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**REACTION WITH BORONATE-BASED** 

**FLUORESCENT PROBE** 

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# Peroxynitrite Is the Major Species Formed from Different Flux Ratios of Co-generated Nitric Oxide and Superoxide

DIRECT REACTION WITH BORONATE-BASED FLUORESCENT PROBE\*S\*

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There is much interest in the nitration and oxidation reaction mechanisms initiated by superoxide radical anion  $(O_2)$  and nitric oxide ('NO). It is well known that  $O_2^{\overline{2}}$  and 'NO rapidly react to form a potent oxidant, peroxynitrite anion (ONOO<sup>-</sup>). However, indirect measurements with the existing probes (e.g. dihydrorhodamine) previously revealed a bell-shaped response to co-generated 'NO and  $O_2^{\overline{}}$  fluxes, with the maximal yield of the oxidation or nitration product occurring at a 1:1 ratio. These results raised doubts on the formation of ONOO<sup>-</sup> per se at various fluxes of 'NO and O2. Using a novel fluorogenic probe, coumarin-7-boronic acid, that reacts stoichiometrically and rapidly with ONOO<sup>-</sup> ( $k = 1.1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ ), we report that ONOO<sup>-</sup> formation increased linearly and began to plateau after reaching a 1:1 ratio of co-generated 'NO and  $O_2^{\overline{2}}$  fluxes. We conclude that ONOO<sup>-</sup> is formed as the primary intermediate during the reaction between 'NO and  $O_2^{\overline{i}}$  co-generated at different fluxes.

Peroxynitrite (ONOO<sup>-</sup>) is an unstable intermediate formed from the diffusion-controlled reaction between nitric oxide ('NO) and superoxide radical anion ( $O_2^{-}$ , Reaction 1,  $k_1 = (0.38 - 1.6) \times 10^{10} \text{ m}^{-1} \text{s}^{-1}$ ) (1–6).

 $NO + O_2^{-} \rightarrow ONOO^{-}$ REACTION 1

The early indication of the occurrence of this reaction in biological systems came from the report on the inhibitory effect of  $O_2^-$  on the activity of endothelium-derived relaxing factor (7). After endothelium-derived relaxing factor identity was established as 'NO (8, 9), its scavenging by  $O_2^-$  was first proposed as a contributing factor to endothelial injury (10). Reaction 1 has great physiological significance as both 'NO and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, the product of dismutation of  $O_2^-$ ) act as important second messengers in redox cell signaling (11, 12).

In the absence of scavengers, ONOO<sup>-</sup> decomposes at neutral pH via protonation to peroxynitrous acid ( $pK_a = 6.7$ , Reaction 2) to yield nitrate ( $NO_3^-$ ) and free radical intermediates:



$$ONOO^- + H^+ \rightleftharpoons ONOOH$$
  
REACTION 2

 $\begin{array}{c} \mathsf{ONOOH} \rightarrow \mathsf{HNO}_3(\sim\!70\%); \ensuremath{\cdot}\mathsf{OH} + \ensuremath{\cdot}\mathsf{NO}_2(\sim\!30\%) \\ \\ \mathrm{REACTION} \ 3 \end{array}$ 

In most biological systems, carbon dioxide is a likely scavenger of ONOO<sup>-</sup>, yielding a short-lived nitrosoperoxycarbonate anion (ONOOCO<sub>2</sub><sup>-</sup>, Reaction 4,  $k_4 = 2.9 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$  (14)). During the decomposition of ONOOCO<sub>2</sub><sup>-</sup>, nitrate and carbon dioxide are formed, as well as nitrogen dioxide radical and carbonate radical anion (Reaction 5) (2, 13, 15).

