formation of a radical (15), implying the oxidation of HE by O$_2^-$ involves a two-step mechanism (Scheme 1).

HO-Etd⁺ can be separated from Etd⁺ by HPLC, providing a specific O$_2^-$ assay (20). However, detection of HO-Etd⁺ by fluorescence microscopy is confounded, because its emission spectrum strongly overlaps the emission of Etd⁺ (20, 21).

We investigated the oxidation of Mito-HE by O$_2^-$ and found Mito-HE was oxidized by O$_2^-$ in a manner similar to HE (Scheme 1). During the course of these investigations, we found...

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that the $O_2^-$-derived product of Mito-HE has a distinct excitation wavelength at 396 nm that was not present for other oxidation products. HO-Etd$^+$ was also selectively excited at this wavelength. The ability to separate the fluorescence of the $O_2^-$-derived product from other less-specific oxidation products can improve the detection and imaging of intracellular $O_2^-$ production.

Results

The Selective Oxidation of Mito-HE. Superoxide generated by xanthine oxidase oxidized Mito-HE to give two closely eluting peaks by reverse-phase HPLC (Fig. 8, which is published as supporting information on the PNAS web site). The later peak was determined to be the two-electron-oxidized form of Mito-HE (3,8-diamino-5-hexyltriphenylphosphonium-6-phenylphenanthridinium, Mito-Etd$^+$) by coelution with standards and mass spectrometry. The first peak contained one additional oxygen and was identified as the hydroxylated product, HO-Mito-Etd$^+$. Oxidation of Mito-HE by hydrogen peroxide, peroxynitrite, hypochlorous acid, or a hydroxyl radical-generating system generated only a small percent of the fluorescence obtained from O$_2^-$ (Table 1, which is published as supporting information on the PNAS web site). The fluorescence emission maximum for HO-Mito-Etd$^+$ is blue-shifted ~20 nm relative to Mito-Etd$^+$ (Fig. 1a), similar to what was reported for the HE superoxide product, HO-Etd$^+$ (20). Interestingly, the excitation spectrum of purified HO-Mito-Etd$^+$ revealed an excitation maximum at 396 nm that was not present for Mito-Etd$^+$ (Fig. 1b). Excitation at 396 nm (Fig. 1c) enhanced the fluorescence emission of HO-Mito-Etd$^+$ by 70% and reduced the spectral overlap of Mito-Etd$^+$ from 40% to 10%. The excitation spectrum of HO-Etd$^+$ also had a distinct excitation at 396 nm that was not present for Etd$^+-$(Fig. 9, which is published as supporting information on the PNAS web site). Therefore, excitation at 396 nm could serve as a more selective means for detecting $O_2^-$ using either HE or Mito-HE.

Detection of Mitochondrial Superoxide. Mito-HE accumulated in isolated mitochondria in a manner that depended on mitochondrial membrane potential, as well as by adsorption to membranes. An ion-selective electrode was used to measure the concentration of Mito-HE in a mitochondrial suspension. Mito-HE (five sequential additions of 1 μM; arrowheads) was added to energized mitochondria, and the mitochondrial uptake of Mito-HE was measured as a decrease in free Mito-HE. The uncoupler trifluoromethoxyphenylhydrazone (0.5 μM) was added to induce mitochondrial uncoupling and Mito-HE release (measured as an increase in free Mito-HE). In a, Mito-HE was added to the chamber before the mitochondria, enabling an estimation of the extent of nonpotential dependent mitochondrial association. In b, mitochondria were present in the chamber before Mito-HE addition.

