Tracking isotopically labeled oxidants using boronate-based redox probes

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Abstract

Reactive oxygen and nitrogen species have been implicated in many biological processes and diseases, including immune responses, cardiovascular dysfunction, neurodegeneration, and cancer. These chemical species are short-lived in biological settings, and detecting them in these conditions and diseases requires the use of molecular probes that form stable, easily detectable products. The chemical mechanisms and limitations of many of the currently used probes are not well understood, hampering their effective applications. Boronates have emerged as a class of probes for the detection of nucleophilic two-electron oxidants. Here, we report the results of an oxygen-18–labeling mass spectrometry study to identify the origin of oxygen atoms in the oxidation products of phenylboronate targeted to mitochondria. We demonstrate that boronate oxidation by hydrogen peroxide, peroxymonocarbonate, hypochlorite, or peroxynitrite involves the incorporation of oxygen atoms from these oxidants. We therefore conclude that boronates can be used as probes to track isotopically labeled oxidants. This suggests that the detection of specific products formed from these redox probes could enable precise identification of oxidants formed in biological systems. We discuss the implications of these results for understanding the mechanism of conversion of the boronate-based redox probes to oxidant-specific products.

Introduction

Reactive oxygen species (ROS), including superoxide (O$_2^-$/HO$_2^-$), hydrogen peroxide (H$_2$O$_2$), and peroxynitrite (ONOO-/ONOOH), have been implicated in (patho)physiological mechanisms in redox biology and medicine (1-4). Both superoxide and H$_2$O$_2$ are relatively slow reacting and/or weak oxidants (4-6) but in biological systems can be converted to more reactive species (Fig. 1), including peroxynitrite (7,8), peroxymonocarbonate (HCO$_3^-$) (9,10), or hypochlorous acid (HOCl) (11), resulting in enhanced redox signaling and/or damage to cell components (5,12,13). Due to the short lifetime of most ROS in biological settings, detection and quantitative analyses of those species have remained a challenge, and development of new probes for redox biology is an active area of research. Most chemical probes used for the detection of cellular oxidants lack selectivity toward a single species. For example, dichlorodihydrofluorescein (DCFH), dihydrodihydroamine-123 (DHR123), and Amplex Red undergo two-electron oxidation to fluorescent dichlorofluorescein, rhodamine, and resorufin, respectively, and nitro blue tetrazolium undergoes four-electron reduction to diformazan, without incorporation of the reactive species detected into the product formed (Fig. 1). This often leads to ambiguity regarding the identity of the species.
detected and prevents tracking of the oxidants using isotope-labeling approach. ROS detection and their unambiguous identification in biological systems requires the use of chemical probes, which upon reaction form species-specific product(s) (14-19). As an example, spin traps react with most radicals by the formation of a covalent bond between the probe and the radical trapped, and the product formed is typically highly specific for the trapped species. Also, the conversion of hydroethidine (HE) into 2-hydroxyethidium (2-OH-E+) has been used to detect O$_2^-$ in cultured cells in vitro and in animal models in vivo (20-27). Other products formed from the HE probe, including diethidium (E$^+$-E$^+$) and 2-chloroethidium (2-Cl-E$^+$), have been proposed as specific marker products of one-electron oxidants and hypochlorous acid, respectively (28,29).

Oxidation of boronate-based probes into phenolic products has been utilized for the detection of H$_2$O$_2$ (30-32). An array of boronate probes, with similar chemical reactivities and a similar mechanism of response to H$_2$O$_2$ but with different modes of detection, has been reported (33-37). Also, fluorogenic boronate probes targeted to various subcellular compartments have been described (31,38-40). Triphenylphosphonium (TPP$^+$)-conjugated phenylboronic acid (called MitoB) was designed for mass-spectrometry-based detection of mitochondrial H$_2$O$_2$ (41-43). Resistance of boronates to heme-catalyzed oxidation makes them good candidates for the detection of oxidants in the in vivo settings. Boronate-based probes are oxidized more than a thousand times faster by HClO and nearly a million times faster by ONOO$^-$ than by H$_2$O$_2$ ($k_{H_2O_2}$ $\sim$ 1 M$^{-1}$s$^{-1}$; $k_{HClO}$ $\sim$ 10$^4$ M$^{-1}$s$^{-1}$; $k_{ONOO^-}$ $\sim$ 10$^6$ M$^{-1}$s$^{-1}$), and the reaction typically involves a minor pathway, with the formation of ONOO$^-$-specific product(s) (7,44-48). Recently, it has been reported that peroxymonocarbonate, the product of the reaction of H$_2$O$_2$ with CO$_2$, reacts with coumarin boronic acid nearly 50 times faster than H$_2$O$_2$ ($k_{HCO_3^-}$ $\sim$ 10$^2$ M$^{-1}$s$^{-1}$) (10).

Although the identities of the oxidation, chlorination, and nitration products of boronate probes have been established in many cases, and the reaction mechanisms have been proposed, the origin of oxygen atoms in the oxidation and nitration products of boronate probes has not been experimentally determined. Understanding the mechanisms of formation of the oxidation products is required for their rigorous use as specific ROS markers in the in vitro and in vivo settings. Also, the potential for selective monitoring of the specific oxidizing species, through use of isotopically labeled oxidant and monitoring isotopic labeling of the specific products, remains to be explored.

Here, we report on the incorporation of an oxygen atom from the biologically relevant two-electron oxidants, including H$_2$O$_2$, HCO$_3^-$, HClO, and ONOO$^-$ in the oxidation and nitration products of the mitochondria-targeted phenyl boronate probe (oMitoPhB(OH)$_2$, Fig. 2). In addition, we demonstrate the involvement of oxygen atoms from superoxide in the formation of the hydroxylated product, 2-OH-E$^+$, during oxidation of hydroethidine by O$_2^-$(Fig. 3), corroborating the proposed mechanism of the conversion of HE into 2-OH-E$^+$.

