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Bioluminescent detection of peroxynitrite with a boronic acid-caged luciferin

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Peroxynitrite, a highly reactive biological oxidant, is formed in pathophysiologic conditions from the nearly diffusion limited reaction of nitric oxide and superoxide radical anion. Peroxynitrite has been implicated as the mediator of nitric oxide toxicity in many diseases, and as an important signaling molecule.¹ Biosensors effective in capturing peroxynitrite in a specific and fast enough manner for detection, along with readout compatible with *in vivo* studies are lacking. Here we report that the boronic acid-based bioluminescent system, PCL-1 (Peroxy-Caged Luciferin-1), previously reported as a chemoselective sensor for hydrogen peroxide,² detects peroxynitrite stoichiometrically with a rate constant of $5.84 \pm 0.05 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and a detection limit of 16 nM, compared to values of $7.8 \pm 0.1 \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$ and 231 nM for hydrogen peroxide. Further, we demonstrate that in the presence of physiologic antioxidants glutathione, albumin and catalase, PCL-1 is a specific sensor for peroxynitrite, reflecting the relative reaction rates of each oxidant with these biocompounds. We also demonstrate the utility of this method in mammalian cells by measuring peroxynitrite generated in under normal culture conditions after stimulation of macrophages with the bacterial endotoxin lipopolysaccharide. Thus, the PCL-1 method for measuring peroxynitrite generation shows superior selectivity over other oxidants for the investigation of peroxynitrite biology in *in vivo* conditions.

Introduction

Peroxynitrite (ONOO^-), the product of the diffusion-limited reaction between nitric oxide (NO) and superoxide radical anion ($\text{O}_2^{\bullet-}$)³, has received considerable attention as the mediator of the deleterious effects of NO overproduction in numerous pathologic conditions^{4,5}, as the toxic product of NO quenching by superoxide radicals in the vasculature^{6,7} and the antiseptic utilized by macrophages to defend the organism from pathogens⁸. Differently from its biologic precursors (NO and $\text{O}_2^{\bullet-}$) peroxynitrite is a powerful oxidant and an efficient nitrating agent capable of altering protein structure^{9,10}, enzyme function^{11–15} and initiating lipid peroxidation¹⁶. Part of the reactivity of peroxynitrite is due to its rapid decomposition to strong free radical oxidants at appreciable yields, either spontaneously at physiological pH¹⁷ or by its reaction with the physiologically ubiquitous carbon dioxide (CO_2)^{18,19}. Expectedly, the high reactivity of peroxynitrite in the physiologic and cellular milieu (which limits its lifetime to fractions of a second, Fig. 1) has

posed a significant challenge to peroxynitrite detection in cells and *in vivo*. Nitrotyrosine, once considered a biomarker of peroxynitrite formation *in cellulo* and *in vivo*²⁰, has been demonstrated to be produced by numerous alternative pathways of biological relevance not requiring the oxidant, dismissing the concept that an unequivocal biomarker for peroxynitrite formation could be found^{21,22}. To date, the very concept that peroxynitrite is produced in biological systems is disputed by numerous investigators, despite abundant indirect evidence, because of the inability to detect its production directly. The detection of peroxynitrite, therefore, requires the development of probes that can compete with the rapid reaction of peroxynitrite with CO₂, peroxiredoxins, glutathione (GSH), and heme proteins (possibly the most significant scavengers of peroxynitrite *in vivo*). Real time signal generation would also be desirable so to avoid purification, isolation and measurement of stable products in complex biological samples that predictably would be produced in extremely low yields.

Boronates are remarkable in their capacity to specifically react with peroxynitrite. Seminal work by Kalyanamaran and colleagues^{23–26} has demonstrated the potential of such compounds to become accurate and sensitive sensors of peroxynitrite in cultured cells when stimulated to elevate both NO and O₂^{•−} levels. Here, we expanded this concept and demonstrate that PCL-1, previously reported as a biosensor for H₂O₂, produces luciferin rapidly and stoichiometrically in the presence of peroxynitrite (schematically represented in Fig. 2). Our findings indicate that PCL-1 becomes a specific sensor of peroxynitrite when in the presence of the biologically ubiquitous molecules CO₂, GSH, albumin, or catalase. This finding indicates that PCL-1 may be used in conjunction with luciferase to detect and quantify peroxynitrite formation *in vivo* in real time. Our studies also indicate that the detection of peroxynitrite by PCL-1 is kinetically favored over the detection of H₂O₂ and that PCL-1 should be considered a selective peroxynitrite sensor when utilized in cells and *in vivo*.