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^-$$
  
REACTION 4

 $\begin{array}{c} \mathsf{ONOOCO}_2^- \rightarrow \mathsf{NO}_3^- + \mathsf{CO}_2(\sim\!65\%); \mathsf{CO}_3^- + `\mathsf{NO}_2(\sim\!35\%) \\ \\ \mathrm{REACTION} \ 5 \end{array}$ 

Due to the occurrence of Reactions 4 and 5, as well as the scavenging by peroxiredoxins or oxyhemoglobin in specific subcellular compartments, the lifetime of ONOO<sup>-</sup> in biological systems is limited to only a few milliseconds (2, 13). The current methodologies for detection of ONOO<sup>-</sup> are based on the detection of radical species formed from ONOO<sup>-</sup> decomposition, *i.e.*  $NO_2$  and  $CO_3^{-}$  or OH, using tyrosine that forms nitrotyrosine  $(TyrNO_2)$  as a marker product of intracellular 'NO<sub>2</sub> and dihydrorhodamine 123 (DHR)<sup>2</sup> as a fluorogenic probe for oxidants ('NO<sub>2</sub>, 'OH,  $CO_3^{-}$ ). However, 'NO<sub>2</sub> radical formed from the ONOO--independent processes, e.g. via myeloperoxidasecatalyzed oxidation of nitrite by H<sub>2</sub>O<sub>2</sub> (16), could make data interpretation more tenuous (17, 18). Additional problems with this indirect approach may arise from alternate mechanisms through which TyrNO<sub>2</sub> can be formed without the involvement of  $NO_2$ radicals (19). DHR can be oxidized to the fluorescent rhodamine molecule by various one-electron oxidants, including compounds I and II of peroxidases (20, 21).



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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S8, Table S1, and references.

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<sup>&</sup>lt;sup>2</sup> The abbreviations used are: DHR, dihydrorhodamine 123; CBA, coumarin-7-boronic acid; CBE, coumarin-7-boronic acid, pinacolate ester; COH, 7-hy-droxycoumarin; PAPA-NONOate, (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate; X, xanthine; XO, xanthine oxidase; SOD, superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; HPLC, high pressure liquid chromatography.

# Quantitation of ONOO<sup>-</sup> from the Reaction between 'NO and $O_2^{-}$



FIGURE 1. Scheme showing the conversion of CBA and CBE into the fluorescent product, COH.

Previous reports suggest that oxidative and nitrative modifications of tyrosine and DHR observed in the presence of cogenerated 'NO and  $O_2^-$  in cell-free and cellular systems displayed a characteristic bell-shaped response with maximal response occurring at a 1:1 ratio of 'NO to  $O_2^-$  (22–26). Although these findings could implicate that ONOO<sup>-</sup> is formed at the maximal yield only at the 1:1 ratio of 'NO/ $O_2^-$ , one could also question the interpretations because of the confounding effects of radical-radical interactions from free radical species derived from ONOO<sup>-</sup> decomposition and probe-derived radicals (tyrosyl radical or rhodamine radical) (22, 27–29). Clearly, there is an urgent need for developing direct probe(s) for ONOO<sup>-</sup> that will enable us to understand the chemical and biological interactions of 'NO with  $O_2^-$ .

We have recently shown that boronic compounds (boronic acids and their esters) react stoichiometrically with ONOO<sup>-</sup>, yielding the corresponding hydroxylated compounds as the major products (30). We have proposed that boronate groups attached to the fluorogenic probes may be used in the detection of ONOO<sup>-</sup> both in cell-free and in cellular systems. As the rate constant of the reaction of arylboronates with ONOO<sup>-</sup> is relatively high ( $\sim 10^6 \text{ M}^{-1} \text{s}^{-1}$  at pH 7.4), it can outcompete other reactions resulting from the decay of ONOO<sup>-</sup> and can be used to monitor ONOO<sup>-</sup> levels under different conditions.

Here we report the development of a novel fluorescent probe for ONOO<sup>-</sup> and resolve a long standing controversy with regard to the identity, reaction profile, and yields of oxidant formed from varying ratios of 'NO to  $O_2^{-}$  fluxes. We synthesized and employed the boronate-based fluorogenic probe, namely coumarin-7-boronic acid (CBA, Fig. 1), to monitor ONOO<sup>-</sup> formed under varying fluxes of  $O_2^-$  and 'NO. Contrary to previous results obtained with tyrosine and DHR probes (22-26), we report in this study, using a boronate probe, that ONOO<sup>-</sup> is the major species formed under various flux ratios of NO and  $O_2^{-1}$ and that there is no bell-shaped response in ONOO<sup>-</sup> formation. We conclude that the bell-shaped response previously reported during the reaction between co-generated 'NO and  $O_2^{\overline{2}}$  is due to the free radical chemistry of the probe employed (tyrosine and dihydrorhodamine), which does not totally reflect the actual yield of ONOO<sup>-</sup> formation.