**Results**

We have investigated the incorporation of oxygen atoms from different biologically relevant nucleophilic oxidants (Fig. 1) capable of oxidizing boronate probes into the products formed. We chose oMitoPhB(OH)$_2$ as a model boronate probe (Fig. 2) because its reactivity toward H$_2$O$_2$, HClO, and ONOO$^-$ has been previously studied in detail and the products characterized (49-51). To demonstrate the formation of $^{18}$O-labeled superoxide, we have also tracked the incorporation of the $^{18}$O atom into the hydroxylation product of hydroethidine.

**Hydrogen peroxide**

Upon oxidation by H$_2$O$_2$, a conversion of oMitoPhB(OH)$_2$ into the phenolic product (oMitoPhOH) occurs (Fig. 4). H$_2$O$_2$ oxidizes the phenylboronate substrate into a phenoxyboronate intermediate that, upon hydrolysis, yields the phenolic product and boric acid. To determine if the phenolic oxygen atom derives from H$_2$O$_2$ or water, we performed the oxidation of oMitoPhB(OH)$_2$ by H$_2$^{16}O$_2$ in H$_2$^{18}O and by H$_2$^{18}O$_2$ in H$_2$^{16}O (Fig. 5). The product detected in the presence of H$_2$^{16}O$_2$ showed the molecular mass of oMitoPh^{16}OH (m/z = 369); in the presence of H$_2$^{18}O$_2$, the product had a molecular mass of 371 (Fig. 5b), attributed to oMitoPh$^{18}$OH. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analyses indicated no formation of oMitoPh$^{18}$OH during the oxidation of the probe by H$_2$^{16}O$_2$ in H$_2$^{18}O, while it
was the predominant product in the presence of H$_{18}$O$_2$ (Fig. 5c). We conclude that during oxidation of boronates by H$_2$O$_2$, the oxygen atom in the phenolic product derives exclusively from H$_2$O$_2$ and not from water.

**Peroxymonocarbonate**

In the presence of CO$_2$, H$_2$O$_2$ is in equilibrium with a more potent oxidant, peroxymonocarbonate (HOOCO$_2^-$) (Fig. 6a) (9,52,53). Formation of this species has been implicated, for example, in the enhanced hyperoxidation of cellular peroxiredoxins and protein tyrosine phosphatase 1B (PTP1B)-mediated signaling cascade observed in the presence of bicarbonate (12,54-56). Recently, it was shown that the rate of oxidation of the coumarin boronate probe in the presence of H$_2$O$_2$ is increased after the addition of bicarbonate (10). Therefore, we tested if the H$_2$O$_2$-derived HCO$_3^-$ incorporates the oxygen atom into oMitoPhB(OH)$_2$ probe.

First, we confirmed that the experimental conditions we used would allow us to detect increased formation of the phenolic product during the reaction of the probe with H$_2$O$_2$ upon addition of NaHCO$_3$. In fact, with increased concentration of NaHCO$_3$, the rate of product formation increased, as determined by LC-MS-based monitoring of the accumulation of oMitoPhOH over the incubation time (Fig. 6b,c). This effect was observed for both H$_{18}$O$_2$ and H$_{16}$O$_2$, when monitoring the $^{16}$O- or $^{18}$O-phenolic products, respectively (Fig. 6c). The relative increase in the yield of the phenolic product in the case of oMitoPh$^{18}$OH was higher than in case of oMitoPh$^{16}$OH, which we attribute to the presence of small amounts of oMitoPh$^{18}$OH but not oMitoPh$^{18}$OH in the probe stock solution. The representative LC-MS chromatograms are shown for both H$_{18}$O$_2$ and H$_{16}$O$_2$, with increased concentrations of NaHCO$_3$ are shown in Fig. 6d,e. Under those conditions, HC$^{16}$O$_2^-$ and HC$^{18}$O$_2^{16}$O$_2^-$ were formed, respectively. Incorporation of an $^{18}$O atom into the phenolic product indicates the involvement of the peroxyl moiety of HOCl$^-$ in the oxidation reaction. Obtained data are consistent with the addition of the deprotonated form of peroxymonocarbonate (CO$_2^-$) to the boronate moiety, with elimination of the carbonate anion and incorporation of an oxygen atom from the peroxy part of the oxidant.

**Hypochlorite**

Boronates are oxidized more than a thousand times faster by HOCl than by H$_2$O$_2$ at neutral pH (44). The product of the reaction is a phenol (or alcohol), which may undergo chlorination in the presence of excess HOCl (47,51,57). To determine the source of the oxygen atom during the conversion of oMitoPhB(OH)$_2$ into oMitoPhOH, we generated H$^{16}$OCl and H$^{18}$OCl in situ from myeloperoxidase (MPO)-catalyzed oxidation of chloride anions by H$_2$O$_2$ and H$_2$O$_2$, respectively (Fig. 7a). To confirm the formation of HOCl in the investigated system, we also performed similar incubations using H$_2^{18}$O$_2$ in the presence of the HE probe, and monitored the chlorination product, 2-Cl-E$^+$ (29).

Incubation of oMitoPhB(OH)$_2$ with H$_2$O$_2$, MPO and potassium chloride (KCl) led to a significant increase in the production of the phenolic product, confirming that HOCl was the major species responsible for oxidation under the conditions used (Fig. 7b). The omission of KCl or MPO resulted in a significantly lower yield of the product. Also, addition of small amounts of dimethyl sulfoxide (DMSO), known to rapidly scavenge HOCl (47,58), led to a significant attenuation of the formation of the phenolic product. Formation of HOCl was further confirmed by the detection of 2-Cl-E$^+$ in analogous systems, using the HE probe instead of the boronate (Fig. 7f). It previously was shown that HOCl and taurine chloramine are able to convert HE into 2-Cl-E$^+$ (29).