![Fig. 2](image-url). Mitochondria were taken up by isolated mitochondria in a manner that depended on mitochondrial membrane potential, as well as by adsorption to membranes. An ion-selective electrode was used to measure the concentration of Mito-HE in a mitochondrial suspension. Mito-HE (five sequential additions of 1 μM; arrowheads) was added to energized mitochondria, and the mitochondrial uptake of Mito-HE was measured as a decrease in free Mito-HE. The uncoupler trifluoromethoxyphenylhydrazone (0.5 μM) was added to induce mitochondrial uncoupling and Mito-HE release (measured as an increase in free Mito-HE). In a, Mito-HE was added to the chamber before the mitochondria, enabling an estimation of the extent of nonpotential dependent mitochondrial association. In b, mitochondria were present in the chamber before Mito-HE addition.
The fluorescence emission of isolated mitochondria incubated with Mito-HE. Each trace was evenly plotted above the previous one for clarity, and the rate of Mito-HE oxidation was calculated from the approximately linear slope of the first 60 sec. (a) Mito-HE (0.8 μM) incubated without mitochondria did not show an increase in fluorescence at 405 nm, which is generally greater than at 514 nm. Instrument parameters were set and held constant to minimize fluorescence from cultured oligodendrocytes incubated with Mito-HE using the two different excitation wavelengths, 405 (blue) and 514 (red). Fluorescence from oligodendrocytes incubated with 15 μM antimycin was oxidized by xanthine oxidase, the fluorescence at 396 nm being the initial oxidation of Mito-HE to a radical.

The two excitation wavelengths can be adapted to more selectively image O2− in living cells. Standard lasers available on confocal microscopes can excite fluorophores at 405 and 514 nm, which are close enough to use in place of 396 and 510 nm. The fluorescence of cultured oligodendrocytes incubated with Mito-HE was more intense using 405-nm excitation yet qualitatively similar to 514-nm excitation (Fig. 4 a–d). Fluorescence colocalized with mitochondria, as visualized with MitoTracker Red (Invitrogen; data not shown). Antimycin treatment increased fluorescence at both excitation wavelengths, supporting the proposal of the carbonyl structure, O=Mito-Etd+.

The rate-limiting step in the competition for O2− was calculated to be 3.9 ± 0.3 × 10^−1 s−1 and was independent of whether fluorescence was measured at excitation/emission wavelength pairs of 396/580 nm (selective for HO-Mito-Etd+), 300/598 nm (selective for Mito-Etd+), or 510/580 nm (which excites both products). This method can be adapted for widefield microscopy as well using a custom filter set (Fig. 10, which is published as supporting information on the PNAS web site).

The doubly charged ion at m/z=646.0 lead to the proposal of the structure of HO-Mito-Etd+. Mass spectrometry of an O2−-oxidized sample of Mito-HE revealed ions at m/z = 316.0 and 630.5 (Fig. 5), which were identified as Mito-Etd+. The doubly charged ion at m/z = 324.0 corresponded to the molecular weight of the hypothesized structure of HO-Mito-Etd+. The singly charged ion at m/z=646.0 lead to the proposal of the structure of HO-Mito-Etd+.
fragmentation of the parent ion at m/z 646.4 supported the proposed structure of O = Mito-Etd⁺ (Fig. 13, which is published as supporting information on the PNAS website).

To determine the extent to which this deprotonation may occur in vivo, we determined the pKₐ of the HO-Mito-Etd⁺ hydroxyl group by measuring fluorescence using 396/579 nm (λₑValueChanged/λₑValue)/λₑValueChanged over a pH range from 1 to 12. The data were fit by using the Henderson–Hasselbalch equation, which revealed two pKₐ values at 2.1 and 10.5, and a third pKₐ was determined to be 0.65 by plotting the fluorescence emission at 795 nm with λₑValueChanged = 396 nm (not shown). Fluorescence of HO-Mito-Etd⁺ was maximal and independent of pH over the physiological range.