## **EXPERIMENTAL PROCEDURES**

*Materials*— $H_2O_2$  was from Fluka, xanthine oxidase (XO), and superoxide dismutase (SOD) from bovine erythrocytes were from Roche Diagnostics, catalase was from Roche Applied

Science, and PAPA-NONOate ((*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate) was from Cayman Co. DHR was from AnaSpec Inc. All other chemicals were from Sigma-Aldrich and were of highest purity available. All solutions were prepared using the deionized water (Millipore Milli-Q system). ONOO<sup>-</sup> was prepared by reacting nitrite with H<sub>2</sub>O<sub>2</sub>, according to the published procedure (31). The concentration of ONOO<sup>-</sup> in alkaline aqueous solutions (pH > 12) was determined by measuring the absorbance at 302 nm ( $\epsilon = 1670$ M<sup>-1</sup>cm<sup>-1</sup>). The pinacolate ester of coumarin boronic acid (CBE) was synthesized following the procedure described elsewhere (32). CBA was prepared by acidic hydrolysis of CBE.

*UV-visible Absorption and Fluorescence Measurements*— The UV-visible absorption spectra were collected using an Agilent 8453 spectrophotometer equipped with a diode array detector and thermostated cell holder. Fluorescence spectra were collected using the PerkinElmer Life Sciences LS 55 luminescence spectrometer. The kinetic absorption and fluorescence measurements were carried out at room temperature using the same instruments.

Determination of  $O_2^{-}$  and 'NO Fluxes—'NO fluxes were determined from the measured rate of the decomposition of PAPA-NONOate by following the decrease of its characteristic absorbance at 250 nm ( $\epsilon = 8.1 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ). Under the conditions used, the 'NO donor decomposed with the rate constant of  $(2.5 \pm 0.2) \times 10^{-4}$  M<sup>-1</sup>s<sup>-1</sup> as determined at 25 °C. The rate of decay of PAPA-NONOate was multiplied by a factor of two to obtain the rate of 'NO release, assuming that two molecules of 'NO are released during the decomposition of one molecule of PAPA-NONOate (33). The stoichiometry of 'NO release was confirmed by performing the oxyhemoglobin assay (34) under the conditions of complete decomposition of PAPA-NONOate (10 and 20  $\mu$ M) in the presence of excess of oxyhemoglobin (60  $\mu$ M). The flux of  $O_2^{\overline{}}$ , generated by xanthine oxidase-catalyzed oxidation of xanthine to uric acid, was determined by monitoring the ferricytochrome c reduction and the increase in absorbance at 550 nm (using a difference in the values of the extinction coefficients between reduced and oxidized cytochrome of 2.1 ·  $10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (35)).

Stopped-flow Measurements—Stopped-flow kinetic experiments were performed on Applied Photophysics 18MX stopped-flow spectrophotometer equipped with photomultipliers for absorption and fluorescence measurements. The thermostatted cell (25 °C) with a 10-mm optical pathway was used for kinetic measurements. For determining the rate constant, the reaction was carried out under pseudo first-order conditions (greater than 10-fold excess of boronate probe over ONOO<sup>–</sup>). For the fluorescence measurements, the cut-off filter (transmitting the light longer than 400 nm) was placed between the cell and the detector.

