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Detection and Differentiation Between Peroxynitrite and Hydroperoxides Using Mitochondria-Targeted Arylboronic Acid

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Abstract

The development of boronic probes enabled reliable detection and quantitative analysis of hydrogen peroxide and peroxynitrite. The major product, in which boronate moiety of the probe is replaced by the hydroxyl group, is however common for both oxidants. Here, we describe how *ortho*-isomer of mitochondria-targeted phenylboronic acid can be used to detect and differentiate peroxynitrite-dependent and peroxynitrite-independent probe oxidation. This method highlights the detection and quantification of both the major, phenolic product and the minor, peroxynitrite-specific nitrated product of probe oxidation.

Keywords

Hydrogen peroxide; Peroxynitrite; Mitochondria-targeted probes; Boronic probes; *o*-MitoPhB(OH)₂; HPLC-MS

1 Introduction

Boronate-based probes were developed over the last decade for the detection of hydrogen peroxide (H₂O₂) and peroxynitrite (ONOO[−]) in biological systems [1,2]. Mitochondria-targeted boronate probes were developed to monitor hydrogen peroxide in mitochondria [3–6]. Boronates react directly and stoichiometrically with both H₂O₂ and ONOO[−] [7, 8]. This is in contrast to more classical fluorogenic probes (e.g., dichlorodihydrofluorescein, DCFH, and dihydrorhodamine, DHR), which need the catalysts (e.g., iron, heme proteins) or react with the products of ONOO[−] decomposition [9, 10]. Additionally, in contrast to boronates, both DCFH and DHR form intermediate radical species that reduce oxygen to superoxide radical anion [10, 11]. However, most boronates lack specificity needed to distinguish between hydrogen peroxide and peroxynitrite in a complex biological system, as discussed below [2, 7, 12]. In a simple system, when bolus amounts of the reactants are quickly mixed, this limitation can be overcome due to significant differences of the reaction rate constants. The rate constant of the reaction of boronates with peroxynitrite is six orders of magnitude higher than that of the analogous reaction with hydrogen peroxide [2, 7]. For example, under the conditions when the reaction with ONOO[−] is completed within 100 ms, the reaction with H₂O₂ was not completed even after 12 h [7]. The situation is, however, different when the oxidants react with the probe in biological systems, with the oxidants continuously produced during the incubation with the probe. Therefore, we proposed the use of specific inhibitors

of oxidants formation and/or specific scavengers of the oxidants to differentiate between different species responsible for oxidation of boronate probes in cells [2, 13]. Recently, we reported a formation of both major, phenolic product (*o*-MitoPhOH) and minor, nitrated product (*o*-MitoPhNO₂) during the reaction of peroxynitrite with *ortho*-isomer of mitochondria-targeted phenylboronic acid (*o*-MitoPhB(OH)₂, Fig. 1) [14]. This particular nitrated product is specific for ONOO[−] reaction and is not produced by any other oxidant. Here, we show how this unique chemistry can be utilized to selectively detect peroxynitrite in cellular systems [15]. The method is based on probing the oxidants (ONOO[−] and H₂O₂) in cells by incubation of cells with *o*-MitoPhB(OH)₂ followed by extraction and HPLC-MS/MS analysis of the products formed. We describe the protocol for probe preparation, processing of biological samples, and HPLC-MS-based analysis of the products formed.

2 Materials

2.1 Components for the Synthesis of *o*-MitoPhB(OH)₂

1. 2 g of triphenylphosphine.
2. 200 ml of anhydrous diethyl ether.
3. 1 g of 2-(bromomethyl)phenylboronic acid.
4. 100 ml of ethanol.
5. Argon gas.

2.2 Cell Incubation Components

1. Cell growth medium (for RAW 264.7 cells): DMEM containing 10 % FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.
2. Assay medium: HBSS supplemented with 25 mM HEPES (pH 7.4) and dtpa (0.1 mM) (*see* Note 1).
3. Solution of 50 mM *o*-MitoPhB(OH)₂ (for the description of synthesis, *see* Subheading 3.1) in DMSO.

