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Detection and identification of oxidants formed during $\cdot\text{NO}/\text{O}_2^{\cdot-}$ reaction: A multi-well plate CW-EPR spectroscopy combined with HPLC analyses

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Abstract

New techniques and probes are routinely emerging for detecting short-lived free radicals such as superoxide radical anion ($\text{O}_2^{\cdot-}$), nitric oxide ($\cdot\text{NO}$) and transient oxidants derived from peroxynitrite ($\text{ONOO}^-/\text{ONOOH}$). Recently, we reported the profiles of oxidation products (2-hydroxyethidine, ethidium, and various dimeric products) of the fluorogenic probe hydroethidine (HE) in the $\cdot\text{NO}/\text{O}_2^{\cdot-}$ system (Zielonka *et al. J. Biol. Chem.* 2012). In this study, we used HPLC analyses of HE oxidation products in combination with continuous wave EPR (CW-EPR) spin trapping with 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline N-oxide (BMPO) to define the identity of the oxidizing species formed in the $\cdot\text{NO}/\text{O}_2^{\cdot-}$ system. EPR spin-trapping technique is still considered as the gold standard for characterization of free radicals and their intermediates. We monitored formation of BMPO-superoxide (BMPO- $\cdot\text{OOH}$) and BMPO-hydroxyl (BMPO- $\cdot\text{OH}$) radical adducts. Simultaneous analyses of results from EPR spin-trapping and HPLC measurements are helpful in the interpretation of the mechanism of formation of products of HE oxidation.

Keywords

spin-trapping; EPR/ESR; HPLC; BMPO; hydroethidine; 2-hydroxyethidium; ethidium; diethidium; superoxide radical anion; nitric oxide; peroxynitrite

Introduction

Hydroethidine (HE) is one of the most widely used probes to monitor intracellular superoxide radical anion ($\text{O}_2^{\cdot-}$) [1-4]. Abundant data now exist in the literature demonstrating the formation of a unique product, 2-hydroxyethidium (2-OH-E^+), formed

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Declaration of interest

The authors report no conflicts of interest.

during HE/O₂^{•−} reaction [5-8]. As O₂^{•−} reacts with •NO at almost a diffusion-controlled rate forming peroxynitrite anion (ONOO[−], *reaction 1*) as a transient and potent oxidant [9], it is crucial to understand the reaction chemistry between peroxynitrite and HE for proper interpretation of intracellular fluorescence formed from HE oxidation. Thus, we embarked on a systematic study of products formed from the reaction between HE and varying fluxes of •NO and O₂^{•−} in a multi-well plate format [3].

Previously, we investigated the products of HE oxidation in the presence of nanomolar (nM/min) fluxes of O₂^{•−} and •NO [3]. Due to the lower sensitivity of EPR spin trapping assays, as compared to the HPLC assay, we were not able to complement those data with similar experiments using spin traps. Therefore we were not able to define the oxidizing species responsible for HE oxidation when O₂^{•−} and •NO were co-generated. To overcome the problem of sensitivity, we report here the results of analogous experiments on HE oxidation, under low micromolar (μM/min) fluxes of O₂^{•−} and •NO. Concomitantly, we monitored radical formation using EPR spin-trapping [10] under the same experimental conditions. We used the spin trap, BMPO, which was originally synthesized and characterized in our laboratory [11]. BMPO was shown to be superior to DMPO in many aspects, including trap and radical adduct stability [11]. In addition, the EPR spectral characteristics are very close to those of DMPO adducts enabling easier characterization of its radical adducts.

As carbon dioxide (CO₂) is believed to be one of major targets of ONOO[−] *in vivo*, forming a short lived nitrosoperoxocarbonate (reaction 2), we also tested the effect of bicarbonate on the pattern of HE oxidation and BMPO spin trapping at different fluxes of •NO and O₂^{•−}.

Results from our concomitant HPLC and spin trapping analyses indicate that one electron oxidants formed from •NO/O₂^{•−} in the presence and absence of bicarbonate react with HE forming both fluorescent and additional non-fluorescent products but not 2-OH-E⁺. These results should enable researchers to better interpret the profiles of oxidation products of HE in cellular and cell-free systems.