*HPLC Analysis*—The CBA and 7-hydroxycoumarin (COH) were separated on an HPLC system Agilent 1100 equipped with fluorescence and UV-visible absorption detectors. Typically, 100  $\mu$ l of sample was injected into the HPLC system equipped with a C<sub>18</sub> column (Alltech, Kromasil, 250 × 4.6 mm, 5  $\mu$ m) equilibrated with 10% acetonitrile (CH<sub>3</sub>CN) (containing 0.1% (v/v) trifluoroacetic acid) in 0.1% trifluoroacetic acid aqueous solution. The compounds were separated by a linear increase in





 $CH_3CN$  phase concentration from 10 to 55% over 15 min at a flow rate of 1 ml/min. Under those conditions, CBA eluted at 9.5 min, and COH eluted at 10.7 min. The concentrations of CBA and COH were calculated based on the peak areas detected by absorptions at 280 and 324 nm, respectively. Although COH can also be detected with a high sensitivity using the fluorescence detection (excitation at 332 nm and emission at 475 nm), at the concentrations used, the absorption detection was sufficient for reliable quantitation. Additionally, under the HPLC conditions used, the concentration of uric acid (2.8 min) and xanthine (3.7 min) could be also quantitated based on the peak areas detected by monitoring the absorption at 280 nm.

*Kinetic Simulations*—The simulation of the inhibitory effect of SOD on the conversion of CBA into COH and on the steadystate concentration of 'NO was carried out using a freely available software, Kintecus, version 3.95 (36). The kinetic model used in this study is a modification of the published model of peroxynitrite decay (37). The list of the chemical reactions, rate constants, and major modifications used in the simulation is shown in supplemental Table S1.

#### RESULTS

Oxidation of Coumarin Boronate by  $ONOO^-$  and  $H_2O_2$ -First, we investigated the stoichiometry and kinetics of the reaction between CBA and ONOO<sup>-</sup> or H<sub>2</sub>O<sub>2</sub>. Both oxidants converted the boronate probe into a fluorescent product that was visually examined under UV light illumination (supplemental Fig. S1). The UV-visible absorption spectra (supplemental Figs. S2 and S3) of the product formed in both reactions indicated the formation of a single species with spectral characteristics similar to that of COH. The fluorescence spectra observed upon oxidation of CBA by ONOO<sup>-</sup> were consistent with the formation of COH as the major product (Fig. 2). Moreover, the intensity of both the excitation and the emission bands increased linearly with increasing ONOO<sup>-</sup> concentration (Fig. 2). The identity of the product was confirmed by HPLC analysis (Fig. 2, insets, and supplemental Fig. S4), showing that the product co-eluted with the authentic standard, 7-hydroxycoumarin, under identical HPLC conditions. The HPLC analysis enabled us to determine the stoichiometry of the reaction, indicating that one molecule of CBA reacts with a molecule of ONOO<sup>-</sup>, producing COH with the overall yield of  $\sim$ 81% (Fig. 3 and supplemental Fig. S4). This finding is similar to what has been previously reported for 4-acetylphenyl and phenylalanine-4-boronic acids (30).

As can be seen in supplemental Fig. S1, the fluorescence of the solutions was observed immediately after mixing with ONOO<sup>-</sup>; however, no significant fluorescence was observed even 30 min after mixing with  $H_2O_2$ , indicating that the reaction was rather slow. As both oxidants resulted in the formation of the same fluorescent product, this is attributed to vastly different rates of oxidation of the probe. We monitored the reaction progress with both oxidants by following the changes in the UV-visible absorption spectra and the increase in the fluorescence intensity during oxidation of CBA to COH. The rate constant of  $1.5 \pm 0.2 \text{ M}^{-1} \text{s}^{-1}$  was determined for the reaction with  $H_2O_2$  at pH 7.4 (supplemental Fig. S5). With ONOO<sup>-</sup>, the stopped-flow technique was used to measure the rate constant.



FIGURE 2. Fluorescent spectral changes during the reaction between CBA and peroxynitrite. Fluorescence (*Fl*) spectra (excitation/emission) were obtained from incubations containing CBA (50  $\mu$ M) and DTPA (50  $\mu$ M) in phosphate buffer (0.45 M, pH 7.4) after the addition of various amouts of ONOO<sup>-</sup> (0-2  $\mu$ M). Emission spectra were collected using excitation at 332 nm; excitation spectra were collected by monitoring the emission intensity at 451 nm. *Insets*, HPLC traces recorded from the incubation mixtures containing CBA (100  $\mu$ M), before (*control, top trace*) and after (*bottom trace*) the addition of ONOO<sup>-</sup> (20  $\mu$ M). The *left panel* represents the signal detected using the absorption (*Abs*) detector set at 300 nm; the *right panel* represents the signal detected using the fluorescence detector with the excitation set at 332 nm and emission at 470 nm. HPLC peaks at 9.5 and 10.7 min correspond to CBA and COH, respectively. *a.u.*, arbitrary units.