**Discussion**

Measurement of intracellular O₂⁻ generation has been hampered by the lack of an assay sensitive enough to compete with endogenous SOD and yet selective for O₂⁻. Zhao et al. (20) have shown that the specificity for detecting O₂⁻ with HE can be greatly improved if the hydroxylated product is distinguished from Etd⁺ by HPLC. However, the overlap in the emission spectra makes separating the two products difficult by fluorescence microscopy (20, 21). In the present study, we have uncovered a simple means to verify the selectivity of O₂⁻ detection with either HE or Mito-HE by comparing fluorescence at two different excitation wavelengths. Excitation at 396 nm allows more selective imaging of the hydroxylated products produced by O₂⁻ and has greater sensitivity than the current practice of 510 nm excitation (Fig. 1). HO-Mito-Etd⁺ was detected by using 396-nm excitation in both isolated mitochondria and cultured cells, and fluorescence was increased with agents that increase O₂⁻ formation, such as antimycin. However, the detection of fluorescence generation from antimycin-stimulated mitochondria was 31% greater at 510- vs. 396-nm excitation, suggesting that Mito-Etd⁺ was being formed by nonsuperoxide-dependent pathways, and O₂⁻ formation was overestimated by using 510-nm excitation. Thus, monitoring fluorescence using both excitation wavelengths can improve selectivity for distinguishing O₂⁻ from other cellular oxidative species.

In 1934, Pauling and Neuman proposed the name superoxide based on the peculiar chemical bonding of KO₂ (23, 24). However, superoxide more generally behaves as a mild reductant under physiological conditions rather than a “super”-oxidizing agent. The initial oxidation of the phenanthridine moiety by O₂⁻ is recognized to generate a radical intermediate (14, 19, 20, 25) and is among the more rapid reactions of O₂⁻ with organic molecules; the rate constant for HE was estimated here to be 2 × 10⁸ M⁻¹s⁻¹, and the oxidation of Mito-HE was twice as fast. This rate is ∼500 times slower than the rate of O₂⁻ scavenging by SOD. However, the second reaction of the Mito-HE radical with O₂⁻ would involve radical–radical coupling to produce a hydroperoxide intermediate and should approach the diffusion limit (>10⁹ M⁻¹s⁻¹). Hence, the Mito-HE radical could efficiently compete for O₂⁻ with SOD in vivo to produce HO-Mito-Etd⁺. The hydroperoxide intermediate can spontaneously rearrange to lose a water molecule, yielding the hydroxylated product (Fig. 7). Hydroxylation appears to be relatively specific for O₂⁻, because other common biological oxidants generated only a small percent of the fluorescent signal generated by O₂⁻ (Table 1). Although the hydroxyl radical in theory could add to the radical to give a hydroxylated product, the hydroxyl radical is a promiscuous oxidant that is far more likely to react with the multitude of other organic molecules in a cell (26).

Most fluorescent probes used to image oxidants in cells, including reduced forms of fluorescein, rhodamine, and Etd⁺, are susceptible to autooxidation through radical intermediates when illuminated (19). These probes can also be oxidized by a variety of intracellular peroxidases, oxidases, or cytochromes to yield radical intermediates that dismutate to give fluorescent products (12, 16–18). Therefore, the detection of the two-electron fluorescent product gives a rather nonspecific...
assay of oxidative stress yet is commonly used as a superoxide indicator. Paradoxically, oxidation of HE by these alternative oxidative mechanisms could enhance $O_2^-$ detection by producing more HE radical, which increases the probability of trapping $O_2^-$ to form HO-Etd$^+$. The detection of HO-Etd$^+$ is generally a semiquantitative assay for intracellular $O_2^-$, because the relative fraction of $O_2^-$ reacting with SOD will be unknown. A second confounding variable can be the endogenous production of nitric oxide competing for $O_2^-$, which may need to be inhibited by nitric oxide synthase inhibitors for $O_2^-$ to be detectable. On the other hand, we have found that the addition of an exogenous nitric oxide donor can inhibit the oxidation of Mito-HE (not shown) and can be used as a control of selectivity for $O_2^-$.

The accumulation of Mito-HE depends upon the mitochondrial membrane potential with an ~10-fold increase in uptake for every 60-mV increase in membrane potential (9). The mitochondrial membrane potential is typically in the range of −140 to −170 mV, which could concentrate Mito-HE in the mitochondria up to 1,000-fold relative to the medium. Such high intramitochondrial concentrations should allow Mito-HE to compete with manganese SOD for $O_2^-$. However, excessive Mito-HE accumulation may stress mitochondria and enhance their sensitivity to inhibitors or other mitochondrial targeted molecules. Potentiometric probes may be added at the end of the incubation with Mito-HE to assess general mitochondrial integrity.