Materials and methods

Chemicals

Hydroethidine (HE) and xanthine oxidase (XO) from cow milk were purchased from Invitrogen and Roche Diagnostics GmbH, respectively. DPTA-NONOate was from Cayman. 5-Tert-butoxycarbonyl-5-methyl-1-pyrroline N-oxide (BMPO) was synthesized as previously described [11]. All other reagents were from Sigma-Aldrich and were of highest purity available. In all samples for EPR and HPLC experiments, we used a mixture of phosphate buffer (50 mM) and dtpa (0.1 mM) solutions for maintaining pH at 7.4 and chelate metal ions. The addition of bicarbonate (NaHCO₃) solution causes pH of the mixture solution to be shifted up to 7.5, and thereby the small amount of acidic NaH₂PO₄ was added to the mixture solution for maintaining pH at 7.4. Hypoxanthine (HX) aqueous solution was

added to achieve a concentration of 0.2 mM. Hydroxyethidium (HE) and BMPO were added for HPLC and EPR experiments, respectively, for probing reactions involving $O_2^{\bullet-}$ and $\bullet OH$ radicals. XO (xanthine oxidase) was added to the resultant solution for initiating incubation before EPR and HPLC measurements.

Determination of $O_2^{\bullet-}$ and $\bullet NO$ fluxes

$\bullet NO$ flux was determined from the rate of decomposition of DPTA-NONOate measured in terms of a decrease in its characteristic absorbance at 250 nm ($\epsilon = 8.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). The determined rate of decay of DPTA-NONOate was doubled to obtain the rate of $\bullet NO$ release, assuming that two molecules of $\bullet NO$ are released during the decomposition of one molecule of DPTA-NONOate [12,13]. The flux of $O_2^{\bullet-}$ generated by XO-catalyzed oxidation of hypoxanthine (HX) was determined by monitoring the increase in absorbance of cytochrome *c* (Fe^{2+}) at 550 nm (using a difference in the values of the extinction coefficients between reduced and oxidized forms of $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) [14,15].

EPR measurements

The CW-EPR measurements were carried out using a Bruker EMX X-band spectrometer at room temperature. Typical spectrometer parameters were: microwave frequency, 9.84 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 Gauss; scan range, 60 G; sweep time, 20.5 seconds; time constant, 1.28 seconds. EPR measurement was initiated 2 min after XO addition and repetitively scanned for 33 min (90 scans). In this experiment, each sample contained dtpa (100 μM), BMPO (200 mM) and HX (0.2 mM) in a phosphate buffer (50 mM, pH=7.4). XO and DTPA-NONOate were added for generating $O_2^{\bullet-}$ and $\bullet NO$, respectively, as described above. The original EPR spectra collected after 30 min of incubation are given in Supplemental Material (Suppl. Figs. S1 and S3). WinSVD program was utilized for singular value decomposition (SVD), enabling us to reduce noise of resultant EPR spectra without loss of temporal information. The SVD analysis was successfully applied previously to investigate the kinetics of trapping of superoxide radical anion by cyclic nitron spin traps [16]. EasySpin 3.1.7 program [17] running on MATLAB 6.5 software was used for spectral simulation and fitting for optimization of hyperfine coupling constants (*hfccs*) of nitrogen and hydrogen nuclei of BMPO adducts. The *hfccs* for BMPO- $\bullet OOH$ and BMPO- $\bullet OH$ adducts were determined for six samples ($O_2^{\bullet-}/\bullet NO$ flux = 0.5/0.0 $\mu\text{M}/\text{min}$, 0.5/0.5 $\mu\text{M}/\text{min}$, 1.5/0.0 $\mu\text{M}/\text{min}$, 1.5/0.5 $\mu\text{M}/\text{min}$, 1.5/0.75 $\mu\text{M}/\text{min}$ and 1.5/1.5 $\mu\text{M}/\text{min}$), as shown in Table 1. For spectral simulation and deconvolution based on EasySpin program, we employed “esfit” function for fitting based on least-squares fitting of the EPR spectra each of which was simulated by “garlic” function suitable for simulating isotropic cw-EPR spectra in solution. Reported *hfccs* [11] were utilized as initial parameters for running EasySpin simulation to deconvolute EPR spectra composed of combination of BMPO- $\bullet OOH$ and BMPO- $\bullet OH$ adducts signals, enabling to optimize *hfccs* and component ratio for both of the adducts including their isomers indicated by symbols (I) and (II) in Table 1.