Materials and methods

Materials

All reagents were purchased from Sigma Aldrich or ACROS unless otherwise stated. Recombinant luciferase was purchased from Promega (Madison, WI). UV-Vis spectra, kinetic data, and bioluminescence data were obtained using a Spectramax M5 spectrophotometer from Molecular Devices (Sunnyvale, CA). PCL-1 was synthesized according to literature procedure², freeze-dried, and stored in aliquots in DMSO at −80 °C protected from light. Purity was assessed via HPLC analysis and identity was confirmed via mass spectroscopy on a Shimadzu IT-TOF mass spectrometer at the UIC Research Resources Center (Fig. S2). Luciferin was quantified via UV-Vis ($\epsilon_{385} = 18200 \text{ M}^{-1}\text{cm}^{-1}$ at pH 12). Hydrogen peroxide solutions were prepared in Millipore water (18.2 mOhm) from a 33% stock solution, and quantified prior to use via UV-Vis ($\epsilon_{240} = 43.6 \text{ M}^{-1}\text{cm}^{-1}$ in deionized water). PCL-1 was quantified prior to use via UV-Vis ($\epsilon_{320} = 19450 \text{ M}^{-1}\text{cm}^{-1}$ in PBS, pH 7.4 with 5% DMSO). Sodium peroxynitrite was generated using a quenched-flow reactor according to literature²⁷. Peroxynitrite solutions in concentrated NaOH were stored over MnO₂ at −80 °C for up to 1 month prior to thaw on ice and quantification by UV-Vis

($\epsilon_{302} = 1670 \text{ M}^{-1}\text{cm}^{-1}$ in 10 mM NaOH). Solutions of peroxynitrite in 1.0 mM NaOH were added directly to buffered reaction mixtures. Solutions of sodium hypochlorite were prepared in PBS buffer (pH 7.4) from a 5% stock.

Determination of reaction rate with stopped-flow spectroscopy

Kinetic studies were performed at 23 ± 1 °C using an Applied Photophysics SX-18 stopped flow mixing unit in single mixing mode. Fluorescence detection was accomplished by irradiating with monochromatic 360 nm light and observing emission greater than 400 nm using a cut-off filter. In the kinetic runs, equal amounts of oxidant and substrate solutions were mixed in 50 mM PBS (pH 7.4), and the increase in fluorescence was followed. Blank studies involving mixing PCL-1 or peroxynitrite solutions with buffer solutions resulted in no appreciable fluorescence signal. For studies of oxidations with peroxynitrite, the signal growth was biphasic (double exponential growth). For studies of oxidations with H_2O_2 , single exponential kinetics was observed.

Luciferase assays

Luciferin formation was quantified by addition of recombinant luciferase to the reaction mixture in a white opaque 96-well plate (Falcon). To a solution of 200 μL PCL-1 (5 μM) in TBS buffer with 5% DMSO (pH 7.4) was added 100 μL of a solution of recombinant luciferase (100 $\mu\text{g}/\text{mL}$) containing 10 mM MgCl_2 and 2 mM ATP in TBS, at pH 8.0. After mechanical shaking for 5 seconds, the bioluminescence was measured at 612 nm in 30 sec increments (100 msec integration time) over 30 minutes. The area under the curve was proportional to the luciferin concentration, and was defined as the photon flux (RLU/sec).

Determination of limit of detection and limit of quantification

Limit of detection was determined according to a $3'$ corresponding to a 99% confidence interval. 20 'blank' wells were prepared by addition 200 μL of 5 μM PCL-1 in 5% DMSO in TBS (pH 7.4) to wells of a white opaque 96-well plate. 20 'sample' wells were prepared by addition of a small amount of oxidant (2–10 times the signal from the blank) to the same solution. This corresponded to 50 nM peroxynitrite and 250 mM for H_2O_2 . After 30 minutes, the 20 wells were treated with a 100 μL solution of recombinant luciferase (100 mg/mol) containing 10 mM MgCl_2 and 2 mM ATP in TBS, at pH 8.0 and bioluminescence was measured at 612 nm every 30 seconds (100 msec integration time) over 30 minutes. Limit of detection was determined following the equation $c_L = ks_{bl}S$; where c_L is the limit of detection, k is the desired confidence interval, s_{bl} is the standard deviation of the blank measurements, and S is the sensitivity. Limit of quantification is defined as 10 times standard deviation of the 20 blank measurements.