Replacement of H$_2^{16}$O$_2$ with H$_2^{18}$O$_2$ in the incubation mixture containing oMitoPhB(OH)$_2$, MPO, and KCl resulted in a switch from oMitoPh$^{16}$OH to oMitoPh$^{18}$OH (Fig. 7c). In the case of both isotopologs, the signal was maximal in a mixture containing H$_2$O$_2$, MPO, and KCl, and decreased upon the addition of DMSO. We conclude that the oxygen atom in the product of oMitoPhB(OH)$_2$ oxidation by HOCl derives from the oxidant.

**Peroxynitrite**

Similar to H$_2$O$_2$, ONOO$^-$ reacts with boronates to form a corresponding phenol as the major product. The rate constant of the reaction, however, is significantly higher (~10$^9$ M$^{-1}$s$^{-1}$ for ONOO$^-$ and ~1 M$^{-1}$s$^{-1}$ for H$_2$O$_2$), and the reaction typically involves a minor pathway, leading to ONOO$^-$-specific minor products (59). The high rate constant
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provides an opportunity to estimate the absolute flux of ONOO\(^-\) in cultured cells (48,60,61). Formation of ONOO\(^-\)-specific products provides an opportunity to selectively monitor ONOO\(^-\) formation in chemical and biological systems (14). We have previously applied this approach to demonstrate the formation of ONOO\(^-\) during the reaction of nitroxy (HNO) with oxygen (62). In the case of oxidation of \(\text{oMitoPhB(OH)}_2\) by ONOO\(^-\), the minor products include cyclo-\(\text{oMitoPh}\) and \(\text{oMitoPhNO}\) (Fig. 8), formed in 10% and 0.5% yields, respectively (51). Although the mechanism of the oxidation of boronates by ONOO\(^-\) has been extensively studied, both experimentally and using theoretical calculations (44,45,47,49), the isotope-labeling studies have not been performed. We decided to test the proposed reaction mechanism by reacting \(\text{oMitoPhB(OH)}_2\) with \(^{18}\text{O}\)-labeled ONOO\(^-\), produced \textit{in situ} from co-generated fluxes of nitric oxide (\(\text{NO}\)) and \(^{18}\text{O}_2\) (7,8). \(\text{NO}\) flux was generated from the decomposition of spermine NONOate, while \(^{18}\text{O}_2\) flux was produced during xanthine oxidase (XO)-catalyzed oxidation of hypoxanthine (HX) in the presence of \(^{18}\text{O}_2\). The identity of \(^{18}\text{O}_2\) has been confirmed using the HE probe and monitoring the incorporation of \(^{18}\text{O}\) atoms into the superoxide-specific product, 2-OH-E (see below). Co-generation of \(^{16}\text{O}_2\) and \(^{18}\text{O}_2\) leads to the formation of \(^{16}\text{ON}^{18}\text{O}^{18}\text{O}\), providing an opportunity to track different oxygen atoms from ONOO\(^-\) during the conversion of \(\text{oMitoPhB(OH)}_2\) into \(\text{oMitoPhOH}\) and \(\text{oMitoPhNO}\). Incubation of \(\text{oMitoPhB(OH)}_2\) with \(^{16}\text{ON}^{18}\text{O}\) led to the formation of the major, phenolic product, which showed the mass \((m/z = 371)\) to be two units higher than when using \(^{16}\text{ON}^{16}\text{O}\) \((m/z = 369, \text{Fig. 9b})\). LC-MS/MS traces of the phenolic products showed no formation of the \(\text{oMitoPhOH}\) in the presence of \(^{16}\text{ON}^{18}\text{O}\), although it was a predominant product when \(^{16}\text{ON}^{18}\text{O}\) was generated (Fig. 9c). In addition, changing the solvent to H\(_2\)\(^{18}\text{O}\) failed to produce \(\text{oMitoPhOH}\) (Fig. S1). These data indicate that the formation of the phenolic product during the reaction of boronates with ONOO\(^-\) is associated with the incorporation of the oxygen atom from the peroxy part of the oxidant. Among the minor, ONOO\(^-\)-specific products formed, cyclo-\(\text{oMitoPh}\) did not change its mass when switching from \(^{16}\text{ON}^{18}\text{O}\) to \(^{16}\text{ON}^{18}\text{O}\) \((\text{Fig. S1})\) as no oxygen atom is incorporated. 

\(\text{Cyclo-oMitoPh}\) was formed in maximal yields when \(\text{NO}\) and \(\text{O}_2\) were co-generated, and its peak intensity was similar for both \(^{16}\text{ON}^{16}\text{O}^{18}\text{O}\) and \(^{16}\text{ON}^{18}\text{O}^{18}\text{O}\) \((\text{Fig. S1})\).