HPLC measurements

HE and its oxidation products were separated and monitored with HPLC using an Agilent 1100 apparatus equipped with an UV-Vis absorption and fluorescence detectors, as described previously [3,18,19]. Typically, 50 μ l of a sample was injected on C₁₈ column (Phenomenex, Kinetex C₁₈, 100 mm $\times$ 4.60 mm, 2.6 μ m) equilibrated with acetonitrile/water mobile phase (20/80 v/v) containing 0.1% trifluoroacetic acid TFA. Compounds were separated by a linear increase of the acetonitrile concentration from 20 to 56 % over 4.5 min. Next, the acetonitrile concentration was increased up to 100 % over 0.5 min and kept at this level for 1.5 min. Before next injection, the column was re-equilibrated with acetonitrile/water mobile phase (20/80 v/v) containing 0.1% TFA for at least 2 min. All analytes were eluted at a flow rate of 1.5 ml/min. Fluorescence detection was implemented at an excitation wavelength of 490 nm and an emission wavelength of 567 and 598 nm. The absorption traces were collected at 290 nm and 370 nm. Each HPLC sample was prepared in the same way as EPR sample, except 50 μ M HE was added instead of BMPO and the samples were protected from light [20]. XO was added 2 min after HE addition and then the resultant incubation mixture was kept in the dark for 15 min followed by HPLC analysis.

Results

Oxidation of hydroethidine by cogenerated $\cdot$ NO and $O_2^{\cdot-}$: HPLC analyses

Hydroethidine was treated with varying fluxes of $\cdot$ NO and $O_2^{\cdot-}$ in a multi-well plate. The flux of $O_2^{\cdot-}$ ranged from 0 to 1.5 μ M/min and $\cdot$ NO from 0 to 1.5 μ M as shown in the plate layout (Fig. 1). Using the HPLC coupled with UV/Vis and fluorescence detection, we identified several oxidation products of HE, the relative distribution of which were dependent on the relative fluxes of $O_2^{\cdot-}$ and $\cdot$ NO. With increasing fluxes of $O_2^{\cdot-}$, 2-OH-E⁺ (peak A) intensity (retention time, 3.25 min) increased which is consistent with our previous report [3,14]. With increasing of $\cdot$ NO fluxes, the peak due to 2-OH-E⁺ disappeared; instead the intensities of other peaks (B, C, D, and E) increased. Peak B results from the oxidation product ethidium (E⁺) while peaks C, D, and E are assigned to homo- and heterodimers (HE-HE, HE-E⁺, E⁺-E⁺) of HE, respectively [21]. As the dimers have been attributed to the one-electron oxidation of HE, these results suggest that co-generated $\cdot$ NO and $O_2^{\cdot-}$ result in radical-mediated oxidation of HE. The oxidation of HE to the dimeric products was also observed in the presence of $\cdot$ NO donor alone in the presence of oxygen. This is consistent with the reported reactivity of nitrogen dioxide radical ($\cdot$ NO₂) towards HE [22]. In an effort to determine the identity of oxidants and their role in HE oxidation mechanism, we performed EPR spin trapping using the BMPO trap.

EPR analyses of radicals formed from varying fluxes of $\cdot$ NO and $O_2^{\cdot-}$

Figure 2 shows EPR spectra of BMPO spin adducts formed under various fluxes of $\cdot$ NO and $O_2^{\cdot-}$ in the sample multiwell plate format similar to those used in HE oxidation (Fig. 1). Under conditions generating only $O_2^{\cdot-}$ and in the absence of $\cdot$ NO, the EPR spectra consist of BMPO- $\cdot$ OOH adduct whose intensity increased as $O_2^{\cdot-}$ flux increased from 0.5 to 1.5 μ M/min (Fig. 2, EPR spectra shown in column 1 of the plate layout). The EPR spectral intensity due to BMPO- $\cdot$ OOH spin adduct first decreased and then completely disappeared with increasing $\cdot$ NO flux. This pattern is similar to that of 2-OH-E⁺ formed from HE/ $O_2^{\cdot-}$