2.3 Cell Extraction Components

1. Cell lysis buffer: 10 ml DPBS containing 0.1 % Triton X-100 spiked with 4 μM ethyltriphenylphosphonium cation (TPP⁺-C₂), place on ice (can be stored at 4 °C) (*see* Note 2).
2. 100 ml of ice-cold acetonitrile.
3. 10 ml of ice-cold acetonitrile containing 4 μM TPP⁺-C₂, place on ice (can be stored at 4 °C).

¹The medium used may be selected according to experimental design and cell culture needs. However, it is preferred that when monitoring extracellular ONOO[−] and/or H₂O₂, the components of the medium capable of scavenging those oxidants should be avoided, if possible. For example, pyruvate in the medium may efficiently compete with boronate probes for H₂O₂.

²Ethyltriphenylphosphonium cation (TPP⁺-C₂) is used as an internal standard to compensate for any sample loss during the preparation of samples for analysis and for any changes in detector sensitivity over the course of batch analysis. If significant changes in the peak area of TPP⁺-C₂ are observed, normalize the results to TPP⁺-C₂ peak area.

4. Protein assay reagent (Bradford reagent).
5. BSA in lysis buffer: 20 mg/ml BSA. Prepare a series of BSA solutions by serial dilutions with the final concentrations of 0.5, 1.0, 1.5, 2, 3, 4, 5, 7, and 10 mg/ml. Keep the solutions on ice.

2.4 HPLC Analysis Components

1. HPLC mobile phase: 0.1 % formic acid in water (mobile phase A) and 0.1 % formic acid in acetonitrile (mobile phase B) (*see* Note 3).
2. Solution of *o*-MitoPhNO₂ (50 mM) in DMSO (*see* Note 4).
3. Solution of *o*-MitoPhB(OH)₂ (50 mM) in DMSO.
4. Solution of *o*-MitoPhOH (50 mM) in DMSO (*see* Note 4).
5. Water-to-acetonitrile (3:1) mixture spiked with 1 μM TPP⁺-C₂. This will be a solvent for the preparation of standards for HPLC calibration.
6. Mixture of the standards of *o*-MitoPhB(OH)₂, *o*-MitoPhOH, and *o*-MitoPhNO₂ (0.1 mM each) in water-to-acetonitrile (3:1) mixture containing 0.1 % formic acid spiked with 1 μM TPP⁺-C₂. Prepare serial dilutions down to 1 nM concentration. Use the solvent for standards prepared in the previous step.

3 Methods

3.1 Preparation of *o*-MitoPhB(OH)₂

1. The preparation of the *o*-MitoPhB(OH)₂ probe should be carried out inside a hood with well-working ventilation!
2. Prepare a solution of triphenylphosphine (1.048 g, 4.0 mmol) in anhydrous diethyl ether (30 ml).
3. Add 2-(bromomethyl)phenylboronic acid (0.856 g, 4.0 mmol) with constant stirring under argon atmosphere.
4. Stir the reaction mixture for 46 h (using magnetic stirrer) at room temperature under argon atmosphere.
5. A crystalline precipitate of bromide salt of *o*-MitoPhB(OH)₂ should be obtained.
6. Filter the suspension. Keep the solid and discard the filtrate.
7. Wash the solid with anhydrous diethyl ether (30 ml). Filter the suspension again and keep the solid.

³LC-MS (preferably UHPLC-MS) grade solvents and formic acid should be used. After preparation, mobile phase should be passed through 0.2 μm filter. Prepare only the amount of mobile phase, which is necessary for the experiment. Do not store the mobile phase for longer than 2–3 days to avoid any growth of the biological matter.

⁴The standards of the products, *o*-MitoPhOH and *o*-MitoPhNO₂, are commercially available but can be also synthesized. The phenolic product can be prepared by reaction *o*-MitoPhB(OH)₂ with hydrogen peroxide, followed by addition of catalase to remove excess H₂O₂. The nitrated product can be synthesized in an analogous protocol as described for *o*-MitoPhB(OH)₂, but starting with 2-nitrobenzyl bromide instead of 2-(bromomethyl) phenylboronic acid.