Formation of the other minor product, \(\text{oMitoPhNO}\), was associated with an increase in the mass of this product by two units \((m/z = 400)\) in the presence of \(^{16}\text{ON}^{18}\text{O}^{18}\text{O}\) as compared with the product formed by \(^{16}\text{ON}^{16}\text{O}^{18}\text{O}\) \((m/z = 398, \text{Fig. 9b})\). This indicates that only one oxygen atom originated from the peroxy \((^{18}\text{O}-\text{labeled})\) part of ONOO\(^-\). Analyses of the LC-MS/MS traces indicate that \(\text{oMitoPhN}^{18}\text{O}^{18}\text{O}\) was formed only when \(^{16}\text{ON}^{18}\text{O}^{18}\text{O}\) was produced (Fig. 9c), and \(\text{oMitoPhN}^{16}\text{O}\) was the product of the reaction with \(^{16}\text{ON}^{16}\text{O}^{18}\text{O}\), even when the reaction was carried out in H\(_2\)\(^{18}\text{O}\) \((\text{Fig. S1})\). The data on the oxidation of \(\text{oMitoPhB(OH)}_2\) by ONOO\(^-\) indicate that the oxygen atoms introduced into the products originate from the oxidant and not from the solvent. These data are consistent with the occurrence of two reaction pathways, including heterolytic and homolytic cleavage of the peroxyl bond in the adduct of ONOO\(^-\) to the boronate probe \((\text{Fig. 10})\). The major pathway, involving a heterolytic cleavage, leads to the formation of the phenolic product, with the oxygen atom incorporated from the peroxy moiety of the oxidant, similar to the reaction with other tested oxidants, H\(_2\)\(_2\), HCO\(_3\), and HOCI. The minor pathway, involving the homolytic cleavage of the peroxy bond, leads to the formation of \(\text{NO}_2\) and a phenyl-type radical, which recombine within the solvent cage to form a nitrobenzene-type product \((\text{oMitoPhNO})\) \((\text{Fig. 10})\). The intramolecular addition of the phenyl radical to the phenyl ring of the TPP\(^+\) moiety yields the cyclic product \((\text{cyclo-\(\text{oMitoPh}\)})\) without incorporating any atom from the oxidant.

\textbf{Superoxide}

The production of \(^{16}\text{ON}^{18}\text{O}^{18}\text{O}\) for the study of the oxidation of boronates by ONOO\(^-\) involved co-generation of \(\text{NO}\) and \(^{18}\text{O}_2\). To confirm the formation of \(^{18}\text{O}_2\) in the incubation mixture containing HX, XO, and \(^{18}\text{O}_2\), we performed the incubation in the presence of the HE probe and monitored the incorporation of \(^{18}\text{O}\) atoms into the 2-OH-E\(^+\) product. HE is the most widely used probe for the detection of \(\text{O}_2\) in biological systems ranging from cultured cells to animals (21,63). In the presence of \(\text{O}_2\), HE is oxidized to 2-OH-E\(^+\), a specific marker product for \(\text{O}_2\) \((\text{Fig. 3})\) (20-24).
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Derivatives of HE for site-specific detection of $O_2^-$ have been reported (64-66). Those probes share the same oxidative chemistry with HE (65). A multistep mechanism of the conversion of HE to 2-OH-E$^+$ has been proposed that involves the oxidation of HE to the HE radical cation (HE$^+$), followed by the reaction of HE$^+$ with $O_2^-$ to form 2-OH-E$^+$ (21,67). This has been supported by pulse radiolysis data, showing the formation and rapid decay of HE$^+$ in the presence of pulse-generated $O_2^-$ (68) and an increase in the yield of 2-OH-E$^+$ by the addition of peroxidase in the presence of a steady flux of $O_2^+$ (67). Here, we provide direct proof of the production of $^{18}O_2^-$ in the HX/XO/$^{18}O_2$ system and the incorporation of the oxygen atom from $O_2^-$ into the product during oxidation of HE to 2-OH-E$^+$.

To follow the oxygen atoms, we incubated HE with $^{16}O_2^-$ or $^{18}O_2^-$, produced during enzymatic oxidation of HX by XO in the presence of $^{16}O_2$ or $^{18}O_2$, and monitored the formation of 2-16OH-E$^+$ and 2-18OH-E$^+$ (Fig. 11). The mass spectra of the products showed m/z values of 330 and 332 when the probe was incubated with $^{16}O_2^-$ or $^{18}O_2^-$, respectively (Fig. 11b). The increase in the mass of the product from $^{18}O_2^-$ is consistent with incorporation of $^{18}O$ into the molecule. The LC-MS/MS traces (Fig. 11c) indicate significant formation of 2-16OH-E$^+$ and negligible formation of 2-18OH-E$^+$ in the presence of $^{16}O_2^-$ (HX/XO/$^{16}O_2$). In the presence of $^{18}O_2^-$ (HX/XO/$^{18}O_2$), only a small peak of 2-16OH-E$^+$ and an intense peak due to 2-18OH-E$^+$ were observed. Furthermore, incubation of HE with $^{18}O_2^-$ in a solvent containing 90% of H$_2^{16}$O led to the formation of 2-16OH-E$^+$ but not 2-18OH-E$^+$ (not shown). These data confirm the formation of $^{18}O_2^-$ in the HX/XO/$^{18}O_2$ system and indicate that during the oxidation and hydroxylation of HE, the oxygen atom in the product originates from $O_2^-$, consistent with a mechanism involving the reaction of HE$^+$ with $O_2^-$ and forming the hydroperoxyl intermediate (Fig. 12).

Discussion
Isotope tracing is a powerful technique in the study of the mechanism of chemical and enzymatic reactions as well as cellular metabolism (69-71). Isotopically labeled oxidants have been used to identify the spin adducts of $O_2^-$ and other oxygen-centered radicals using an EPR spin trapping technique (72). EPR spin trapping, however, is only useful for the detection of radical species and has only limited applicability to detect intracellular ROS. Oxygen tracing in other probes used for cellular oxidants has not been reported.

In this study, we have investigated the origin of the oxygen atom in the products of the reaction of mitochondria-targeted boronate probe with four biologically relevant, two-electron oxidants: hydrogen peroxide, peroxyxymonocarbonate, hypochlorite, and peroxynitrite. The results support the previously proposed mechanisms of the probes’ oxidation and formation of the specific products, and provide a solid foundation for the use of those products for identification and tracking isotopically labeled oxidants.