Isolation of mouse bone marrow-derived macrophages

All experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee at University of Illinois, Chicago. Bone marrow was aspirated from femurs and tibias of 6–12 week old mice using complete RPMI medium and cultured in 10% L929 cell supernate. L929 cells (ATCC) were cultured in complete DMEM (Cellgro) for up to 3 weeks and culture supernate was then sterile filtered and frozen until

use, diluted to 10% in RPMI medium (Cellgro). All media was made complete with 10% FBS, penicillin, streptomycin, and glutamine (Gibco)). Cells were cultured 4–6 days prior to plating for experiments in opaque, white microtiter plates (Falcon) for analysis on a Wallac Victor2 luminometer (PerkinElmer).

Quantification of BH₄

Cells were washed with PBS and lysed in 50mM phosphate buffer (pH=2.6) containing 0.1 mM DTT and 0.1 mM DTPA, then centrifuged at 13,000 rpm, 4°C for 15 min. After centrifugation, supernant was transferred into a Millipore Amicon Ultra 3K centrifugal filter and spun down at 13,000 rpm, 4°C for 15 min. The flow-through samples were analyzed on a Beckman System Gold HPLC system equipped with UV, fluorescence detectors and ESA Coulochem III, using a Synergi Polar-RP (Phenomenex, 4μm, 250×4.6mm) eluted with 50mM phosphate buffer (pH=2.6, 0.1 mM DTT and 0.1 mM DTPA) at flow rate of 1.0 ml/min. BH₄ was detected on ESA Coulochem III using a high-sensitivity 5011A dual channel detector. Channel one was set at -160 mv to verify the reversibility of oxidized BH₄. Channel two was set at 270 mV for quantification of BH₄.

Results and discussion

Peroxy-caged luciferin-1 (PCL-1) detects peroxynitrite rapidly and stoichiometrically

In light of recent reports of superior reactivity of arylboronic acids with peroxynitrite over hydrogen peroxide^{23–25} (10⁴–10⁶-fold increases), we investigated the chemoselectivity of a boronic acid-based hydrogen peroxide sensor reported by Chang, et. al.² PCL-1 has been previously reported to be insensitive towards nitric oxide (NO), superoxide radical anion (O₂^{•-}), HOCl and singlet oxygen but has not been evaluated in the context of peroxynitrite production.

Treatment of PCL-1 with 1 equivalent of peroxynitrite, or *in-situ* generated peroxynitrite from combination of NO and O₂^{•-} from 3-morpholinosydnonimine (SIN-1), for 60 minutes while following A₃₈₅ resulted in an electronic absorption spectrum which overlays with that of authentic luciferin (Fig. 3A, Fig S1). To confirm the formation of luciferin, recombinant luciferase was added to the reaction mixture and bioluminescence was recorded as outlined in the Materials and methods section (Fig. S3). We observed peak bioluminescence after addition of approximately 6 μM peroxynitrite, consistent with a stoichiometric 1:1 ratio of sensor to peroxynitrite (Fig. 3B). With a bioluminescent output, we calculated a detection limit of 16 nM for peroxynitrite after incubation at 37 °C for 30 minutes, compared to that of 231 nM with hydrogen peroxide (Table 1).

Kinetics studies of reactions of PCL-1 with peroxynitrite and hydrogen peroxide were performed by stopped-flow mixing methods with fluorescence detection. In these studies, solutions of substrate and oxidant were mixed in PBS buffer at pH 7.4 and the increase in fluorescence from luciferin was followed. Fig. 3C shows results for reactions of peroxynitrite and H₂O₂ with PCL-1, respectively. Reactions of peroxynitrite with PCL-1 were biphasic, whereas reactions of hydrogen peroxide with PCL-1 were clearly pseudo-first-order.

The reaction of 10 μM ONOO^- with 20 μM PCL-1 had observed rate constants of $k_{\text{obs}1} = 8.76 \pm 0.02 \times 10^{-2} \text{ s}^{-1}$ and $k_{\text{obs}2} = 1.56 \pm 0.07 \times 10^{-3} \text{ s}^{-1}$. We assume that the faster reaction reflects the reaction of authentic ONOO^- with PCL-1 and that the slower reaction, detectable due to the sensitivity of fluorescence based methods, is from reaction of traces of H_2O_2 with nitrite, the by-product of the ONOO^- reaction, to regenerate ONOO^- that reacts with more PCL-1. From $k_{\text{obs}1}$, the second-order rate constant for reaction of ONOO^- with PCL-1 is $k = 5.84 \pm 0.05 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$.