FIGURE 3. **Stoichiometry of the reaction between CBA and ONOO**<sup>-</sup>. The concentrations of CBA and COH calculated from HPLC data were obtained from incubations containing CBA (100  $\mu$ M), 20  $\mu$ M DTPA, and ONOO<sup>-</sup> (0–200  $\mu$ M) in phosphate buffer (0.1 M, pH 7.4). *Error bars* indicate S.D.

As shown in Fig. 4, the disappearance of the absorption band responsible for CBA (as monitored at 286 nm) was accompanied by the build-up of the absorption (monitored at 370 nm) and fluorescence (excitation at 332 nm, emission at > 400 nm)



## Quantitation of ONOO<sup>-</sup> from the Reaction between 'NO and $O_2^-$



FIGURE 4. **Kinetics of the reaction between CBA and ONOO**<sup>-</sup>. *A–C*, stopped-flow kinetic traces obtained after mixing CBA (20  $\mu$ M) and ONOO<sup>-</sup> (10  $\mu$ M) in phosphate buffer (0.25 M, pH 7.4), using the absorption detector at 286 nm (*A*) and 370 nm (*B*) or using the fluorescence detector (*C*, excitation at 332 nm, emission at > 400 nm). *a.u.*, arbitrary units. *D*, the dependence of the pseudo first-order rate constant of COH formation (as detected by fluorescence) on the CBA concentration; [ONOO<sup>-</sup>] = 0.1  $\mu$ M.



FIGURE 5. Detection of ONOO<sup>-</sup> formed from co-generated superoxide and nitric oxide fluxes. The fluorescence intensity (excitation at 332 nm, emission at 450 nm) of the reaction mixtures consisting of CBA (100  $\mu$ M) with or without X/XO (O<sub>2</sub><sup>-</sup> flux: 2  $\mu$ M/min) and PAPA-NONOate ('NO flux: 2  $\mu$ M/min) was measured over a period of 30 min. The reaction mixtures contained catalase (0.1 kU/mI) and DTPA (10  $\mu$ M) in phosphate buffer (100 mM, pH 7.4). *a.u.*, arbitrary units.

bands for COH. Based on the rate of the product build-up, the rate constant of (1.1  $\pm$  0.2)  $\times$  10<sup>6</sup>  $\rm M^{-1}s^{-1}$  was calculated for oxidation of CBA by ONOO<sup>-</sup>. Thus, ONOO<sup>-</sup> reacts with the coumarin boronate at least a million times faster than H<sub>2</sub>O<sub>2</sub>.

Oxidation of Coumarin Boronate by Co-generated 'NO and  $O_2^-$ —To investigate whether the same probe can be oxidized similarly by 'NO and  $O_2^-$  generated simultaneously, we monitored the rate of oxidation of CBA to COH in incubation mixtures containing xanthine (X) and XO as a source of steady flux of  $O_2^-$  and PAPA-NONOate as a source of 'NO flux (Fig. 5). The

uric acid formed from xanthine oxidation interfered with tyrosine nitration assay (38). However, the reaction between  $ONOO^-$  and CBA outcompetes not only the self-decomposition of  $ONOO^-$  but also its reaction with uric acid (the reported value of the apparent rate constant for the reaction of urate monoanion with ONOOH is  $155 \text{ M}^{-1}\text{s}^{-1}$  at pH 7.4 (39)). The slower reaction between CBA and H<sub>2</sub>O<sub>2</sub> generated by XO and by dismutation of O<sup>-</sup><sub>2</sub> was completely mitigated using the catalase enzyme. The oxidation of CBA to COH occurred only in the presence of both O<sup>-</sup><sub>2</sub> and 'NO, and virtually no oxidation of the probe into fluorescent product was observed in the presence of either O<sup>-</sup><sub>2</sub> or 'NO alone (Fig. 5). This is attributed to the reaction between CBA and ONOO<sup>-</sup> formed *in situ*. These results suggest that it is feasible to monitor in "real time" the formation of ONOO<sup>-</sup>, using the boronate probe.

