Peroxynitrite-induced luminol chemiluminescence

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Vascular endothelial cells, smooth muscle cells, macrophages, neutrophils, Kupffer cells and other diverse cell types generate superoxide ($O_2^{\cdot-}$) and nitric oxide ($\cdot NO$), which can react to form the potent oxidant peroxynitrite anion ($ONOO^-$). Peroxynitrite reacted with luminol to yield chemiluminescence which was greatly enhanced by bicarbonate. The quantum chemiluminescence yield of the $ONOO^-$ reaction with luminol in bicarbonate was approx. 10^{-3} . Chemiluminescence was superoxide dismutase-inhibitable, indicating that $O_2^{\cdot-}$ was a key intermediate for chemiexcitation. $O_2^{\cdot-}$ appears to be formed secondarily to the reaction of a bicarbonate–peroxynitrite com-

plex with luminol, yielding luminol radical and $O_2^{\cdot-}$. Luminol radical reacts with $O_2^{\cdot-}$ to form the unstable luminol endoperoxide, which follows the light-emitting pathway. Neither $\cdot NO$ nor $O_2^{\cdot-}$ alone were capable of directly inducing significant luminol chemiluminescence in our assay systems. These results suggest that $ONOO^-$ can be a critical unrecognized mediator of cell-derived luminol chemiluminescence reported in previous studies. In addition, it is shown that bicarbonate can participate in secondary oxidation reactions after reacting with $ONOO^-$.

INTRODUCTION

Vascular endothelial cells, smooth muscle cells, macrophages, neutrophils, Kupffer cells and a growing list of other cell types generate superoxide ($O_2^{\cdot-}$) [1] and nitric oxide ($\cdot NO$) [2]. Different mediators stimulate the simultaneous production of $\cdot NO$ and $O_2^{\cdot-}$, including interferon γ [3], calcium ionophores [2,4], lipopolysaccharide [5,6] and phorbol esters [4,7]. Thus concomitant $\cdot NO$ and $O_2^{\cdot-}$ generation may be enhanced in a variety of pathophysiological situations such as ischaemia–reperfusion, acute inflammatory processes, atherosclerosis, bacterial infections and sepsis.

Both $\cdot NO$ and $O_2^{\cdot-}$ are free-radical species that rapidly react with each other in aqueous solution at pH 7.4, yielding peroxynitrite anion ($ONOO^-$) ($k_2 = 3.7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) [8]. Several observations support the *in vivo* formation of $ONOO^-$. First, the half-life of endothelial-derived $\cdot NO$ is doubled in the presence of superoxide dismutase (SOD), suggesting that $O_2^{\cdot-}$ is involved in its degradation [9]. Second, inhibitors of $\cdot NO$ synthase increase detectable $O_2^{\cdot-}$ release from macrophages [10]. Third, decomposition of the sydnone, SIN-1, to products including $\cdot NO$ and $O_2^{\cdot-}$ yields a species with $\cdot OH$ -like reactivity, inferring the intermediate formation of $ONOO^-$ [11,12]. Finally, $ONOO^-$ has been directly detected as a product of phorbol myristate acetate-activated rat lung alveolar macrophages, which produce 100 pmol of $ONOO^-$ /min per 10^6 cells [13].

Peroxynitrite ($pK_a = 6.8$ [14]) is an unstable species at physiological pH ($t_{1/2} < 1 \text{ s}$), protonating to peroxynitrous acid ($ONOOH$) which spontaneously decomposes to $\cdot NO_2$ and $\cdot OH$ in 20–30% yield:

The remaining $ONOOH$ will directly isomerize to nitrate (NO_3^- , [11]).

Peroxynitrite may be an important mediator of free-radical-dependent toxicity [11–14] because of its strong oxidizing properties towards different biomolecules, including protein and non-

protein thiols [14], deoxyribose [11] and membrane phospholipids [15]. In addition to its role in oxidation reactions, $ONOO^-$ can also nitrate free or protein-associated tyrosine [16].

Luminol chemiluminescence has been widely used to detect the production of reactive oxygen species (i.e. $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$) from enzyme, cell and organ systems [17–19] and has been useful for examining the kinetics and reaction mechanisms of oxygen-radical processes [17,20]. In order to yield light, luminol has to undergo a two-electron oxidation and form an unstable endoperoxide. This luminol endoperoxide decomposes to an excited state, 3-aminophthalic acid, which relaxes to the ground state by emitting photons [18,21]. In most cases of luminol chemiexcitation in biological systems, $O_2^{\cdot-}$ is a key intermediate [22,23], but alternative pathways of chemiexcitation not requiring $O_2^{\cdot-}$ have been described [17,24].