The reaction with 0.1 M H_2O_2 with 20 μM PCL-1 was cleanly pseudo-first-order. The observed rate constant was $k_{\text{obs}} = 7.8 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$. This gives a second-order rate constant of $k = 7.8 \pm 0.1 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$, which is five-orders of magnitude lower than the rate constant for ONOO^- reaction with PCL-1. A six order of magnitude difference in reactivity was previously found for the rate constants for oxidation of coumarin-7-boronic acid by ONOO^- and H_2O_2 ²⁴. The coumarin-7-boronic acid (CBA) oxidation rate constants are $1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $1.5 \text{ M}^{-1} \text{ s}^{-1}$ for reactions with ONOO^- and H_2O_2 , respectively, factors of 200 and 20 times greater than the respective rate constants for reactions of PCL-1. In CBA, oxygen substitution on the aromatic ring bonded to boron is expected to withdraw electron density increasing the positive charge on boron and its reactivity with the nucleophilic oxidant.

We detected nearly quantitative yield of luciferin from treatment of PCL-1 with excess H_2O_2 and SIN-1, and slightly more than 80% yield with simultaneous treatment with DEANO and KO_2 (Fig 4A, PCL-1+NO+ $\text{O}_2^{\bullet-}$). Treatment with DEANO (NO-donor) or KO_2 ($\text{O}_2^{\bullet-}$ source) alone did not result in appreciable yield of luciferin. In addition, we show that PCL-1 is not a substrate for the recombinant luciferase. Importantly, we show that catalase inhibits only H_2O_2 -derived luciferin formation but not that produced by SIN-1 (peroxynitrite donor).

Addition of glutathione or albumin blunts hydrogen peroxide detection PCL-1 in vitro

The environment inside most cellular compartments is reducing, a status granted by the multiple antioxidant systems that rapidly detoxify oxidants in cells, such as thioredoxins, glutathione/glutathione reductase redox systems^{28,29} and catalase. Thus, to test whether PCL-1 serves as a sensor of either H_2O_2 or peroxynitrite in *in vivo* relevant conditions, we measured the yield of luciferin from PCL-1 in slight excess to added H_2O_2 , SIN-1, and NaONOO, each in the presence of increasing amounts of glutathione ($k_{\text{ONOO}} = 1.4 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ ⁽³⁰⁾) and bovine serum albumin ($k_{\text{ONOO}} = 8.31 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ ⁽³¹⁾) via UV-Vis spectroscopy. Competition experiments were performed at pH 8.5 to take advantage of stronger absorbance of luciferin product than at pH 7.4 ($\epsilon_{385} = 8156 \pm 252 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 8.5 and $\epsilon_{385} = 1200 \pm 92 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 7.4).

In the case of BSA, a dose dependent decrease in luciferin yield was observed when H_2O_2 was used as the oxidant, resulting in only ~4–5% yield luciferin in the presence of plasma levels of BSA (~50 mg/mol). In stark contrast, we detected ~20% and 38% yield from the same concentration of SIN-1 and NaONOO, respectively, in the presence of 50 mg/mol BSA (~1.0 mM) (Fig. 4B, experiment repeated at pH 7.4, Fig. S5). Similarly, GSH, which is present at mM concentrations in cells, significantly reduced H_2O_2 -induced conversion of

PCL-1 into luciferin at the concentration of 50 μM (Fig. 4C) and abrogated conversion when used at the physiologically relevant concentrations of 1–10 mM. Peroxynitrite was capable of producing substantial yield of luciferin across the entire range of concentrations tested (Fig 4C).

A major scavenger for cellular peroxynitrite is dissolved carbon dioxide. This critical component of the blood plasma bicarbonate/ CO_2 buffer system has been shown to scavenge peroxynitrite, with a rate constant of $5.8 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$.³² We measured the yield of luciferin upon treatment of PCL-1 with bolus peroxynitrite or SIN-1 in the presence of increasing partial pressured of dissolved CO_2 , tuned with solutions of various concentrations of bicarbonate buffer held at pH 7.4 (Fig. 4D). After conversion of reaction solutions to pH 8.5 for quantification, we show that even with concentrations of HCO_3^- of 100 mM, nearly double that of normal human patient blood (10–40 mM), we detected 82 % and 71 % yield luciferin after addition of bolus peroxynitrite or SIN-1, respectively demonstrating further that the sensor can detect peroxynitrite in the presence of GSH, albumin, catalase and CO_2 .