reaction with increasing $\cdot\text{NO}$ flux. In the presence of $\text{O}_2^{\cdot-}$ and with increasing $\cdot\text{NO}$ flux, the EPR spectra of BMPO- $\cdot\text{OH}$ adduct began to appear. In this regard, the BMPO- $\cdot\text{OH}$ spin adduct formation parallels the behavior of HE dimeric products (C, D and E), also increased with increasing the flux of $\cdot\text{NO}$. However, in contrast to HE dimers, BMPO- $\cdot\text{OH}$ adduct is not observed when $\cdot\text{NO}$ levels were higher than that of $\text{O}_2^{\cdot-}$ (upper right quadrant). This is consistent with decreased stability of DMPO- $\cdot\text{OH}$ adduct in the presence of $\cdot\text{NO}$ and $\cdot\text{NO}_2$ [23]. The EPR parameters and the extent of contribution of BMPO- $\cdot\text{OOH}$ and BMPO- $\cdot\text{OH}$ adducts determined under different fluxes of $\text{O}_2^{\cdot-}$ and $\cdot\text{NO}$ are given in Table 1 and shown in Suppl. Fig. 2. As described in the caption of Table 1, the isomer (I) ratio is 45% for DMPO- $\cdot\text{OOH}$ analogous to that reported previously [11] while the isomer (I) ratio ranged from 21% to 40% for BMPO- $\cdot\text{OH}$. The examples of the simulated EPR spectra of the adducts and the comparison of the original spectra and simulated spectra of the mixtures are presented in Suppl. Fig. S5. and S6. The spectral intensity and pattern were not affected by catalase (100 U/ml) added exogenously (not shown). This indicates that H_2O_2 is not responsible for formation of the BMPO-adduct, and that this adduct is most likely generated from transient oxidants generated from $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$.

Effect of bicarbonate on oxidation of hydroethidine by cogenerated $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$: HPLC analysis

Next we investigated the effect of bicarbonate on HE oxidation by varying fluxes of $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ as shown (e.g., Fig. 1, plate layout). ONOO^- reacts rapidly with CO_2 (being in equilibrium with HCO_3^-) to form the nitrogen dioxide radical $\cdot\text{NO}_2$ and the carbonate radical anion ($\text{CO}_3^{\cdot-}$) (Reaction 2). Both $\cdot\text{NO}_2$ and $\text{CO}_3^{\cdot-}$ are one-electron oxidants capable of oxidizing HE to the dimeric products. $\text{O}_2^{\cdot-}$ alone in the presence of bicarbonate oxidized HE to form 2-OH- E^+ as the major product. In the presence of increasing fluxes of $\cdot\text{NO}$, there was a steady increase in E^+ and dimeric products (HE-HE, HE- E^+ and E^+-E^+), indicating that bicarbonate plus $\cdot\text{NO}/\text{O}_2^{\cdot-}$ also induces formation of a one-electron oxidant(s) converting HE to E^+ and the characteristic dimeric products (Fig. 3).

Effect of bicarbonate on BMPO-radical adducts formed from $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$

The plate layout and incubation conditions are similar to those used in Figure 2 except that bicarbonate was present. Similar to the lack of the effect of bicarbonate on the yield of 2-OH- E^+ , the BMPO- $\cdot\text{OOH}$ spin adduct formation was not significantly affected by the addition of NaHCO_3 . In the presence of bicarbonate, the contribution of BMPO- $\cdot\text{OH}$ spin adduct was consistently higher in all samples as compared to samples without NaHCO_3 (Suppl. Fig. 2 and 4, Table 1). Further studies are required to explain this effect. In the presence of $\cdot\text{NO}$ flux, the signal intensity due to BMPO- $\cdot\text{OH}$ spin adduct became predominant, with the signal intensity ratio of first signal (3485G) to second signal (3495G) gradually varying from 1:1 to 1:1.5 with time. The variation in the peak intensity ratio is clearly recognized, especially in the presence of higher $\text{O}_2^{\cdot-}$ flux. In the presence of $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$, the EPR spectra consist of both BMPO- $\cdot\text{OOH}$ and BMPO- $\cdot\text{OH}$ adducts (Fig. 4 and Suppl. Fig. 4 and 7), depending on the ratio of $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$. As shown in Suppl. Fig. 7, the monitoring of the second peak attributable to BMPO- $\cdot\text{OOH}$ adduct (marked by an arrow) made it possible for easy characterization of the adduct formation under different fluxes of $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$. Similar to experimental conditions without bicarbonate, addition of

catalase (100 U/ml) had no effect on the EPR spectral intensity (not shown). Previous reports suggest that cyclic nitron traps (DMPO) react with carbonate radicals to ultimately form the corresponding hydroxyl adduct [24,25]. We conclude that under the incubation conditions (Fig. 4) bicarbonate induces the formation of carbonate radicals, although our attempts to detect the BMPO-carbonate adduct were unsuccessful.