New insights into the selective detection of peroxynitrite
Although initially assumed to be completely selective (specific) for $H_2O_2$, boronate-based probes also respond to other biologically relevant nucleophilic oxidants, including $HCO_3^-$, HOCl, ONOO$, and amino acid hydroperoxides (44,57,73). The main oxidation product in case of all the listed oxidants is the corresponding phenol. In the presence of excess HOCl or ONOO$, the phenolic product may undergo chlorination or nitration, respectively, providing an opportunity to identify the oxidant by profiling the products formed (14,44). As an example, in the presence of HOCl, the peroxo-caged luciferin probe is converted not only to luciferin but also to chloroluciferin (47). The reaction of boronate probes with ONOO$^+$ is of special interest, as this reaction typically proceeds via two pathways of the decomposition of peroxynitrite adduct to the boronate: (i) major pathway (~85%-90%) involving heterolytic cleavage of the peroxyl bond, leading to the formation of the phenolic product; and (ii) minor pathway (10%-15%), involving a homolytic cleavage of the peroxyl bond, with the formation of phenyl-type radical and $\cdot NO_2$, which upon recombination form nitrobenzene-type product (Fig. 10) (45). We have proposed using that product as a specific marker for ONOO$^+$ (50), and with such an approach, we demonstrated the formation of ONOO$^-$ during the reaction of HNO with oxygen (O$_2$) (62). In the case of the oMitoPhB(OH)$_2$ probe, the nitrated product, oMitoPhNO$_2$, accounts for only 0.5% of ONOO$^-$.
consumed. The other minor product, cyclo-oMitoPh, formed at 10% yield, is due to a rapid intramolecular addition of the phenyl-type radical to one of the phenyl rings of the TPP* moiety (Fig. 10) (51). Both minor products have been detected in macrophages stimulated to produce ONOO* (51) and can be used as specific marker products for intracellular ONOO*.

Detection of ONOO* in cells has remained a challenge, as most methods were based on the nitrate and/or oxidative properties of ONOO*-derived radicals (e.g., 'OH, "NO2, CO3") (74). However, the same radical species can be formed in biological systems in ONOO*-independent reactions. For example, although nitrated tyrosine residues are commonly used as an endogenous marker of ONOO*, the same product is formed by "NO2 from the MPO-catalyzed oxidation of nitrite by H2O2. Dihydrorhodamine, a fluorogenic probe used for ONOO* detection, cannot distinguish the two pathways of "NO2 either. Boronate probes, including oMitoPhB(OH)2, provide the first chemical tool to distinguish these two nitration pathways (75). Formation of the cyclic and nitrobenzene-type products from oMitoPhB(OH)2 occurs in the presence of ONOO* but not in the presence of MPO/H2O2/NO2 (51). This shows that monitoring the conversion of oMitoPhB(OH)2 into cyclo-oMitoPh and oMitoPhNO2 products can be used to selectively detect ONOO* formed in cell-free and cellular systems. Although other boronate probes may not form the cyclic product during the reaction with ONOO*, in most cases they produce nitrobenzene-type minor products. These products may be used to confirm the identity of the oxidant detected. For example, a new boronate probe recently was developed to detect ONOO* in amyloid beta aggregates (76). The minor product(s) formed during the reaction of the probe with ONOO* should be characterized and high-performance liquid chromatography (HPLC)- or LC-MS-based profiling should accompany fluorescence measurements, which report the yield of the phenolic product. This product is common for various nucleophilic oxidants, as exemplified here by H2O2, HCO3-, HClO, and ONOO*. Amino acid- and protein-based hydroperoxides also oxidize boronate probes to the phenolic products (73).

Oxidation of aromatic boronates involves initial formation of phenoxyboronic acid, followed by its hydrolysis into phenolic product (Fig. 4). The results obtained in this study demonstrate that during the oxidation of boronates by H2O2, HCO3-, HClO, or ONOO*, the oxygen atom in the phenolic product derives from those oxidants, not from water. In the case of HCO3- and ONOO*, the oxygen atoms in these oxidants are not equivalent, and the data obtained support the mechanism involving the nucleophilic addition of CO32- or ONOO* to the boron atom, via their peroxyl moieties, followed by elimination of a carbonate or nitrite anion, respectively (Fig. 10). Also, in the case of the formation of nitrobenzene-type product, the pattern of labeling of oMitoPhNO2 during the reaction of oMitoPhB(OH)2 with ONOO* provides insight into the mechanism of the minor pathway of the reaction. Incorporation of only one oxygen-18 atom into the nitrated product from 18ON18NO18O is consistent with the initial homolytic cleavage of the peroxy bond in the adduct, formation of phenyl-type radical and "NO2, and recombination of both radicals (Fig. 10).

2-Hydroxyethidium as a specific marker for O2-•

The HPLC or LC-MS-based analysis of 2-OH-E* is regarded as a “gold standard” of the detection of O2-• in biological systems (77). However, the utility of 2-OH-E* as the marker of cellular O2-• recently has been questioned, based on the lack of increase of its amount in HepG2 cells treated with H2O2 or rotenone (78). However, the ability of those treatments to induce O2-• generation in the used cell model has not been shown. Numerous reports demonstrate the utility of HE, MitoSOX Red, and hydropropidine, when coupled with HPLC-based analyses, to detect O2-• in different cellular models, as reviewed elsewhere (20,21,63). In those reports, 2-OH-E*, 2-OH-Mito-E*, and 2-OH-Pr* were used as specific marker products for O2-•. Hydroxylation of ethidium-based probes remains a method of choice for the detection of O2-• in cell-free and cellular systems (17,22,64-66,79).