Superiority of PCL-1 as a detector of peroxynitrite over H_2O_2 in plasma or in the presence of red blood cells

Production of luciferin from PCL-1 depends on the nucleophilic reaction of either peroxynitrite (ONOO^-) or peroxide (HOO^-) anions with the boronate group of PCL-1 to release the pre-immobilized phenol that decays generating luciferin (Fig. 2). In principle, although slower due to the low abundance of HOO^- at physiologic pH, both peroxynitrite and H_2O_2 reacting with PCL-1 will quantitatively produce luciferin at equivalent yields in the absence of competing reactants that consume H_2O_2 or peroxynitrite at near neutral pH provided enough time for the completion of the reactions is provided. The question that arises is whether under biologically relevant conditions H_2O_2 will be stable enough to slowly convert PCL-1 into luciferin before being degraded by competing reactants. To address this question we devised an experiment in which SIN-1, authentic peroxynitrite, H_2O_2 produced in flow (by glucose/glucose oxidase) or added as a bolus reacted with PCL-1 in plasma or in buffer to which red blood cells (RBCs) were added at low density (1–10%), compared to 50–60% in blood. Since *in vivo*, it is impossible to find environments devoid of biologic fluids containing proteins or antioxidants or cells, this experiment was designed to test whether H_2O_2 would be stable enough, even in buffer to which only 1% of red blood cells were added to produce enough luciferin, thereby furnishing sufficiently strong grounds to defy our conclusion that under *in vivo*-relevant conditions, PCL-1 is an exclusive detector of peroxynitrite. We found (Fig. 5) that when matched flows of either H_2O_2 (produced by glucose (5 mM)/glucose oxidase) or peroxynitrite (produced by SIN-1) set to 1 $\mu\text{M}/\text{min}$ for 15 minutes were produced in the presence of PCL-1, only under complete absence of cells was PCL-1 able to detect H_2O_2 . In fact, even at 1% density (v/v) RBCs were able to completely ablate PCL-1 conversion to luciferin by glucose/glucose oxidase. Curiously, RBC also considerably reduced PCL-1 conversion to luciferin induced by SIN-1 (Fig. 5). To explain this surprising finding we hypothesized that release of superoxide dismutase by trace hemolysis of RBC induced by the oxidants, or quenching of the freely diffusible NO by hemoglobin, could account for the inhibition of peroxynitrite formation. Thus, in Fig. 5B, we repeated the experiments utilizing bolus addition of authentic H_2O_2 or peroxynitrite.

Qualitatively, the findings in Fig. 5B confirmed the results shown in Fig. 5A in the sense that H_2O_2 , in the presence of 1% RBC (v/v) was unable to convert PCL-1 to luciferin, while the conversion of PCL-1 to luciferin was nearly quantitative when peroxynitrite was used. In Fig. 5C, further experiments are shown demonstrating that the yield of luciferin produced when PCL-1 is exposed to glucose/glucose oxidase is considerably lower than that achieved when SIN-1 is used, reflecting the fairly distinct kinetics of the reactions between the oxidants and PCL-1. Predictably, the use of catalase or the repetition of the experiment in cell-free plasma completely ablated luciferin production from PCL-1 induced by glucose/glucose oxidase. In the case of SIN-1 only the addition of superoxide dismutase (MnSOD) that prevents peroxynitrite production by scavenging superoxide radicals was able to ablate luciferin production. This experiment indicated that in aqueous solution the only plausible scenario where PCL-1 functions as a biosensor of both peroxynitrite and H_2O_2 is in cell free buffer.

Detection of iNOS-dependent production of peroxynitrite in bone marrow-derived macrophages under normal culture conditions