Luminol chemiluminescence is observed during the respiratory burst of macrophages and neutrophils, which has been commonly attributed to the production of $O_2^{\cdot-}$ and H_2O_2 [18]. Nevertheless, there are instances where more potent oxidation reactions are needed to explain observed chemiluminescence yields [25]. For example, myeloperoxidase liberated from macrophage granules greatly enhances the reactivity of H_2O_2 towards luminol by forming an oxo-haem oxidant species [18]. Also, $\cdot NO$ -derived intermediates contribute to luminol chemiluminescence in phorbol ester-activated Kupffer cells and may react via $ONOO^-$ [7].

Herein we report that $ONOO^-$, a newly described biological oxidant of emerging significance [26–28] induced SOD-inhibitable luminol chemiluminescence. Furthermore, it is shown that bicarbonate can participate in secondary oxidative reactions after reacting with $ONOO^-$.

EXPERIMENTAL

Materials

Cysteine, cystine, uric acid, mannitol, dimethyl sulphoxide (DMSO), horseradish peroxidase type VI and tetranitromethane were obtained from Sigma. Potassium superoxide, *cis*-dicyclo-

Abbreviations used: SOD, superoxide dismutase; DMSO, dimethyl sulphoxide.

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hexano-18-crown-6 and tetrafluoroborate nitronium were from Aldrich. H_2O_2 and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were obtained from Fluka and Cu/Zn SOD was generously provided by Grunenthal GmbH.

Peroxynitrite was synthesized in a quenched-flow reactor as previously described [11,14,15]. Fresh solutions of potassium superoxide (0.1 M) were prepared daily in DMSO and 18-crown-6 as described elsewhere [29]. A 1.7 mM $\cdot\text{NO}$ solution was prepared by extensive bubbling of $\cdot\text{NO}$ gas in anaerobic deionized water contained in a gas sampling tube.

Inactivation of Cu/Zn SOD

SOD inactivation by H_2O_2 [30] was performed by incubating 2.0 $\mu\text{g}/\text{ml}$ SOD (2100 units/mg) with 1.0 mM H_2O_2 in 50 mM sodium pyrophosphate, pH 10.0, at 25 °C for 30 min, with residual H_2O_2 removed by Sephadex G-25 chromatography. SOD activity was determined by measuring the inhibition of cytochrome c^{3+} reduction by xanthine plus xanthine oxidase [31]. SOD was approximately 86 % inactivated by this method.

Chemiluminescence measurement

Chemiluminescence studies were performed in a SLM DMX-1000 fluorimeter by closing the excitation window and maximally opening the emission slit width to 16 nm. Reactions were initiated by injection of ONOO^- directly to cuvettes with continuous stirring through a syringe adapted to the instrument. Light emission was simultaneously recorded by computer interface. Reported chemiluminescence is the integrated light emission from a 5 s time interval, unless otherwise stated. When studying the effect of different oxygen tensions on chemiluminescence yield, reactions were performed with continuous oxygen, air or nitrogen bubbling at 1–2 ml/min in a 3 ml cuvette volume.

Spectrophotometric analysis

Absorbance measurements and spectra were performed with a Gilford Response spectrophotometer. Luminol oxidation was measured by absorbance decrease at 350 nm ($\epsilon = 7200 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and 300 nm ($\epsilon = 6800 \text{ M}^{-1} \cdot \text{cm}^{-1}$). For ONOO^- concentrations utilized in this paper, there was no significant interference of the ONOO^- decomposition products NO_3^- and NO_2^- on luminol oxidation determination, because of the extremely low absorption coefficients of these species in the 300–350 nm region compared with luminol (NO_2^- , $\epsilon_{350} = 23 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{300} = 10 \text{ M}^{-1} \cdot \text{cm}^{-1}$; NO_3^- , $\epsilon_{350} = 0$ and $\epsilon_{300} = 9 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Data reported herein are the average of three independent determinations performed in a one-day experiment. Each experiment was repeated on a minimum of 4 different days and similar results were obtained with a maximum 10 % variation.