Careful optimization of conditions is necessary when using Etd$^+$-based probes, and several caveats must be considered. Mitochondrial depolarization may reduce the efficiency of $O_2^-$ detection due to the decreased uptake of Mito-HE. Mito-HE itself, even at low (1- to 10-μM) concentrations, may disrupt membrane potential or increase mitochondrial permeability. We have observed a rapid loss of fluorescence from mitochondria and subsequent redistribution to the nucleus after incubation with as little as 2 μM Mito-HE. The optimal concentration of Mito-HE should be determined empirically in each cell type and varied in our experience from 0.1 to 2.5 μM. Because DNA is necessary to enhance the fluorescence of HO-Mito-Etd$^+$, fluorescence might be limited by the amount of mitochondrial DNA, particularly in rho-naught cells or other pathological conditions where mitochondrial DNA has been deleted. Fluorescence intensity could artificially increase because of photooxidation. Photooxidation may be minimized by using the lowest possible concentration of Mito-HE and reducing the exposure to light throughout an experiment. Autofluorescence of cells will also be higher using 405-nm excitation and therefore should be controlled in microscopy experiments.

Because HE and Mito-HE accumulate to different extents within cells depending upon membrane potential, differences in fluorescence intensity between the two probes may not be directly compared to assess $O_2^-$ production between the two compartments. Higher concentrations of HE may be needed to achieve cytosolic concentrations similar to those of Mito-HE in mitochondria. Also, it seems possible that HE could be oxidized by mitochondrially generated $O_2^-$. Selective inhibitors would be needed to uncover the source of $O_2^-$. Exact quantitation of products using fluorescence microscopy is fraught with difficulties, and therefore HPLC methods (20, 25) quantifying the relative amount of hydroxylated and nonhydroxylated products should be used in conjunction with fluorescence experiments when possible. If intracellular oxidation of Mito-HE to Mito-Etd$^+$ should become 10- to 20-fold greater than HO-Mito-Etd$^+$, a majority of fluorescence from 396-nm excitation could be due to Mito-Etd$^+$. However, monitoring the oxidation at both 396 and 510 nm will reveal this potential artifact.

In conclusion, with judicious choice of conditions and an appreciation for the limitations of these probes, we have shown that the oxidation of HE to HO-Etd$^+$ and Mito-HE to HO-Mito-Etd$^+$ can be a sensitive indicator to monitor dynamic changes of endogenous $O_2^-$ generation.

**Materials and Methods**

MitoSOX Red mitochondrial superoxide indicator (Mito-HE), Mito-Etd$^+$ HE, and ethidium were obtained from Invitrogen–Molecular Probes. The purity of Mito-HE was ascertained by HPLC (as described below), because Mito-Etd$^+$ can be a contaminant in Mito-HE. If Mito-Etd$^+$ contamination was ≥10%, Mito-HE was either purified by HPLC or reduced by an equimolar amount of NaBH$_4$ (50 nmol in ethanol), with the reaction quenched by the addition of 120 nmol HCl. Recombinant human Cu, Zn SOD was expressed in Escherichia coli (27). Xanthine oxidase activity was assayed by the reduction of cytochrome c ($\Delta A_{550} = 21 \text{ mM}^{-1}\text{cm}^{-1}$) (28). Mito-HE was dissolved in DMSO to 5 mM and stored in the dark at −20°C for a maximum of 3 days. Mito-HE and all ethidium derivatives are carcinogenic and should be detoxified by reacting solutions diluted to ≤0.5 mg/ml ethidium with 0.2 volume of 5% hypophosphorous acid and 0.12 volume of fresh 0.5 M sodium nitrite, mixing carefully and venting the nitrogen gas evolved. DMSO penetrates gloves and skin and should also be used with caution.

