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On the use of peroxy-caged luciferin (PCL-1) probe for bioluminescent detection of inflammatory oxidants *in vitro* and *in vivo* – Identification of reaction intermediates and oxidant-specific minor products

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ABSTRACT

Peroxy-caged luciferin (PCL-1) probe was first used to image hydrogen peroxide in living systems (Van de Bittner et al., 2010 [9]). Recently this probe was shown to react with peroxynitrite more potently than with hydrogen peroxide (Sieracki et al., 2013 [11]) and was suggested to be a more suitable probe for detecting peroxynitrite under *in vivo* conditions. In this work, we investigated in detail the products formed from the reaction between PCL-1 and hydrogen peroxide, hypochlorite, and peroxynitrite. HPLC analysis showed that hydrogen peroxide reacts slowly with PCL-1, forming luciferin as the only product. Hypochlorite reaction with PCL-1 yielded significantly less luciferin, as hypochlorite oxidized luciferin to form a chlorinated luciferin. Reaction between PCL-1 and peroxynitrite consists of a major and minor pathway. The major pathway results in luciferin and the minor pathway produces a radical-mediated nitrated luciferin. Radical intermediate was characterized by spin trapping. We conclude that monitoring of chlorinated and nitrated products in addition to bioluminescence *in vivo* will help identify the nature of oxidant responsible for bioluminescence derived from PCL-1.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have emerged as important mediators of cellular signaling and damage [1–3]. ROS and RNS comprise of different species of very diverse chemical reactivity, lifetime and target specificity in extracellular and intracellular milieu [4,5]. The term 'ROS' typically refers to superoxide radical anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), lipid peroxy radicals ($LOO^{\bullet}$), lipid hydroperoxides (LOOH), and singlet oxygen ($^1O_2$) and 'RNS' refers to peroxynitrite ($ONOO^-$) and nitrogen dioxide radical ($^{\bullet}NO_2$). Rigorous identification of those species is crucial for understanding their role in cellular signaling and pathology.

Molecular imaging of ROS/RNS is an emerging area of research in redox and free radical biology [6–8]. Bioluminescence or fluorescence modalities are typically used. Peroxy-caged luciferin (PCL-1) (Fig. 1) is one of the first cell-permeable small molecular weight probes used to image ROS in living systems [9,10]. H_2O_2 slowly reacts with PCL-1 probe ($k = 1.2\ M^{-1}\ s^{-1}$ [11]) to form luciferin *in situ* that is oxidized by the luciferase enzyme (using ATP as a co-factor) emitting a green bioluminescent signal [9,10] (Fig. 1). Upon oxidation, PCL-1 probe eliminates the *para*-quinone methide (QM), with the formation of luciferin which gets oxidized to oxyluciferin in luciferase-transfected cells generating bioluminescence (Fig. 1). It was shown that administration of bolus H_2O_2 to mice over-expressing luciferase increased the bioluminescent signal from PCL-1 [9,10]. Subsequently, PCL-1 was shown to be oxidized to luciferin in the presence of hypochlorite (HOCl) and $ONOO^-$ [11]. Importantly, it was shown that in the presence of plasma, the probability of oxidation of PCL-1 probe by H_2O_2 is very low; however, under this condition, the probe was still oxidized by

Abbreviations: AA-OOH, amino acid hydroperoxide; DIPPMPO, diisopropoxyphosphoryl-5-methyl-1-pyrroline N-oxide; DPI, diphenyleneiodonium; HO-Bz-OH, 4-hydroxybenzyl alcohol; L-NAME, L-N^G-Nitroarginine methyl ester; $LOO^{\bullet}$, lipid peroxy radicals; LOOH, lipid hydroperoxide; Luc-Cl, chloroluciferin; MNP, 2-methyl-2-nitroso propane; MRM, multiple reaction monitoring; PCL-1, peroxy-caged luciferin; Pr-OOH, protein hydroperoxide; QM, *para*-quinone methide; RNS, reactive nitrogen species; ROS, reactive oxygen species; SIM, single ion monitoring

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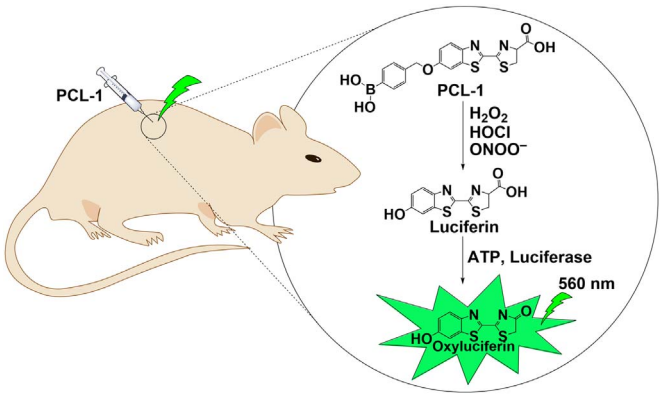

Fig. 1. A hypothetical scheme showing the application of PCL-1 probe for bioluminescent detection of H_2O_2, HOCl or $ONOO^-$ in luciferase-transfected mice tumor xenografts.

$ONOO^-$. This is consistent with our previous reports demonstrating that $ONOO^-$ reacts directly and rapidly with boronate probes forming corresponding phenols as the major products. This brings into question the identity of the oxidant(s) responsible for *in vivo* bioluminescence measurements using PCL-1 probe.