Discussion

Hydroethidine: A versatile probe for intracellular oxidant formation

Over twenty years ago it was proposed that conversion of HE to ethidium (red fluorescent) can be used as an intracellular assay for superoxide radical anion formation [26]. In recent years, it became evident that $O_2^{\bullet-}$ reacts with HE to form a characteristic hydroxylated product, 2-OH- E^+ , and not E^+ [5]. Recently, we have shown that 2-OH- E^+ is the only superoxide-derived product over a wide range of $O_2^{\bullet-}$ fluxes (high $\mu M/min$ to low nM/min) [27]. We proposed that although 2-OH- E^+ is only formed from the interaction between HE or HE-derived radical and $O_2^{\bullet-}$, other oxidants do react with HE to form E^+ [18,19]. These oxidants include peroxynitrite-derived species, hydroxyl radical, carbonate radical, higher oxidants derived from peroxidases and singlet oxygen [19]. Most recently, HOCl was shown to react with HE forming a characteristic chlorinated product [28]. Unlike other dye-derived radicals (dichlorodihydrofluorescein and dihydrorhodamine radical) [2,29], HE-derived radical does not react with oxygen forming artifactually dye-mediated superoxide and hydrogen peroxide [19]. In addition, the fluorescent and non-fluorescent oxidation products and dimers derived from HE radical have been detected and characterized [21]. Formation of a dimeric molecule from a monomeric probe is invariably consistent with a one-electron oxidation process. Unlike the hydrocyanine dyes for which the free radical chemistry with $O_2^{\bullet-}$ and other oxidants remains unknown and its specificity to be tested [30], the mechanisms of ROS reaction with HE-based dyes is much better understood and the product specificity towards $O_2^{\bullet-}$ has been rigorously tested, and verified [19].

In this study, we focused on peroxynitrite ($ONOO^-/ONOOH$) as it is formed as a ubiquitous species in various pathophysiological conditions [31]. Results indicate that one-electron oxidants ($\bullet OH$, $\bullet NO_2$, or $CO_3^{\bullet-}$) formed from $\bullet NO/O_2^{\bullet-}/HCO_3^-$ interact with HE to yield fluorescent oxidation product (E^+) and dimeric products (HE-HE, HE- E^+ , and E^+-E^+). These findings are particularly significant when interpreting HE-derived “red fluorescence” that is often equated to intracellular $O_2^{\bullet-}$ [32,33]. We have previously described the overlapping characteristics of fluorescence signals of 2-OH- E^+ ($O_2^{\bullet-}$ derived product) and E^+ (non-specific oxidation product) and concluded that fluorescence microscopy can't be used to detect and monitor intracellular $O_2^{\bullet-}$ [19,34]. However, we and others have consistently noticed intracellular formation of E^+ in most experiments [27,34,35]. In other works, exogenously-added SOD or manipulation of intracellular SOD levels by transfection experiments decreased the HE-derived “red fluorescence” [36]. These reports gave a false sense of security and confidence in red fluorescence assay for $O_2^{\bullet-}$. These reports have reassured, without any consideration to numerous reports to the contrary [19,33], the overall conclusion that intracellular red fluorescence accurately measures intracellular $O_2^{\bullet-}$ [36]. The present results indicate that one-electron oxidants formed from $\bullet NO/O_2^{\bullet-}$ or $\bullet NO/$

$O_2^{\bullet-}/HCO_3^-$ interaction can react with HE forming E^+ and other products. We demonstrated that SOD prevents generation of $ONOO^-$ by activated macrophages [3]. We anticipate that by preventing $ONOO^-$ formation SOD will decrease intracellular formation of one-electron oxidants. The inhibitory effects of SOD on 2-OH- E^+ (superoxide-specific product) and on E^+ and dimers can be distinguished with HPLC analyses, but not with fluorescence spectroscopy alone. Clearly, with increased understanding of the oxidation mechanism of HE, it is now feasible to detect additional products which may allow us to offer a more rational explanation for these dichotomous results. Additionally, the use of peroxynitrite scavengers and probes (e.g. boronate compounds) may help in identification of the oxidants formed (3,14,15).