To investigate the relative contribution of  $H_2O_2$  and ONOO<sup>-</sup> in the conversion of CBA into COH in this system, we tested the effect of catalase and SOD on the fluorescence increase in incubations containing X/XO system with or without 'NO donor (Fig. 6). In the absence of the 'NO donor, the fluorescence signal was inhibited by catalase but not by SOD. This indicates that H<sub>2</sub>O<sub>2</sub> is the primary oxidant responsible for the observed increase in the fluorescence intensity obtained in the absence of the 'NO donor. The addition of the 'NO donor to the incubation caused an increase in the rate of formation of the fluorescence product, which was partly inhibited by SOD and not by catalase. This observation suggests that the oxidation of CBA to COH was  $H_2O_2$ -independent and  $O_2^{-}$  and 'NOdependent oxidant formation in this system. In the presence of the 'NO donor, maximal inhibition was observed when both catalase and SOD were present in the system, indicating that H<sub>2</sub>O<sub>2</sub> produced by SOD is also contributing to the oxidation of CBA to COH. For the quantitative analysis of the amount of CBA consumed and COH formed, we used the HPLC method. As can be seen in Fig. 6C, the effect of SOD and catalase on the amount of COH formed during a 30-min incubation period closely followed the fluorescence results. The concentration of COH formed (50  $\mu$ M) in incubations containing the 'NO donor and X/XO system is lower than the theoretical yield of ONOO<sup>-</sup> (66  $\mu$ M, based on the assumption of a steady flux of 2.2  $\mu$ M/min of  $O_2^{-}$  and 'NO), giving the net yield of 76%, which is reasonably close to  $\sim$ 81% detected in the presence of a bolus addition of ONOO<sup>-</sup>.

Effect of Variation of  $\text{`NO}/\text{O}_2^-$  Flux Ratios on the Yield of  $ONOO^-$ —The fluorescence intensity changes were monitored over a 5-min period in incubation mixtures containing CBA and a constant flux of  $\text{O}_2^-$  (2  $\mu$ M/min) and different fluxes of `NO (0–12  $\mu$ M/min) by varying the concentrations of `NO donor, PAPA-NONOate (Fig. 7*A*). At low rates of `NO generation (<2  $\mu$ M/min), the rate of increase in the fluorescence intensity was proportional to the `NO flux as `NO concentration was the limiting factor in ONOO<sup>-</sup> generation. At rates of `NO generation higher than that of  $\text{O}_2^-$  (>2  $\mu$ M/min), the rate of fluorescence increase reached a plateau and remained unchanged up to a 6-fold excess of `NO (Fig. 7*B*). At longer duration of incubation (30 min) and gradually increasing ratios of `NO to  $\text{O}_2^-$  fluxes from 1 to 8, a slow decline in the yield of COH was noted as determined by HPLC (Fig. 7*C*). However, we also observed that





FIGURE 6. Detection of ONOO<sup>-</sup> formed from co-generated superoxide and nitric oxide fluxes; effects of superoxide dismutase and catalase (CAT). A, the time course of the fluorescence intensity increase (excitation at 332 nm, emission at 450 nm) measured in reaction mixtures containing CBA (100  $\mu$ M) and DTPA (10  $\mu$ M) in phosphate buffer (100 mM, pH 7.4) with or without X (200  $\mu$ M), XO (O<sup>-</sup><sub>2</sub> flux: 1.2  $\mu$ M/min), and PAPA-NONOate ('NO flux: 2.7  $\mu$ M/min). B, effects of SOD (1 mg/ml) and catalase (100 units/ml) on the total change in the fluorescence intensity after 30 min of incubation. Error bars indicate S.D. a.u., arbitrary units. C, COH concentrations in the reaction mixtures measured by HPLC after a 30-min incubation under similar conditions but with 'NO and O<sup>-</sup><sub>2</sub> fluxes of 2.2  $\mu$ M/min and SOD concentration of 0.2 mg/ml. Open and solid symbols/bars represent the values obtained from mixtures in the absence and presence of PAPA-NONOate, respectively.