We have demonstrated that in addition to H_2O_2, other biologically-relevant oxidants, including HOCl and $ONOO^-$, are able to oxidize aromatic boronates to the corresponding phenolic products [12–15]. More recently, we have shown that selected amino acid hydroperoxides (AA-OOH) and protein hydroperoxides (Pr-OOH) also oxidize boronic compounds [16]. With all of the oxidants tested so far, the mechanism appears to be similar; a 1:1 stoichiometry between oxidant and boronate probes was observed, resulting in the same major product. However, the main difference is the rate constant of the reaction between different oxidants and the boronate probe. The rate constants varied from 10^0, 10^1, 10^4 to 10^6 M^{-1} s^{-1} for H_2O_2, AA-OOH, HOCl and $ONOO^-$, respectively [13,16]. Therefore, depending on the experimental settings, boronates may be used to detect different oxidants by monitoring the reaction products. Unlike other listed oxidants, $ONOO^-$ oxidizes boronic compounds in two pathways: major (~90%), non-radical pathway, leading to the corresponding phenol; and minor (~10%), radical pathway, forming a phenyl-type radical, nitrogen dioxide ($^{\bullet}NO_2$) and stable products formed from them [12,13,17]. We propose that these $ONOO^-$-specific products may serve as specific markers for $ONOO^-$. By determining the $ONOO^-$-specific products, we recently confirmed the formation of $ONOO^-$ from nitroxyl (HNO) reaction with O_2 [18] and tested the effect of inhibition of NADPH oxidase on the production of $ONOO^-$ by activated macrophages [19].

Here we investigate in detail the products formed from the oxidative and nitrative chemistry of PCL-1 that will help to better interpret *in vivo* bioluminescence results. We compared the products formed during the oxidation of PCL-1 by H_2O_2, HOCl and $ONOO^-$, the likely *in vivo* inflammatory oxidants. As several oxidant species react with PCL-1 to generate bioluminescence, the oxidant-specific minor product(s) may be used to confirm the identity of ROS/RNS species.

2. Materials and methods

2.1. Chemicals, preparation of solutions

PCL-1 probe was synthesized as described below. D-Luciferin (potassium salt) was purchased from Gold Biotechnology. H_2O_2 and HOCl were from Sigma-Aldrich. $ONOO^-$ was synthesized as described elsewhere [12] and stored at −80 °C. L-NAME and DPI were from Cayman. All other chemicals were from Sigma-Aldrich and were of highest purity available. The stock solutions of $ONOO^-$, HOCl and H_2O_2 were prepared freshly each day and the concentration was determined by spectrophotometry, using the extinction coefficients values of 1.7×10^3 M^{-1} cm^{-1} (at 302 nm, in 0.1 M NaOH), 350 M^{-1} cm^{-1} (at 292 nm, in 0.1 M NaOH) and 39.4 M^{-1} cm^{-1} (at 240 nm, in water), respectively. PCL-1 stock solution (1 mM) was typically prepared using ethanol (EtOH) as a solvent to minimize scavenging of HOCl by DMSO, a solvent typically used for boronate probes. Of the four organic solvent (EtOH, acetonitrile, DMSO and DMF) tested for interference with HOCl-induced oxidation of coumarin boronic acid (CBA) to hydroxycoumarin (COH), EtOH exhibited the smallest inhibitory effect (Suppl. Fig. 1). For the spin trapping of phenyl radical, DMSO was used to prepare the stock solution of PCL-1 to avoid scavenging of phenyl radical by EtOH. 5-Diisopropoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DIPPMPO) was synthesized according to the published procedure [20].

2.2. HPLC analyses

HPLC analyses of PCL-1 and its oxidation products were performed using Agilent 1100 HPLC equipped with UV–vis absorption and fluorescence detectors. The compounds were loaded onto Kinetex C_{18} column (Phenomenex, 100 mm × 4.6 mm, 2.6 μm) equilibrated with 10% of acetonitrile in water, containing 0.1% trifluoroacetic acid. The products were eluted by an increase of the acetonitrile concentration from 10-100% over 7 min. The flow rate was kept at 1.5 mL/min. PCL-1 and the products containing luciferin moiety were detected by monitoring absorbance at 330 nm, and the product of water added to QM was detected at 220 nm. Additionally, luciferin was also monitored using the fluorescence detector with the excitation set at 330 nm and emission set at 520 nm.

2.3. LC-MS analyses

LC-MS analyses of PCL-1, its oxidation products and spin adducts were performed using Shimadzu LC-MS 8030 triple quadrupole mass detector coupled to Shimadzu Nexera 2 UHPLC system. The reaction mixture was injected on Cortecs C_{18} column (Waters, 50 mm × 2 mm, 1.6 μm) equilibrated with 10% of acetonitrile in water containing 0.1% of formic acid. The compound was eluted by increasing the acetonitrile concentration in the mobile phase from 10-80% over 4 min. The flow rate was set at 0.5 mL/min, and the flow was diverted to waste during the first minute and after 4 min, counting from the time of injection. PCL-1, luciferin, Luc-Bz-NO_2, Luc-Bz-H and Luc-Cl were detected as positive ions using multiple reaction monitoring (MRM) mode, using the primary/fragment ion pairs of 415 > 135, 281 > 235, 416 > 234, 371 > 91 and 315 > 269, respectively. Luc-Bz-OH was detected in positive mode using single ion monitoring (SIM), set at the *m*/*z* value of 387.

2.4. EPR spin-trapping

EPR spin trapping experiments were performed using Bruker EMX EPR spectrometer, as reported previously [17]. The instrument parameters were as follows: scan range, 150 G; time constant, 1.28 ms; scan time, 84 s; modulation amplitude, 1 G; modulation frequency, 100 kHz; receiver gain, 1×10^5; and microwave power, 20 mW. The spectra shown are the averages of 5 scans.