The free radical chemistry of phenanthridine-based dyes is very similar as has been reported earlier [19]. Recently, we reported a water-soluble probe, hydropropidine (HPr^+), for extracellular trapping of $O_2^{\bullet-}$. Analogous to HE, HPr^+ also forms a characteristic product, 2-OH- Pr^{++} , upon reaction with $O_2^{\bullet-}$ [37]. We indicated that other one electron oxidation chemistry of HPr^+ is also similar to that of HE. The chemistry of Mito-SOX (or Mito-HE), a HE analog that is conjugated to a triphenylphosphonium cation, is again similar to that of HE. It is now possible to target HE moiety to various subcellular and extracellular locations for site-specific oxidant detection. However, the researchers have to keep in mind the basic chemical reactivity of the HE molecule, in relation to the specific subcellular location. For example, because of increased peroxidatic activities (e.g., cytochromes and other heme proteins) in mitochondria, Mito-SOX is more prone to undergo one-electron mediated oxidation, forming characteristic fluorescent (Mito-ethidium) and other dimeric oxidation products [21]. Also, analogous to rhodamine cation [1], the oxidized product of Mito-SOX (Mito-ethidium) could translocate to mitochondria [21,37]. Reaction chemistry between Mito-SOX or HPr^+ and $^{\bullet}NO/O_2^{\bullet-}$ is expected to be similar to that of HE, indicating that in the presence of co-generated $^{\bullet}NO$ and $O_2^{\bullet-}$, the fluorescence signal from those probes may be due to non-specific oxidation products, and still remain inhibitable by SOD.

BMPO-derived radical adducts: interaction with $CO_3^{\bullet-}$ radical anion

We have previously reported that $CO_3^{\bullet-}$ radical anion reacts with DMPO to ultimately form the DMPO- $^{\bullet}OH$ adduct [25]. In this study, we used BMPO to detect $O_2^{\bullet-}$, $^{\bullet}OH$, and $CO_3^{\bullet-}$ formed from $^{\bullet}NO/O_2^{\bullet-}$ and $^{\bullet}NO/O_2^{\bullet-}/CO_2$ interaction. As expected, we detected the BMPO- $^{\bullet}OH$ adduct under both conditions. However, we attribute this to BMPO reaction with $^{\bullet}OH$ or $CO_3^{\bullet-}$. Although we were unable to detect the BMPO- $CO_3^{\bullet-}$ adduct, it is likely that BMPO- $CO_3^{\bullet-}$ undergoes hydrolysis similar to the DMPO- $CO_3^{\bullet-}$ adduct, resulting in the formation of the corresponding hydroxyl adduct (25,38,39).

Conclusions

In this study, using the combined EPR spin-trapping and HPLC-detection of HE-derived products, we report the following:

1. In the absence of $^{\bullet}NO$, HE reacts with $O_2^{\bullet-}$ to form 2-OH- E^+ as the major product. This was supported by the concomitant detection of the BMPO- $^{\bullet}OOH$ adduct by spin trapping.

2. With increasing $\cdot\text{NO}$ levels, 2-OH- E^+ formation drastically decreased; E^+ and HE-derived dimeric products increased. Concomitantly, the EPR spectral intensity due to BMPO- $\cdot\text{OOH}$ decreased and that of BMPO- $\cdot\text{OH}$ formed from trapping of $\cdot\text{OH}$ increased.
3. In the presence of $\cdot\text{NO}/\text{O}_2^{\cdot-}/\text{HCO}_3^-$, and with increasing $\cdot\text{NO}$ flux, 2-OH- E^+ formation decreased whereas the formation of E^+ and HE-dimeric products increased; the EPR spectra observed under these conditions are consistent with the formation of the BMPO- $\cdot\text{OH}$ adduct presumably formed from a transient adduct resulting from the interaction between $\text{CO}_3^{\cdot-}$ and BMPO.
4. The combined EPR spin trapping and HPLC-based determination of HE-derived products is essential for fully corroborating the mechanism of probe oxidation in $\cdot\text{NO}/\text{O}_2^{\cdot-}$ system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HPLC analyses of oxidation products of hydroethidine generated from varying fluxes of $\bullet\text{NO}$ and $\text{O}_2^{\bullet-}$

Hydroethidine (50 μM) was incubated with different fluxes of $\bullet\text{NO}$ and $\text{O}_2^{\bullet-}$ in phosphate buffer (50 mM, pH 7.4) containing hypoxanthine (0.2 mM) and dtpa (0.1 mM). Incubations were performed in a multi-well plate format as shown. Fluxes of $\bullet\text{NO}$ and $\text{O}_2^{\bullet-}$ were varied by carefully adjusting the amount of XO and/or the $\bullet\text{NO}$ donor. HPLC measurements were performed 15 min after adding XO and the $\bullet\text{NO}$ donor. The HPLC traces recorder by absorption detector set at 290 nm are shown. Peaks (A-E) indicate the following oxidation products: A, 2-OH- E^+ ; B, E^+ ; C, HE-HE; D, HE- E^+ , and E, E^+-E^+ .