RESULTS

Luminol chemiluminescence spectra and quantum yield

Addition of peroxynitrite stimulated luminol chemiluminescence in both bicarbonate and phosphate buffer. Emission spectra were similar for both buffers, with λ_{em} maximum = 425 nm (Figure 1), which was used as the λ_{em} for further studies. Maximal light-emission intensity in phosphate buffer was 0.3–0.5 % of that in

bicarbonate buffer at pH 10.5 (Figure 1). The proportion of chemiluminescence yield in phosphate buffer, with respect to bicarbonate buffer, was increased by lowering pH. At pH 7.5, the luminol chemiluminescence yield of ONOO^- reaction in phosphate buffer became 15 % of that in bicarbonate buffer. The addition of H_2O_2 (10, 50 and 200 μM) to reaction mixtures containing 400 μM luminol and 100 μM ONOO^- in either bicarbonate or phosphate buffers at pH 10.5 did not have a significant effect on chemiluminescence yield or the time course of light emission (results not shown).

The quantum yield of ONOO^- -induced chemiluminescence (quanta/molecules of ONOO^- consumed) was determined at

Figure 1 Chemiluminescence spectra for luminol- ONOO^- reactions

Peroxynitrite (100 μM) was added to reaction mixtures containing 400 μM luminol in 50 mM bicarbonate (●) or phosphate (□), pH 10.5, at 25 °C. Maximal light intensities obtained at different emission wavelengths are reported. Results are means $\pm$ S.E.M.

Figure 2 Luminol chemiluminescence as a function of ONOO^- concentration

Reactions were started by addition of 10 μM (Δ), 25 μM ($\blacktriangle$), 50 μM ($\circ$) or 100 μM ($\bullet$) ONOO^- to reaction mixtures containing 400 μM luminol in 50 mM bicarbonate, pH 10.5, at 25 °C. k_d values were 0.020 s^{-1} , 0.024 s^{-1} , 0.022 s^{-1} and 0.021 s^{-1} for 10, 25, 50 and 100 μM ONOO^- respectively. Results are means $\pm$ S.E.M.

Figure 3 Luminol chemiluminescence as a function of pH

Reactions were started by addition of $100\ \mu\text{M}$ ONOO^- to a reaction mixture containing $400\ \mu\text{M}$ luminol and $50\ \text{mM}$ bicarbonate, pH 10.0 (●), 9.5 (▲), 8.5 (○) and 7.5 (+) at $25\ ^\circ\text{C}$. k_d values were $0.17\ \text{s}^{-1}$, $0.45\ \text{s}^{-1}$, $1.11\ \text{s}^{-1}$ and $1.20\ \text{s}^{-1}$ respectively. Chemiluminescence was integrated every $0.2\ \text{s}$.

pH 10.5 with luminol in excess of ONOO^- ($400\ \mu\text{M}$ luminol, $100\ \mu\text{M}$ ONOO^-). Quantum yields were approx. 10^{-3} in bicarbonate buffer and $< 10^{-5}$ in phosphate buffer. A secondary standard of persulphate/ H_2O_2 was used as our reference system [32].

Time course of luminol chemiluminescence

Luminol chemiluminescence induced by ONOO^- reached peak intensity by $5\ \text{s}$ at pH 10.5 and was followed by an exponential decay (Figure 2). The emission decay followed first-order kinetics

and was quantified as the first-order rate constant k_d [17,20]. Chemiluminescence intensity was proportional to ONOO^- concentration, with the calculated k_d independent of ONOO^- . Light-emission decay in bicarbonate buffer was zero order with respect to luminol in concentrations up to $1\ \text{mM}$. No further increase in $100\ \mu\text{M}$ ONOO^- -induced chemiluminescence yield was obtained with concentrations of luminol greater than $400\ \mu\text{M}$. Alternatively, chemiluminescence was strongly influenced by pH, with k_d increasing at lower pH (Figure 3). Addition of a second portion of ONOO^- to reaction mixtures after chemiluminescence ceased resulted in similar chemiluminescence yields. Thus quenching by ONOO^- and luminol reaction byproducts did not artifactually influence the ONOO^- -induced luminol chemiluminescence.

Chemiluminescence decay rates and peak intensities depended on bicarbonate concentration, with k_d directly proportional to bicarbonate (Figure 4a). Total chemiluminescence yields (area below the curve) were similar for all bicarbonate concentrations at a particular pH (Figure 4b). In contrast, the potassium phosphate concentration of phosphate-buffered systems did not influence the rate of luminol chemiluminescence decay. No chemiluminescence was observed after addition of tetranitromethane ($200\ \mu\text{M}$), nitronium tetrafluoroborate ($200\ \mu\text{M}$), nitrate ($100\ \mu\text{M}$), nitrite ($100\ \mu\text{M}$), nitric oxide (up to $200\ \mu\text{M}$) or potassium superoxide (up to $2.5\ \text{mM}$) to $400\ \mu\text{M}$ luminol in $50\ \text{mM}$ bicarbonate, pH 10.5, at $25\ ^\circ\text{C}$. Luminol chemiluminescence from $\text{O}_2^{\cdot-}$ only occurs when $\text{O}_2^{\cdot-}$ reacts with luminol radical, the formation of which requires prior reaction with stronger oxidants.