In order to validate the utility of PCL-1 in detecting peroxynitrite in cells under normal culture conditions, we stimulated mouse bone marrow-derived macrophages with bacterial LPS (lipopolysaccharide) for 22 hours. In macrophages, LPS induces the expression of iNOS, a high output NO synthase that has been proposed to produce peroxynitrite in chronic inflammatory diseases^{33,34}. We added PCL-1 to the culture for two hours to allow for the reaction with peroxynitrite, and quantified luciferin generated over this period by addition of recombinant luciferase. As shown in Fig. 6, a nearly two-fold increase of luciferin production is observed over the basal value of unstimulated cells. Macrophages from iNOS-knockout mice deficient in iNOS yielded no signal above baseline (Fig. 6). This demonstrates that the species contributing to luciferin production was produced in an iNOS-dependent manner. Taken together with the fact that PCL-1 is insensitive to NO and $\text{O}_2^{\bullet-}$, the iNOS dependence of luciferin production from PCL-1 in the presence of macrophages permitted us to conclude peroxynitrite is produced from iNOS without further stimulation for the activation of $\text{O}_2^{\bullet-}$ production. Indeed, we confirmed this possibility using gp91phox deficient macrophages obtained from gp91phox-knockout mice. Stimulation of gp91phox deficient cells produced nearly the same results as wild type cells demonstrating that in this system NADPH oxidase is not required for peroxynitrite production (at least not at 22h post LPS stimulation, Fig. 7). To our knowledge, this is the first detection of peroxynitrite in a cellular system that was not activated to produce NO and $\text{O}_2^{\bullet-}$ simultaneously from different sources. This observation further argues for the superiority of PCL-1 over other methodologies for peroxynitrite detection that have required a 'double-hit' type of stimulation to maximize the concentration of the oxidant. It also suggests that PCL-1 can detect peroxynitrite production even in the presence of excess NO.

We also investigated the cause of iNOS-dependent peroxynitrite formation in stimulated macrophages. L-arginine supplementation slightly increased peroxynitrite production from iNOS in LPS-stimulated macrophages indicating that substrate deprivation is not a cause of iNOS dysfunction in this system (Fig. 7). Media supplementation with sepiapterin, a precursor of tetrahydrobiopterin (BH_4) synthesis efficiently dampened peroxynitrite

formation indicating that cofactor depletion produces iNOS uncoupling and consequentially peroxynitrite. Curiously, supplementation with authentic BH_4 bolstered peroxynitrite production either in the presence or in the absence of L-arg. According to previously published studies^{35,36} we hypothesized that the rapid oxidation of BH_4 by reactive nitrogen species continuously produced by the activated macrophages produced BH_2 accumulation which has been shown to further uncouple a closely related NOS isoform eNOS, elevating peroxynitrite levels. Consistent with the hypothesis that peroxynitrite formation under our experimental conditions arises from cofactor deficiency, in Fig. 7B, we show that BH_4 supplementation contrary to sepiapterin does not increase intracellular BH_4 concentrations in macrophages. Thus, we reason based on our data and cited studies that in the absence of enough substrate (L-arg) or cofactor (intracellular BH_4) iNOS withdraws electrons from NADPH but is unable to complete the full catalytic cycle, thus releases electrons directly to molecular oxygen, increasing $\text{O}_2^{\bullet-}$ and consequentially peroxynitrite production. Our evaluation of the activated macrophage system with PCL-1 not only permitted to demonstrate this principle but to determine the cause of peroxynitrite formation and estimate the extent to which substrate and/or cofactor can blunt this effect.

Conclusions

Recent discoveries established boronates as a first generation of biological peroxynitrite detectors. This study aimed at defining whether PCL-1, a boronate-based compound previously characterized as a H_2O_2 biosensor, could be used as a tool for the detection and quantification of peroxynitrite. It also aimed at determining how specific, sensitive and potentially useful PCL-1 is towards serving as a biosensor for either H_2O_2 and/or peroxynitrite in *in vivo*-relevant conditions.

Taken together with previous data our studies demonstrated that PCL-1 fulfills all the requirements to be classified as an exceptionally efficient peroxynitrite sensor in a variety of biologically relevant systems. Fig. 3 and Fig. 4 showed that PCL-1 reacts rapidly with peroxynitrite to produce luciferin. PCL-1 competes with several important biologic scavengers of peroxynitrite used at concentrations that are representative of most pathophysiologic conditions. Importantly, because it releases luciferin, PCL-1 is likely to be instrumental for the real time imaging of peroxynitrite formation *in vivo*.

PCL-1 is insensitive towards NO, superoxide radical anion $\text{O}_2^{\bullet-}$, HOCl (shown in Fig. S4) and singlet oxygen². Among the biologically relevant oxidants the only one that presented appreciable reactivity towards PCL-1, other than peroxynitrite, was H_2O_2 . In the particular case of H_2O_2 , we confirmed, in this study, that *in vitro* and in the absence of GSH, albumin or catalase, PCL-1 is quantitatively converted to luciferin. Nevertheless, the much higher reactivity of H_2O_2 with biological antioxidants present at millimolar concentrations in cells and in biological fluids makes it very unlikely that any appreciable reaction of PCL-1 with H_2O_2 in cells or *in vivo* will occur. Indeed, we showed that in the presence of GSH, albumin or catalase, H_2O_2 -mediated conversion of PCL-1 to luciferin is negligible while enough luciferin is produced by peroxynitrite reactivity with PCL-1 under all tested conditions to permit the detection of the oxidant. Also, we did not observe significant degradation of PCL-1-derived luciferin over the course of experimentation, even in the absence of added