under excess of 'NO, the extent of conversion of xanthine into uric acid was also decreased with increasing 'NO flux (Fig. 7*C*). This may be due to 'NO-dependent inhibition of the enzyme (40-42). Assuming that the rate of  $O_2^{-}$  generation is proportional to XO activity, and therefore, to the rate (and the yield) of uric acid formation, we normalized the amount of COH ("corrected") to the yield of uric acid (and thus to  $O_2^{\overline{}}$ ). In the presence of excess 'NO, the rate of  $O_2^{-}$  production was the limiting factor in COH formation. The dependence of the corrected yield of COH on the ratio of  $NO/O_2^-$  fluxes is shown in Fig. 7D. The HPLC data are in agreement with the fluorescence results, indicating that the yield of ONOO<sup>-</sup> is constant under conditions of NO formation rate that exceeds the rate of  $O_2^{-}$  production by 8-fold. From these results, we conclude that ONOO<sup>-</sup> is formed as a major species during simultaneous generation of 'NO and  $O_2^{-}$  and that ONOO<sup>-</sup> formation increased linearly up to a 1:1 ratio of  $NO/O_2^{-}$  flux, which then began to plateau. In contrast to results shown in Fig. 7, different results were obtained when DHR was used as a detection probe for ONOO<sup>-</sup>. The rate of fluorescence increase of rhodamine 123 formed from DHR was monitored at different 'NO/ $O_2^{-1}$  ratios (supplemental Fig. S6). In agreement with the previous reports (23-26), a bell-shaped response was observed under these conditions. Clearly, the DHR-based detection method is not reliable for quantitative analysis of ONOO<sup>-</sup> formed from  $NO/O_2^{-}$  reaction.

## DISCUSSION

CBE has previously been reported to react with H<sub>2</sub>O<sub>2</sub>, resulting in the formation of a highly fluorescent COH (umbelliferone) (32). In this study, we show that coumarin-7-boronic acid reacts rapidly and stoichiometrically with ONOO<sup>-</sup> to give COH as the major product ( $\sim$ 81%). This is similar to what had been published with simple arylboronates, indicating that the reaction between boronates and ONOO<sup>-</sup> is quite general (30). The rate of reaction between CBA and ONOO<sup>-</sup> is at least a million times faster than between CBA and H<sub>2</sub>O<sub>2</sub>, and even at low micromolar concentrations, the boronate probes can effectively compete with the self-decomposition of ONOO<sup>-</sup> at neutral pH. To our knowledge, this is the first report using a fluorogenic probe that reacts directly and stoichiometrically with ONOO<sup>-</sup>. The probe can successfully scavenge ONOO<sup>-</sup> added as a bolus or formed from co-generated 'NO and  $O_2^{-}$ , thus allowing for real-time monitoring of ONOO<sup>-</sup> formation in biological systems.

To confirm the identity of the oxidant trapped, we tested the effects of SOD and catalase on the yield of the fluorescent product formation (Fig. 6). The results are consistent with the oxidation of the probe by an oxidant formed from interaction of 'NO and  $O_2^-$ . From our earlier studies, we can conclude that  $ONOO^-$  but not nitrogen oxides ('NO, 'NO<sub>2</sub>) is responsible for oxidation of the boronate probe (30). The lack of a total inhibition of the oxidation by SOD, even in the presence of catalase and at a high concentration of SOD (up to 1 mg/ml), can be explained by the dynamic competition between 'NO and SOD for  $O_2^-$  as follows. SOD effectively competes with 'NO in removing  $O_2^-$ , causing a rise in the steady-state concentration of 'NO that can again effectively compete with SOD for  $O_2^-$  (43, 44).