The presented results demonstrate that the specificity of 2-OH-E* for O2-• derives from incorporation of an oxygen atom from this species. Together with the pulse radiolysis data on the oxidation of HE by pulse-generated O2-• (68), the 2:1 stoichiometry of the reaction (67), and the lack of incorporation of oxygen from water—observed in this study and during oxidation of HE by Fremy’s salt (80)—the obtained data are consistent with the
mechanism shown in Fig. 12. Initial oxidation of HE by the protonated form of $O_2^-$ (hydroperoxyl radical, $HO_2^-$) produces a radical cation of HE, which rapidly reacts with $O_2^-$ to form a hydroperoxide, containing an oxygen atom from $O_2^-$ (Fig. 12). This product must undergo rapid transformation in aqueous solutions to 2-OH-E$^+$, as no intermediates have been detected by HPLC analyses.

**Concluding remarks**

In summary, we demonstrated that the oxygen atoms in the oxidation and nitration products of the boronate probe, oMitoPhB(OH)$_2$, originate from the corresponding oxidants, H$_2$O$_2$, HCO$_2^-$, HOCl, or ONOO$^-$. Also, in the case the conversion of the HE probe into 2-OH-E$^+$, oxygen comes from $O_2^-$.

The presented data indicate that it is possible to track isotopically labeled oxidants by monitoring the incorporation of the isotopes into the oxidation/nitration products using the boronate and hydroethidine probes. Because no incorporation of the atoms from ROS/reactive nitrogen species occurs in most other commonly used probes, including DCFH, DHR123, Amplex Red, and NBT, they cannot be used for such purposes. The results obtained also corroborate the mechanisms of the conversion of HE into 2-OH-E$^+$ by $O_2^+$ and of oxidation and nitration of boronate-based probes, proposed previously based on product analyses, EPR experiments, and density functional theory calculation. We expect that oxygen-18 labeling studies, using $^{18}$O$_2$, H$_2^{18}$O, and H$_2$O$_2^{18}$O, may also be used in cell-free or cellular systems to reveal whether metal-induced hydrolysis and/or high-valent iron-oxo species may contribute to the hydroxylation of the probes (81). The reaction of the probes with $^{18}$O-labeled oxidants also can be used to prepare isotopically labeled standards of the oxidation products. Furthermore, H$_2^{18}$O$_2^-$ or H$_2^{18}$O$_2^{18}$O$_2^-$-mediated oxidation of boronates represents a convenient route for the synthesis of $^{18}$O-labeled alcohols and phenols.

**Experimental procedures**

**Materials**

*Ortho-*MitoPhB(OH)$_2$ and its oxidation and nitration products were synthesized, as described previously (49-51). The stock solution of oMitoPhB(OH)$_2$ (0.1 M) was prepared in DMSO and stored at −20°C. The HE probe was obtained from Invitrogen (Carlsbad, CA, USA). The stock solution of HE (20 mM) was prepared in deoxygenated DMSO under argon atmosphere and stored at −80°C. The standards of the oxidation products were synthesized, as described previously (22,82). For experiments involving HOCl, both probes were dissolved in ethanol (EtOH) to avoid the scavenging effect of DMSO on HOCl,(47) Water-$^{18}$O (97% oxygen-18), H$_2^{18}$O$_2$ (90% oxygen-18), $^{18}$O$_2$ (97% oxygen-18), HX, XO, superoxide dismutase (SOD), and catalase were obtained from Sigma-Aldrich (St. Louis, MO, USA). MPO was from Calbiochem.

**Determination of the flux of $O_2^-$**

$O_2^-$ was generated from the XO-catalyzed oxidation of HX in a phosphate buffer solution (25 mM, pH = 7.4) containing 0.1 mM diethylenetriamine pentaacetic acid (dtpa). The solution was continuously purged with O$_2$. The flux of $O_2^-$ was determined, as described previously (7,83,84), by performing the incubation in the presence of ferricytochrome c (50 µM) and monitoring the rate of its reduction following an increase in absorbance at 550 nm ($\Delta \varepsilon = 2.1 \times 10^4$ M$^{-1}$ cm$^{-1}$) (85). Superoxide dismutase completely blocked the reduction of ferricytochrome c under the conditions used.

**Determination of the flux of $^\cdot$NO**

$^\cdot$NO was generated from the thermal decomposition of spermine NONOate in a phosphate buffer (25 mM, pH = 7.4) containing 0.1 mM dtpa. The flux of $^\cdot$NO was determined, as described previously (7,84), by monitoring the rate of decay of spermine NONOate following a decrease in absorbance at 252 nm ($\varepsilon = 8 \times 10^5$ M$^{-1}$ cm$^{-1}$). The release of two molecules of $^\cdot$NO per one molecule of spermine NONOate consumed was assumed in the calculations (86).

**Oxidation of oMitoPhB(OH)$_2$ by H$_2$O$_2$**

To analyze the product of oxidation of oMitoPhB(OH)$_2$ by H$_2$O$_2$, oMitoPhB(OH)$_2$ (20 µM) was incubated at room temperature with H$_2$O$_2$ (10 mM) for 20 min in a phosphate buffer (25 mM, pH = 7.4) containing 0.1 mM dtpa. When performing the reaction in water-$^{18}$O, the final concentration of H$_2^{18}$O was 90% (by vol.).
Oxidation of oMitoPhB(OH)$_2$ by HCO$_3^-$

Oxidation oMitoPhB(OH)$_2$ by HCO$_3^-$ was studied by incubation of the probe with H$_2$O$_2$ in phosphate-buffered (0.1 M) aqueous solution containing dtpa (0.1 mM) in the presence of NaHCO$_3$ (25 and 50 mM). To maximize the involvement of HCO$_3^-$ in probe oxidation, the probe concentration was lowered to 1 µM, the H$_2$O$_2$ concentration was lowered to 50 µM, and the pH was adjusted to 7.0.

Oxidation of oMitoPhB(OH)$_2$ by HOCl

To study oxidation of oMitoPhB(OH)$_2$ by HOCl, the probe (50 µM, from a stock solution in EtOH) was incubated with H$_2$O$_2$ (0.1 mM), KCl (0.1 M) and MPO (20 mM) for 15 min at 25 °C in a phosphate-buffered (0.1 M) aqueous solution. Where indicated, DMSO was added (final concentration of 0.2% v/v) to scavenge HOCl.