Mito-HE and its oxidation products were separated by using C$_{18}$ reverse-phase HPLC (Supelco [Bellefonte, PA] column, 15 cm × 4.6 mm, 5 μM) and a photodiode array detector. The mobile phase was H$_2$O/CH$_3$CN in 0.1% formic acid and 0.1% trifluoroacetic acid, using a linear gradient from 30% to 35% organic over 40 min. Fluorescence spectra of 1.0 μM HO-Mito-Etd$^+$, Mito-Etd$^+$, HO-Etd$^+$, and Etd$^+$ were obtained by using samples that were incubated for 15 min at 37°C with 1 mg/ml salmon sperm DNA, on a SLM Aminco (San Jose, CA) 8100c Fluorometer. The extinction coefficients for HO-Mito-Etd$^+$ and Mito-Etd$^+$ were determined to be $\varepsilon_{478} = 9,400 \text{ M}^{-1}\text{cm}^{-1}$ and $\varepsilon_{488} = 5,800 \text{ M}^{-1}\text{cm}^{-1}$, which were nearly identical to those reported for HO-Etd$^+$ (29) and Etd$^+$.

A SpectraMAX (San Jose, CA) Gemini fluorometer equipped with a 96-well plate reader was used for the SOD competition assays and also to compare fluorescence emission of Mito-HE oxidized by several oxidants. Assays were performed in 100 mM potassium phosphate buffer with 100 μM diethylenetriaminepentaacetic acid, pH 7.4, 37°C. To determine rate constants, fluorescence emission was plotted as a function of the fractional inhibition ($f_i$) by the addition of SOD, as described by Eq 1.

$$f_i = k_{ed}[\text{HE}]/(k_{ed}[\text{HE}] + k_{i}[\text{SOD}]) \quad [1]$$

The rate of $O_2^-$ scavenging by SOD ($k_i$) has been determined as 1.8 × 10$^{-3}$ M$^{-1}$s$^{-1}$ (30).

Superoxide was generated by xanthine oxidase, where the added volume of xanthine oxidase suspension was adjusted to generate 0.2 mM $O_2^-$ per sec with 0.5 mM xanthine, as measured by cytochrome c reduction. Alternatively, 10 μM Mito-HE was reacted with five 100-μM additions of peroxynitrite ± 25 mM ammonium bicarbonate with immediate vortexing, 100 μM hydrogen peroxide, or 100 μM hypochlorous acid. Hydroxyl radical was generated by using xanthine oxidase, 0.5 mM xanthine, and 10 μM Fe$^{3+}$-EDTA. All assays were repeated at least three times and are reported ± standard deviation.

The uptake of Mito-HE into isolated mitochondria was determined by using an electrode selective for the triphenylphosphonium cation (31, 32). Rat livers were homogenized in 250 mM sucrose/5 mM Tris-HCl (pH 7.4)/1 mM EGTA, followed by differential centrifugation (33). The electrode was constructed as described (31), and the voltage was measured relative to an Ag/AgCl reference electrode. The electrode was calibrated by the sequential addition of 5 × 1 mM reduced Mito-HE to a
sucrose

The membrane potential was subsequently dissipated with 0.5 ml). Mitochondria were energized with succinate (10 mM), and 30°C. The respiration buffer was 225 mM mannitol of protein per ml) were incubated with 10 mM succinate of pH

Primary-oligodendrocyte cultures were prepared from 1-day-old Sprague–Dawley rat pups, as reported (36). Cultured cells were transferred to the heated stage (37°C) of a Zeiss (Oberkochen, Germany) LSM510 confocal microscope with constant 5% CO2. Live cells were imaged with a 63× oil immersion objective by using either 405- or 514-nm laser excitation. A single field of view was used throughout an entire experiment and corrected for autofluorescence by collecting a 405-nm excitable signal before labeling with Mito-HE. Image acquisition conditions were kept constant for comparison between ± antimycin. Cells were incubated with 0.1 μM Mito-HE for 15 min, washed, and incubated in supplemented L15 media (36). Images were obtained immediately after Mito-HE addition and every 10 min thereafter. In some experiments, antimycin (15 μM) was added immediately after Mito-HE incubation. After confocal imaging of HO-Mito-Etd+, cells were incubated with 3.6 nM MitoTracker Deep Red (Invitrogen, Carlsbad, CA) to visualize mitochondrial colocalization and general integrity.