# Quantitation of ONOO<sup>-</sup> from the Reaction between 'NO and $O_2^-$



FIGURE 7. **Detection of ONOO**<sup>-</sup> **formed from co-generated superoxide and nitric oxide fluxes; effects of varying NO/O**<sup>-</sup>/<sub>2</sub> **ratios on product formation.** *A*, the time course of the increase in the fluorescence intensity (excitation at 332 nm, emission at 450 nm) obtained at room temperature from the incubation mixtures containing CBA (100  $\mu$ M), 10  $\mu$ M DTPA, 100 units/ml catalase, xanthine (200  $\mu$ M), XO (O<sup>-</sup>/<sub>2</sub> flux: 2  $\mu$ M/min), and different concentrations of PAPA-NONOate ('NO flux: 0–12  $\mu$ M/min) in phosphate buffer (0.1 m and pH 7.4). 'NO fluxes ( $\mu$ M/min) are: *a*, 0; *b*, 0.75; *c*, 1.5; *d*, 2.25; *e*, 3; *f*, 6; *g*, 9; *h*, 12. *a.u.*, arbitrary units. *B*, rate of increase in the fluorescence intensity versus 'NO flux. *Error bars* indicate S.D. *C*, HPLC measurements of COH and uric acid formed after 30 min of inclusation under similar conditions but with an O<sup>-</sup>/<sub>2</sub> flux of 2.2  $\mu$ M/min. *D*, the concentration of COH after correcting for the inactivation of the enzyme, XO. Note that in *panels C* and *D*, the *x* axis scale is not linear.

This proposal was indeed confirmed by the kinetic simulations of the inhibitory effects of SOD, yielding the same degree of inhibition of CBA oxidation by the 'NO/ $O_2^-$  system, as noted experimentally (supplemental Figs. S7 and S8). Additionally, the kinetic simulations predicted an increase in the steady-state 'NO levels upon the addition of SOD, as reported previously (43). The reaction pathways involved in the oxidation of CBA to COH and the inhibitory effects of SOD and catalase in the present study are shown in Fig. 8.

We used the coumarin boronate to resolve the long standing controversy regarding the yield of ONOO<sup>-</sup>, formed under different fluxes of 'NO and  $O_2^-$ . Previous studies indicate that in cell-free systems, tyrosine nitration/oxidation and dihydrorhodamine oxidation are maximal only when the rates of generation of 'NO to  $O_2^-$  are equal and that the probe oxidation and nitration declined drastically with increasing flux of either species (22, 24, 45). The net effect is a bell-shaped response of the yield of reaction product *versus* the flux of one species at the constant flux of the other co-reactant. A bell-shaped response in DHR oxidation in the presence of various ratios of 'NO to  $O_2^-$  fluxes in cellular systems was also reported (26). These findings called into question the nature of the identity of the oxidant formed from the reaction of 'NO with  $O_2^-$  under various ratios of both species. The lack of availability of a chemical probe that would react quickly and stoichiometrically with ONOO<sup>-</sup> hampered the progress in the understanding of the chemistry of the interaction of 'NO with  $O_2^-$ . Both tyrosine and dihydrorhodamine react with the free radical product(s) of ONOO<sup>-</sup> decomposition rather than with ONOO<sup>-</sup> itself, complicating the analysis of the primary events in the interaction



# Quantitation of ONOO<sup>-</sup> from the Reaction between 'NO and O<sup>-</sup><sub>2</sub>



between 'NO and  $O_2^-$ . In this study, we used the fluorogenic boronic probe, which reacts directly with ONOO<sup>-</sup>, outcompeting the decomposition of ONOO<sup>-</sup> into the radical products. Our results directly prove that ONOO<sup>-</sup> is the primary product of the reaction of  $O_2^-$  with 'NO over a wide range of 'NO to  $O_2^$ fluxes. Thus, the previously reported bell-shaped responses, which do not accurately reflect the chemistry of  $O_2^-$ /'NO interaction, are due to free radical-dependent oxidation and nitration of the probe molecules (tyrosine and dihydrorhodamine) and reactions of probe-derived radicals with 'NO and  $O_2^-$ . The ongoing research indicates that the boronate-based fluorogenic probes can be used for real-time monitoring of ONOO<sup>-</sup> generation in cellular systems (46).

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