Figure 2. EPR analyses of spin-trapped BMPO-adducts

Experimental conditions are identical to those described in Fig. 1, except that instead of HE, BMPO (200 mM) was present in the incubation under otherwise similar conditions. EPR measurements were performed 30 min after the addition of XO and/or the [•]NO donor. Experimental parameters are given in Materials and Methods section.

Figure 3. HPLC analyses of HE-derived oxidation products generated from varying $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ fluxes in the presence of bicarbonate

Experimental conditions are similar to those described in Fig. 1 except in the presence of bicarbonate (25 mM). Similar to Fig. 1, HPLC measurements were performed 15 min after the addition of XO and/or the $\cdot\text{NO}$ donor and the chromatograms recorded at 290 nm (absorption detector) are shown. Peaks designations are the same as in Fig. 1.

Figure 4. EPR analyses of BMPO-adducts formed from varying $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ fluxes in the presence of bicarbonate

Experimental conditions are similar to those described in Fig. 2 except in the presence of bicarbonate (25 mM). EPR analyses were performed 30 min after the addition of XO and/or the $\cdot\text{NO}$ donor.

Table 1Optimized *hfcc* values and component ratios of two isomers for BMPO-OOH and BMPO-OH.

	O ₂ ^{•-} flux (μM /min)	[•] NO flux (μM /min)	BMPO-OOH Ratio	BMPO-OH Ratio	BMPO-OOH : BMPO-OH
- NaHCO ₃	0.5	0	(I) 55%	(I) 26%	82 : 18
			(II) 45%	(II) 74%	
	0.5	0.5	-	(I) 40%	0 : 100
			-	(II) 60%	
	1.5	0	(I) 55%	(I) 21%	86 : 14
			(II) 45%	(II) 79%	
	1.5	0.5	(I) 55%	(I) 24%	69 : 31
			(II) 45%	(II) 76%	
	1.5	0.75	(I) 56%	(I) 22%	44 : 56
			(II) 44%	(II) 78%	
	1.5	1.5	-	(I) 37%	0 : 100
			-	(II) 63%	
+ NaHCO ₃	0.5	0	(I) 56%	(I) 26%	63 : 37
			(II) 44%	(II) 74%	
	0.5	0.5	-	(I) 28%	0 : 100
			-	(II) 72%	
	1.5	0	(I) 55%	(I) 22%	65 : 35
			(II) 45%	(II) 78%	
	1.5	0.5	(I) 55%	(I) 23%	48 : 52
			(II) 45%	(II) 77%	
	1.5	0.75	(I) 55%	(I) 26%	3 : 97
			(II) 45%	(II) 74%	
	1.5	1.5	-	(I) 29%	0 : 100
			-	(II) 71%	

Optimized *hfcc* values of BMPO-OOH are as follows: (I) a(N)=13.3G, a(H_β)=12.1G: (II) a(N)=13.3G, a(H_β)=9.7G. The ratio of BMPO-OOH isomers (I) and (II) is the same as that reported previously [11], i.e., (I) 55%, (II) 45%.

(Reported *hfcc* values of BMPO-OOH are as follows: (I) a(N)=13.4G, a(H_β)=12.1G: (II) a(N)=13.37G, a(H_β)=9.42G. [3])

Optimized *hfcc* values of BMPO-OH are as follows: (I) a(N)=14.2G, a(H_β)=15.7G, a(H_γ)=0.6G: (II) a(N)=14.1G, a(H_β)=12.8G, a(H_γ)=0.7G.

(Reported *hfcc* values of BMPO-OH are as follows: (I) a(N)=13.47G, a(H_β)=15.31G, a(H_γ)=0.62G: (II) a(N)=13.56G, a(H_β)=12.30G, a(H_γ)=0.66G. [3])