8. Dissolve the solid in ethanol and precipitate the compound back by addition of diethyl ether (1:1). Filter the powder and keep the solid. Repeat this step once more.
9. Leave the solid to dry. A white powder should be obtained with the melting point of 225–226 °C. Test the identity and purity of the synthesized probe (*see* Note 5).

3.2 Cell Incubation with the Probe

1. Prepare the cells according to the experimental conditions to be tested for peroxynitrite formation (*see* Note 1).
2. Add *o*-MitoPhB(OH)₂ to obtain the final concentration of 50 μM (*see* Notes 6 and 7).
3. Incubate the cells for 1 h.
4. Collect an aliquot of the medium (0.1 ml) in 1.5 ml microcentrifuge tube and freeze in liquid nitrogen.
5. Remove the rest of medium and wash the cells twice with ice-cold DPBS.
6. Add 1 ml of ice-cold DPBS and harvest the cells, place the cell suspension in 1.5 ml microcentrifuge tube, and spin down the cells by quick centrifugation. Remove the supernatant and freeze the cell pellet in liquid nitrogen.
7. Frozen cell pellets and media can be stored at –80 °C for at least 1 week before analysis.

3.3 Extraction of the Products

3.3.1 Cell Pellets

1. Preload one set of 1.5 ml microcentrifuge tubes with 0.1 ml of acetonitrile and place on ice.
2. Preload a second set of 1.5 ml microcentrifuge tubes with 0.1 ml of 0.1 % formic acid in water and place on ice.
3. Prepare a clear-bottom 96-well plate for protein assay and place on ice.
4. Place the tubes with frozen cell pellets on ice.
5. Add 0.2 ml of the lysis buffer spiked with TPP⁺-C₂ (4 μM) and lyse the cells by ten syringe strokes using a 0.5 ml insulin syringe with the needle 28 G × 0.5 in. (0.36 mm × 13 mm).

⁵Purity of synthesized *o*-MitoPhB(OH)₂ should be tested by HPLC and the compound repurified, if needed. The identity should be confirmed by nuclear magnetic resonance (NMR) analysis. ¹H NMR (Bruker 250 MHz, DMSO-d₆) δ_{ppm}: 8.18 (s, ²H, *B*-OH), 7.91–7.98 (m, ⁷H), 7.72–7.79 (m, ⁶H), 7.34–7.38 (m, ²H), 7.07–7.12 (m, ¹H), 5.43 (d, ²H, *J* = 15.5 Hz, *CH*₂). Mass spectrometry analysis: 396.1561 ([*M* – 1]⁺, 22 %), 397.1529 ([*M*]⁺, 100 %), 398.1568 ([*M* + 1]⁺, 25 %), 399.1569 ([*M* + 2]⁺, 4 %) [14].

⁶The concentration of *o*-MitoPhB(OH)₂ used for probing of ONOO[–] should be chosen so as it does not interfere with mitochondrial function. We did not observe significant effects of 50 μM *o*-MitoPhB(OH)₂ on the rate of oxygen consumption by RAW 264.7 cells.

⁷When exposing cells to *o*-MitoPhB(OH)₂, it is preferred to add the medium containing the probe, rather than directly adding a solution of concentrated *o*-MitoPhB(OH)₂ in DMSO, to avoid local exposure of cells to high concentrations of DMSO.

6. Transfer 0.1 ml of the cell lysates into the tubes containing ice-cold acetonitrile, vortex for 10 s, and place on ice. Transfer $3 \times 2 \mu\text{l}$ of the cell lysate aliquots into 3 wells on 96-well plate for the protein assay.
7. Incubate the mixtures of cell lysates with acetonitrile for 30 min on ice.
8. During incubation, measure the protein concentration in the cell lysates using Bradford assay and plate reader with absorption detection (*see* Note 8).
9. Vortex the tubes again for 5 s and centrifuge for 30 min at $20,000 \times g$ at 4 °C.
10. Place the tubes back on ice and transfer 0.1 ml aliquots of the supernatants into the second set of tubes, containing 0.1 % formic acid in water.
11. Vortex the tubes for 5 s and centrifuge for 15 min at $20,000 \times g$ at 4 °C.
12. Transfer 0.15 ml of the supernatants into HPLC vials preloaded with conical inserts, seal the vials, and place on ice. Once all solutions have been transferred, place the vials in HPLC autosampler precooled to 4 °C.