antioxidants (Fig. S1). Further evidence that PCL-1 is specific for peroxynitrite detection under *in vivo* relevant conditions came from experiments shown in Fig. 5, where the compound was reacted with H₂O₂ and peroxynitrite, either authentic or produced in flow. In the experiments shown in Fig. 5 evidence was gathered to demonstrate that only in pure buffer, H₂O₂ can efficiently convert PCL-1 into luciferin. Reactions performed in biological fluid (plasma) or performed in the presence of freshly prepared RBC at exceedingly low concentrations (1%, v/v) completely eliminated H₂O₂-derived while not reducing the yield of luciferin produced by authentic peroxynitrite. Comparisons between the effects of RBC in incubations where SIN-1 or peroxynitrite were used together with the measured rate constants for the reaction of peroxynitrite with PCL-1 indicate that trace amounts of intracellular contents (presumably superoxide dismutase and hemoglobin, which interfere with peroxynitrite formation from NO and O₂^{•-}) contributed to dampened luciferin production by SIN-1. Although the type of cells used in this study was of a particular type (RBCs are rich in hemoglobin, and antioxidant enzymes), the extremely low % of cells resuspended in solution argue for the fact that PCL-1 utilization for H₂O₂ assessments in cells and *in vivo* would be challenging and certainly would require controls employing systems devoid of NOS activity and replicates in the presence of catalase.

Importantly, we took advantage of this novel direct technique to assess peroxynitrite to demonstrate that the oxidant is generated by long-term activated macrophages from NOS. Indeed, previous studies that showed peroxynitrite formation in cellular systems utilized mechanisms of peroxynitrite formation in which iNOS expression was induced prior to a second stimulation typically with phorbol-myristate-acetate (PMA)³⁷ that rapidly elicits O₂^{•-} by NADPH oxidases. In our experiments controls utilizing gp91phox excluded the possibility that O₂^{•-} from NADPH oxidases is required for peroxynitrite formation what together with parallel experiments using iNOS devoid cells strongly indicate that peroxynitrite can be produced from a single source, i.e. uncoupled iNOS.

In summary, our results show that PCL-1 is a specific, sensitive, quantitative and versatile biosensor for the detection of peroxynitrite. It is likely to become instrumental in the elucidation of NO/O₂^{•-} and peroxynitrite roles in health and disease.

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Fig. 1. Boronic acids react with peroxynitrite on par with cellular targets

The lifetime of peroxynitrite in cells is determined by rapid reaction with dissolved carbon dioxide, activated cysteine residues and metalloproteins. Boronic acids offer a bio-orthogonal reactive group and fast reaction rate. Rate constants were obtained between 25 °C and 37 °C in the pH range 7.0 to 7.5. (Boronic acids (^{23,24}), carbon dioxide (³⁸), nitric oxide (³⁹), peroxiredoxins (^{40,41}), aconitase (¹²), hemethiolate proteins(⁴²), glutathione(³⁰), decomposition (³⁹), protein tyrosine phosphatases (¹³).

Fig. 2.

Proposed mechanism for detection of peroxynitrite by a peroxy-caged luciferin (PCL-1), and release of luciferin (adapted from ref ²).

Fig. 3. PCL-1 reacts rapidly with peroxynitrite and converts stoichiometrically to Luciferin

A) Electronic absorption spectrum of Luciferin (5 μM) and PCL-1 (5 μM) in TBS (pH 8.5) along with treatment of PCL-1 with either peroxynitrite (10 μM) or SIN-1 (100 μM) for 60 minutes at 37 $^{\circ}\text{C}$. Where applicable, spectral contributions from oxidants were subtracted via a parallel blank experiment containing no PCL-1. **B)** Plot of photon flux vs. [peroxynitrite] after treatment of 6.1 μM PCL-1 in TBS (pH 7.4) with increasing concentrations of peroxynitrite for 30 minutes and subsequent addition of recombinant luciferase, according to the Materials and methods section. **C)** Fluorescence signal increase from reaction of 10 μM PCL-1 with 20 μM peroxynitrite and 0.1 M hydrogen peroxide. The reactions were performed at 23 ± 1 $^{\circ}\text{C}$ in 50 mM phosphate buffer (pH 7.4). The experimental data is shown as grey lines, and the fits (double exponential for ONOO^- and single exponential for H_2O_2) are dashed black lines. Residuals for the data and the fits are shown below the growth plots.