Oxidation of oMitoPhB(OH)$_2$ by ONOO$^-$

To react oMitoPhB(OH)$_2$ with ONOO$^-$, oMitoPhB(OH)$_2$ (20 µM) was incubated with spermine NONOate (200 µM, generating 0.2 µM/min ‘NO), HX (200 µM), and XO (0.1 mU/mL, 0.2 µM O$_2$ /min) in an O$_2$-saturated phosphate buffer (25 mM, pH = 7.4) containing 0.1 mM dtpa and 5 kU/mL catalase. The deoxygenated stock solutions of all components were mixed under argon atmosphere in a hypoxic chamber (final reaction volume: 200 µL). The incubation was started immediately after mixing by passing oxygen gas ($^{16}$O$_2$ or $^{18}$O$_2$) through the solution for 10 min, followed by 20 min further incubation at room temperature. Incubation in water-$^{18}$O was performed in the presence of 90% (by vol.) of H$_2^{18}$O.

Chlorination of HE by HOCl

The reaction of HE with HOCl and the formation of 2-Cl-E$^+$ was investigated in the presence of H$_2$O$_2$, MPO, and KCl under conditions identical to those described above for the oMitoPhB(OH)$_2$ probe but using HE (50 µM, from a stock solution in EtOH).

Oxidation of HE by O$_2$-$^-$

Conversion of HE into 2-OH-E$^+$ was studied by incubation of HE (20 µM) with HX (200 µM) and XO (0.1 mU/mL, 0.2 µM O$_2$ /min) in an oxygen-saturated phosphate buffer (25 mM, pH = 7.4) containing 0.1 mM dtpa and 5 kU/mL catalase. To better control the type of O$_2$ isotopolog present in the solutions, samples were first deoxygenated to remove $^{16}$O$_2$ and then reoxygenated using $^{16}$O$_2$ or $^{18}$O$_2$. The deoxygenated stock solutions of all components were mixed under argon atmosphere in a hypoxic chamber (final reaction volume: 200 µL). The incubation was started immediately after mixing by passing O$_2$ gas ($^{16}$O$_2$ or $^{18}$O$_2$) through the solution for 10 min, followed by 20 min further incubation at room temperature. To stop the incubation, SOD was added (final concentration: 0.1 mg/mL) and the sample was taken for LC-MS/MS analysis. The addition of SOD at the beginning of incubation resulted in complete inhibition of 2-OH-E$^+$ formation. When using water-$^{18}$O as a solvent, the final concentration of H$_2^{18}$O was 90% (by vol.).

LC-MS/MS analysis of oMitoPhB(OH)$_2$ oxidation products

The oxidation products of oMitoPhB(OH)$_2$ were analyzed using a Shimadzu Nexera2 ultra-HPLC system equipped with UV-visible absorption and LC-MS8030 mass spectrometry detectors (Columbia, MD, USA). The presence of a positive charge (due to the presence of the TPP$^+$ moiety) allows a sensitive detection by mass spectrometry, as reported previously for the MitoB probe (41-43). The incubation mixture was injected into a Raptor Biphenyl column (Restek, Bellefonte, PA, USA; 100 mm × 2.1 mm, 2.7 µm) equilibrated with a mobile phase containing 80% water, 20% MeCN, and 0.1% formic acid. The products were eluted by increasing the content of the MeCN (0.1% formic acid) from 20% to 60% over 5.5 min. The mobile phase flow rate was 0.5 mL/min. Detection events included continuous scanning of the spectra of the eluate, as well as detection of the specific oxidation products in an MRM mode. MRM transitions were as follows: 397>135 for oMitoPhB(OH)$_2$, 369>107 for oMitoPh$^{18}$OH, 371>263 for oMitoPh$^{18}$OH, 398>262 for oMitoPhN$^{16}$O$_2$, 400>262 for oMitoPhN$^{18}$O$^{18}$O, and 351>183 for cyclo-oMitoPh. The MRM transitions of other oxidation products have been reported elsewhere (50,51,79).

LC-MS/MS analysis of HE oxidation products

Detection of HE oxidation products, including 2-Cl-E$^+$ and 2-OH-E$^+$, was performed using a
Shimadzu Nexera2 ultra-HPLC system equipped with UV-visible absorption and LC-MS8030 mass spectrometry detectors. The reaction mixture was injected into a Raptor Biphenyl column (Restek, Bellefonte, PA, USA; 100 mm × 2.1 mm, 2.7 µm) equilibrated with the mobile phase containing 90% water, 10% acetonitrile (MeCN), and 0.1% formic acid. The products were eluted by increasing the content of the organic mobile phase (MeCN, 0.1% formic acid) from 10% to 65% over 4.5 min at the flow rate of 0.4 mL/min. Detection events included continuous scanning of the spectra of the eluate, as well as detection of the specific oxidation products in a multiple reaction monitoring (MRM) mode. MRM transitions for 2-Cl-E+, 2-16OH-E+, and 2-18OH-E+ were 348>320, 330>300, and 332>302, respectively. The MRM transitions of other oxidation products were as previously reported (19,29,79,87).