3.3.2 Media

1. Preload one set of 1.5 ml microcentrifuge tubes with 0.1 ml of 0.1 % formic acid in water and place on ice.
2. Place the tubes with frozen media on ice.
3. Add 0.1 ml of the ice-cold acetonitrile spiked with $\text{TPP}^+\text{-C}_2$ (4 μM) to each tube, vortex for 10 s, and place on ice.
4. Incubate the mixtures of media with acetonitrile for 30 min on ice.
5. Vortex the tubes again for 5 s and centrifuge for 30 min at $20,000 \times g$ at 4 °C.
6. Place the tubes back on ice and transfer 0.1 ml aliquots of the supernatants into the tubes containing 0.1 % formic acid in water.
7. Vortex the tubes for 5 s and centrifuge for 15 min at $20,000 \times g$ at 4 °C.
8. Transfer 0.15 ml of the supernatants into HPLC vials preloaded with conical inserts, seal the vials, and place on ice. Once all solutions have been transferred, place the vials in HPLC autosampler precooled to 4 °C.

3.4 HPLC-MS/MS Analysis of the Extracts

1. Install the column Kinetex Phenyl-Hexyl 50 mm $\times$ 2.1 mm, 1.7 μm (Phenomenex) in the HPLC-MS/MS system. The column should be equipped with a UHPLC column filter or guard column to extend the column lifetime.
2. Equilibrate the column with the mobile phase (75 % of mobile phase A and 25 % mobile phase B).

⁸If plate reader with absorption detection is not available, protein measurements can be carried out using regular spectrophotometer. The volume of cell lysate needed for the assay may be higher, depending on the volume of the spectrophotometer cell.

3. Set up the HPLC-MS/MS method and detection parameters according to Tables 1 and 2, respectively (*see Note 9*).
4. Test the system by three injections of standards (10 μ M) for the reproducibility of retention times and peak intensities for all analytes and internal standard (TPP⁺-C₂), as shown in Fig. 2.
5. Run the analysis of the batch of samples.
6. Include the system and column wash with water-to-methanol (1:1) mixture at the end of batch.
7. Quantify each analyte based on the specific multiple reaction monitoring (MRM) transitions and calibration curves constructed in the concentration range relevant to the samples analyzed (*see Note 10*).
8. When appropriate, normalize the concentrations of analytes to the protein levels in cell lysates, as determined by Bradford method.
9. Increase in peak intensities of both *o*-MitoPhOH and *o*-MitoPhNO₂ (Fig. 3) indicates the formation of ONOO⁻, while formation of *o*-MitoPhOH, but not *o*-MitoPhNO₂ (Fig. 4), indicates the presence of other oxidants, most commonly H₂O₂ (*see Note 11*).

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⁹To protect the detector, only portion of eluate is flowed into mass detector. This is achieved by using a diverter valve, which directs the flow into waste before 0.7 min and after 2.5 min after injection. Between 0.7 and 2.5 min, the flow is directed into the detector and the signals recorded.

¹⁰For each compound, two MRM transitions (pairs of parent ion/daughter ion) are recorded. The primary (dominant) transition is used for quantification, while the secondary (reference) transition is used for confirmation of the identity of the analyte. If the ratio of reference to dominant MRM transitions (Table 2) is outside the range allowed (typically $\pm 30\%$), the peak is rejected and not used for quantification. Due to similar *m/z* values of *o*-MitoPhB(OH)₂ and *o*-MitoPhNO₂, there is a small peak of the boronate appearing in the channel of the nitro derivative (Fig. 2, a peak at 1.55 min), which may show up as the dominant peak, when *o*-MitoPhB(OH)₂ is in high excess comparing to *o*-MitoPhNO₂ (Fig. 3). This peak is not however observed in the reference channel, exemplifying the usefulness of the reference transitions for peak identification.