Fig. 3. PCL-1 detects peroxynitrite selectively and in the presence of physiologic milieu components. **A)** Plot of yield of luciferin after treatment of PCL-1 (5 μM) with H_2O_2 (100 μM) or SIN-1 (100 μM), with or without catalase (500 $\mu\text{g}/\text{mL}$), NO (66 mM DEANO)), or $\text{O}_2^{\bullet-}$ (100 μM KO_2) or both DEANO and KO_2 for 60 minutes, and subsequent addition of recombinant luciferase to the reaction mixture according to Materials and methods. Data were normalized to bioluminescence obtained from an identical treatment of authentic luciferin (5 μM). **(B–D)** Plot of total luciferin obtained (A_{385}) from treatment of 25 μM PCL-1 with either H_2O_2 , SIN-1, or NaONOO (24 μM) for 90 minutes in the presence of increasing concentrations of BSA, glutathione, and CO_2 relative to condition with no

oxidant. In all cases a spectral contributions from oxidants were subtracted via a parallel blank experiment containing no PCL-1.

Fig. 4. Conversion of PCL-1 to luciferin by H₂O₂ and peroxynitrite in cell-free and RBC enriched milieu

A) Glucose (5mM)/glucose oxidase (titrated to produce H₂O₂ at 1μM/min) and SIN-1 (2.4 μM) were incubated with PCL-1 (2.5μM) at 25 °C for 15 minutes at pH 7.4. Reactions were stopped by the addition of catalase (to incubations containing G/GO at 50μg/ml) or MnSOD (50μg/ml added to incubations containing SIN-1). Prior to GO or SIN-1 addition, freshly prepared RBC washed 5 × with cold PBS was added at the indicated concentrations. Experiments are averages of three independent replicates. **B)** PCL-1 (2.5μM) was reacted with peroxynitrite (2.5μM) and H₂O₂ (2.5μM) in phosphate buffer pH 7.4 at 25°C. Plasma was collected after whole blood centrifugation and stored on ice until use. **C)** Same as A but incubations were performed for 1 h in plasma or in phosphate buffer pH 7.4 in the presence the indicated compounds. Luminescence was measured by mixing resulting incubations with luciferase (100μg/ml) in 10 mM MgCl₂ and 2 mM ATP in TBS, pH 8.0.

Fig. 5. Detection of iNOS-derived peroxynitrite in mouse bone marrow derived macrophages
 Plot of bioluminescence upon treatment of bone marrow-derived macrophages from wild-type or iNOS^{-/-} mice with LPS (50 ng/mL) for 22 hours, addition of PCL-1 (5 μ M) for an additional 2 hours, and finally addition of recombinant luciferase (100 μ g/mL) and quantification of bioluminescence at 612 nm over 2 hours. (***) = $p < 0.005$)

Fig. 6. Peroxynitrite detection in activated BMDM macrophages

Plot of bioluminescence upon treatment of bone marrow-derived macrophages with LPS (50 $\mu\text{g/mL}$) for 22 hours, addition of PCL-1 (5 μM) for an additional 2 hours, and finally addition of recombinant luciferase (100 $\mu\text{g/mL}$) and quantification of bioluminescence over 2 hours. (***) = $p < 0.005$). Different supplementations and cell types are marked in the figure. L-NAME was 1 mM.

Fig. 7. Quantification of intracellular tetrahydrobiopterin in BMDM

Cells were washed with PBS 4 times and lysed; Debris was immediately separated by filtration and the BH₄ content quantified by HPLC coupled to electrochemical detection. BH₄ and sepiapterin were 100 μM supplemented in the medium 24 h prior to lysis. LPS was 50 ng/ml. Experiment was performed under the exact same conditions of the experiment shown in Fig. 6.

Table 1

Rate constants and bioluminescent limit of detection and limit of quantification for PCL-1.

Oxidant	Rate Constant ($M^{-1}sec^{-1}$) [*]	L.O.D. ^{**} (nM)	L.O.Q. ^{***} (nM)
ONOO ⁻	$5.84 \pm 0.05 \times 10^3$	16	56
H ₂ O ₂	$7.8 \pm 0.1 \times 10^{-2}$	231	763

* Rates determined at 23 °C in 50 mM PBS, pH 7.4

** Limit of detection, as defined in Materials and methods.

*** Limit of quantification, as defined in Materials and methods.