Data Availability
All data presented and discussed are contained within the manuscript or in the supporting information.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.
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FOOTNOTES

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The abbreviations used are: 2-chloroethidium, 2-Cl-E⁺; 2-hydroxyethidium, 2-OH-E⁺; acetonitrile, MeCN; dichlorodihydrofluorescein, DCFH; dimethyl sulfoxide, DMSO; diethidium, E⁺-E⁺; diethylenetriamine pentaacetic acid, dtpa; ethanol, EtOH; hydroethidine, HE; HE radical cation, HE•⁺; hypoxanthine, HX; liquid chromatography with tandem mass spectrometry, LC-MS/MS; mitochondria-targeted phenyl boronate probe, oMitoPhB(OH)₂; multiple reaction monitoring, MRM; myeloperoxidase, MPO; protein tyrosine phosphatase 1B, PTP1B; reactive oxygen species, ROS; superoxide dismutase, SOD; triphenylphosphonium cationic moiety, TPP⁺; xanthine oxidase, XO
Figure 1. In contrast to commonly used redox probes DCFH, DHR123, Amplex Red, and NBT, spin traps, boronate-based probes, and HE incorporate atoms from the oxidants into the products formed.

Figure 2. Conversion of oMitoPhB(OH)$_2$ boronate probe in the phenolic product, oMitoPhOH, in the presence of nucleophilic two-electron oxidants.

Figure 3. Conversion of HE into 2-OH-E$^+$ by O$_2^-$.

Figure 4. Oxidation of the oMitoPhB(OH)$_2$ probe by H$_2$O$_2$. 
Figure 5. Incorporation of an oxygen atom into the phenolic product during the oxidation of oMitoPhB(OH)$_2$ by H$_2$O$_2$. (a) Chemical structures of the products; (b) online mass spectra of the products; and (c) LC-MS/MS traces of the phenolic products containing $^{16}$O (left panel) or $^{18}$O (right panel). LC-MS/MS analyses were performed after incubation (20 min) of oMitoPhB(OH)$_2$ (20 µM) alone (control), with H$_2^{16}$O$_2$ (10 mM) in H$_2^{18}$O (90%), or with H$_2^{18}$O$_2$ (10 mM) in H$_2^{16}$O.
Figure 6. NaHCO$_3$-enhanced oxidation of oMitoPhB(OH)$_2$ by H$_2$O$_2$ and incorporation of an oxygen atom from HCO$_4^-$ into the phenolic product. (a) Chemical scheme of the formation of HCO$_4^-$ and acid-base equilibria involved; (b) dynamics of the formation of oMitoPhOH in the absence and presence of NaHCO$_3$; (c) relative increase in the yield of oMitoPhOH after 1-h incubation of the probe with H$_2$O$_2$ or H$_2$O$_2$ in the absence and presence of NaHCO$_3$; and (d,e) LC-MS/MS traces of the phenolic products containing $^{16}$O (d) or $^{18}$O (e) atoms. LC-MS/MS analyses were performed after incubation (1 h) of oMitoPhB(OH)$_2$ (1 µM) alone (control), with H$_2$O$_2$ (50 µM, d), or with H$_2$O$_2$ (50 µM, e). All solutions contained 0.1 M phosphate buffer and 0.1 mM dtpa, and the pH of the solutions was adjusted to 7.0.
Figure 7. Incorporation of an oxygen atom into the phenolic product during oxidation of oMitoPhB(OH)2 by HOCl. (a) Method generating HOCl; (b,c) LC-MS/MS traces of the phenolic products containing 16O (b) or 18O (c). LC-MS/MS analyses were performed after incubation (15 min) of oMitoPhB(OH)2 (50 µM) alone (control), with H216O2 (0.1 mM, b), or with H218O2 (0.1 mM, c) in the presence or absence of MPO (20 nM) and KCl (0.1 M). All solutions contained 0.1 M phosphate buffer, pH 7.4. (d) Scheme of the conversion of HE into 2-Cl-E+. (e) Online mass spectrum of 2-Cl-E+ detected from reaction of HE with bolus HOCl. (f) Confirmation of HOCl generation using 2-Cl-E+ marker product. All experimental conditions were the same as described above, but the oMitoPhB(OH)2 probe was replaced by HE (50 µM).
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Figure 8. Oxidation of the oMitoPhB(OH)₂ probe by ONOO⁻.

Figure 9. Incorporation of an oxygen atom into the phenolic and nitrated products during oxidation of oMitoPhB(OH)₂ by ONOO⁻. (a) Chemical structures of the products; (b) online mass spectra of the detected products; and (c) LC-MS/MS traces of the phenolic products containing ¹⁶O (left panel) or ¹⁸O (right panel). LC-MS/MS analyses were performed after incubation (30 min) of oMitoPhB(OH)₂ (20 µM) alone (control), or with in situ-generated ON¹⁶O¹⁶O⁻ or ON¹⁸O¹⁸O⁻. ON¹⁶O¹⁶O⁻ and ON¹⁸O¹⁸O⁻ were produced by cogenerated fluxes of •NO (0.2 µM/min) and ¹⁶O₂⁻ or ¹⁸O₂⁻ (0.2 µM/min), respectively.
Figure 10. Proposed mechanism of incorporation of oxygen atoms into the oxidation and nitration products of oMitoPhB(OH)$_2$ from H$_2$O$_2$, HCO$_4^-$, HOCl, and ONOO$^-$. 
Figure 11. Incorporation of an oxygen atom into the hydroxylated product during oxidation of HE in the presence of $\text{O}_2^-$. (a) Chemical structures of the products; (b) online mass spectra of the products; and (c) LC-MS/MS traces of 2-OH-E$^+$ containing $^{16}\text{O}$ (left panel) or $^{18}\text{O}$ (right panel). LC-MS/MS analyses were performed after incubation (30 min) of HE (20 µM) alone (control) or with $^{16}\text{O}_2^-$ or $^{18}\text{O}_2^-$ (0.2 µM $\text{O}_2^-$/min, generated from HX/XO and $^{16}\text{O}_2$ or $^{18}\text{O}_2$, respectively).

Figure 12. Proposed mechanism of incorporation of oxygen atom from $\text{O}_2^-$ into 2-OH-E$^+$ during oxidation of the HE probe.
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