To determine the chemical structure of the oxidation product, samples were directly injected onto the electrospray interface of an LC-Q Classic ion trap mass spectrometer in positive ion mode. The mobile phase was 25% 10 mM ammonium acetate and 75% acetonitrile with 5 μl/min flow rate. The source voltage was 2.72 kV, and the capillary voltage was 32.7 V.

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<table>
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<th>Oxidant</th>
<th>AUC</th>
<th>Mito-HE λ&lt;sub&gt;ex&lt;/sub&gt;=396nm</th>
<th>HE λ&lt;sub&gt;ex&lt;/sub&gt;=396nm</th>
<th>Mito-HE λ&lt;sub&gt;ex&lt;/sub&gt;=510nm</th>
<th>HE λ&lt;sub&gt;ex&lt;/sub&gt;=510nm</th>
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<td>Superoxide (O&lt;sub&gt;2&lt;/sub&gt;•&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>3.52</td>
<td>100 ± 5.5%</td>
<td>100 ± 35%</td>
<td>100 ± 5.3%</td>
<td>100 ± 21%</td>
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<tr>
<td>Peroxynitrite (ONOO&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>8.33</td>
<td>2.2 ± 0.3%</td>
<td>1.5 ± 0.5%</td>
<td>3.0 ± 0.9%</td>
<td>0.6 ± 0.2%</td>
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<tr>
<td>Nitrosoperoxy carbonate (ONOOCO&lt;sub&gt;2&lt;/sub&gt;•)</td>
<td>8.33</td>
<td>2.1 ± 0.4%</td>
<td>1.6 ± 0.6%</td>
<td>1.6 ± 0.4%</td>
<td>1.2 ± 0.3%</td>
</tr>
<tr>
<td>Hydrogen peroxide (H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>100</td>
<td>6.4 ± 0.5%</td>
<td>6.2 ± 2.2%</td>
<td>10 ± 3.8%</td>
<td>6.7 ± 1.4%</td>
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<tr>
<td>Hydroxyl Radical (•OH)</td>
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<td>4.4 ± 0.2%</td>
<td>1.3 ± 0.5%</td>
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<td>Hypochlorous bleach (HOCl)</td>
<td>100</td>
<td>4.8 ± 0.2%</td>
<td>3.3 ± 1.2%</td>
<td>8.4 ± 0.1%</td>
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Table 1. Selectivity of oxidation of Mito-HE and HE. Oxidant exposure (μM•min) represents the area under the curve (AUC) or integrated amount of oxidant exposure over time to account for differences in half life decay of oxidants. A significant concentration of DMSO (28 mM) from the Mito-HE stock, as well as urate, xanthine or xanthine oxidase were all present in the hydroxyl radical generating system and will therefore have competed with the reaction of Mito-HE for hydroxyl radical. The increase in fluorescence of 10 μM Mito-HE upon oxidant exposure was measured using λ<sub>ex</sub>=396 and λ<sub>ex</sub>= 510 nm with λ<sub>em</sub> = 580 nm. Mito-HE appears to be selectively oxidized by superoxide. For example, a 28-fold greater exposure to hydrogen peroxide was required to obtain just 10% of the superoxide-induced fluorescent signal.
Relative Fluorescent Units
Supplemental Figure 3.

Exc = 387 nm

Exc = 484 nm
Figure 11a.

Figure 11b.
Calculated m/z = 315.89

Calculated m/z = 630.78 amu
Supplemental Figure 6.

(a) Relative Intensity graph with peaks at m/z = 302.3, 345.2, and 384.2.

(b) Compound structures with m/z values:
- m/z = 384.2
- m/z = 345.2
- m/z = 302.3