¹¹The identity of the oxidizing species can be tested by the application of specific inhibitors and/or scavengers [13, 15]. Confirmation of peroxynitrite involvement may be obtained by testing the inhibitory effects of inhibitors of nitric oxide synthase, for example, L-NAME, as shown in Fig. 3. Similarly, the inhibitory activity of catalase on the yield of *o*-MitoPhOH (Fig. 4) indicates the involvement of H₂O₂.

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Fig. 1. Scheme of oxidation of *o*-MitoPhB(OH)₂. *o*-MitoPhB(OH)₂ is oxidized to *o*-MitoPhOH by both ONOO⁻ and H₂O₂. However, *o*-MitoPhNO₂ is formed only in reaction between *o*-MitoPhB(OH)₂ and ONOO⁻ as the minor product

Fig. 2. HPLC-MS/MS detection of *o*-MitoPhB(OH)₂, *o*-MitoPhOH, and *o*-MitoPhNO₂. The chromatograms have been obtained with the described method by the injection of 10 μ l of the mixture of *o*-MitoPhB(OH)₂, *o*-MitoPhOH, and *o*-MitoPhNO₂ (1 μ M each). For each compound, two MRM transitions are shown: dominant (*solid lines*) and reference (*dashed lines*). The traces have not been scaled and thus represent the actual intensities of each MRM transition in the equimolar mixture

Fig. 3.

HPLC-MS/MS analysis of the extracts of RAW 264.7 macrophages activated to produce peroxynitrite. To produce peroxynitrite, RAW 264.7 cells were pretreated overnight with interferon- γ (IFN, 50 U/ml) and lipopolysaccharide (LPS, 1 μ g/ml) followed by addition of phorbol 12-myristate 13-acetate (PMA, 1 μ M) in the presence or absence of L-NAME (1 mM) [13]. During stimulation with PMA, *o*-MitoPhB(OH)₂ (50 μ M) was present and incubated for 1 h. Cell pellets were collected and processed as described in the protocol. The intensities of *o*-MitoPhOH signals were multiplied by a factor of 5 and intensities of *o*-MitoPhNO₂ signals were multiplied by 1,000 to fit the same scale as of *o*-MitoPhB(OH)₂. *Solid lines* represent the dominant transitions used for quantification, and the *dashed lines* represent the reference transitions used for confirmation of peak identity

Fig. 4.

HPLC-MS/MS analysis of the media of neutrophil-like cells activated to produce hydrogen peroxide. To produce hydrogen peroxide, HL60 cells differentiated for 4 days with all-*trans* retinoic acid were stimulated with PMA (1 μ M) in the presence and absence of catalase (1 kU/ml) and co-treated with *o*-MitoPhB(OH)₂. Aliquots of media were collected after 30 min and processed and analyzed as described in the protocol, but with twofold lower concentration of the internal standard (TPP⁺-C₂). The intensities of the peaks for different analytes have been scaled in the same manner as described for Fig. 3

Table 1

HPLC method parameters

Flow rate	0.5 ml/min		
Gradient	0 min	75 % A	25 % B
	2 min	68.3 % A	31.7 % B
	2.5 min	0 % A	100 % B
	3 min	0 % A	100 % B
	3.5 min	75 % A	25 % B
Diverter valve	0 min	Waste	
	0.7 min	Detector	
	2.5 min	Waste	

Table 2

MS/MS detection parameters

Analyte	Dominant MRM transition	Reference MRM transition	Reference MRM to dominant MRM intensities ratio	Retention time (min)
TPP ⁺ -C ₂	291.10 > 182.95	291.10 > 107.95	0.55	1.12
<i>o</i> -MitoPhB(OH) ₂	397.00 > 135.00	397.00 > 379.05	0.80	1.55
<i>o</i> -MitoPhOH	369.00 > 107.10	369.00 > 183.05	0.40	1.85
<i>o</i> -MitoPhNO ₂	397.90 > 262.05	397.90 > 351.10	0.25	1.98