Net luminol oxidation was measured spectrophotometrically at 350 and $305\ \text{nm}$ and was ONOO^- concentration-dependent (Table 1), with about 5 – 8 and 3 – 6 mol of ONOO^- consumed per mol of oxidized luminol at pH 7.5 and 10.5 respectively. Luminol oxidation yields were greater at pH 10.5 than at pH 7.5 , were greater at lower ONOO^- concentrations and were similar for bicarbonate and phosphate buffers. There was no effect of bicarbonate concentration (50 – $400\ \text{mM}$) on oxidation yield (results not shown).

Figure 4 k_d as a function of bicarbonate concentration

(a) Assay conditions were: $100\ \mu\text{M}$ ONOO^- , $400\ \mu\text{M}$ luminol and 50 – $400\ \text{mM}$ bicarbonate at pH 10.5 (▲) or 9.5 (●) at $25\ ^\circ\text{C}$. k_d was determined as the first-order constant of exponential decay for each buffer concentration. (b) Chemiluminescence records were obtained in $50\ \text{mM}$ (○) and $300\ \text{mM}$ (△) bicarbonate, pH 10.5. Results are means $\pm$ S.E.M.

Table 1 Luminol oxidation as a function of ONOO⁻ concentration

Peroxynitrite (100–400 μ M) was added to 400 μ M luminol in 50 mM bicarbonate, pH 10.5 or 7.5, at 25 °C and incubated for 10 min. Data represent means $\pm$ S.D. ($n = 4$).

[ONOO ⁻] (μ M)	Luminol (μ M)	
	pH 10.5	pH 7.5
0	400 $\pm$ 5	400 $\pm$ 5
100	366 $\pm$ 8	379 $\pm$ 6
250	340 $\pm$ 8	364 $\pm$ 5
500	307 $\pm$ 7	333 $\pm$ 12
750	274 $\pm$ 5	305 $\pm$ 20
1000	243 $\pm$ 4	274 $\pm$ 20

Figure 5 Effect of Cu/Zn SOD and oxygen on luminol chemiluminescence

Assay conditions were: 100 μ M ONOO⁻, 400 μ M luminol with no SOD (●), 0.23 units/ml native SOD, (Δ, 3.75 nM), 2.3 units/ml native SOD (+, 37.5 nM), 0.27 units/ml inactivated SOD (○, protein equivalent 37.5 nM) and 2.7 units/ml inactivated SOD (□, protein equivalent 2.7 units/ml) in 50 mM bicarbonate, pH 10.5, at 25 °C. The inset shows the effect of oxygen on chemiluminescence. Assay conditions were as above with no SOD present. Reactions were saturated with air (●), nitrogen (▽) or oxygen (□).

Effect of Cu/Zn SOD

SOD caused a dose-dependent inhibition of luminol chemiluminescence and a small increase in k_d (Figure 5). When H₂O₂-inactivated SOD replaced native SOD, the inhibition of light emission corresponded to that expected for the residual SOD activity remaining in the inactivated enzyme preparation (Figure 5). A significantly greater SOD activity than that used to inhibit chemiluminescence only partially inhibited luminol oxidation (Table 2). With the experimental conditions of Figure 5, cupric sulphate (1 and 20 μ M) inhibited luminol chemiluminescence by 70% and 95% respectively. The copper chelate Cu/EDTA (1:1.1, mol/mol; up to 200 μ M) did not inhibit light emission.

Reactions conducted in 100% oxygen-saturated bicarbonate buffer, pH 10.5, increased both chemiluminescence yield and

Table 2 Effect of antioxidants on luminol oxidation

Assay conditions were as described in Figure 6. Incubations were performed for 10 min at 25 °C. Data represent means $\pm$ S.D. ($n = 3$). Superscripts denote significance of difference from control (a) and ONOO⁻ (b)-treated (no addition) condition after ANOVA and analysis by Duncan's multiple-range test ($P < 0.05$).

Condition	Luminol (μ M)
Control	396 $\pm$ 2
+ ONOO ⁻	
No addition	371 $\pm$ 2 ^a
+ 100 mM mannitol	372 $\pm$ 3 ^a
+ 100 mM DMSO	372 $\pm$ 3 ^a
+ 3 mM cystine	371 $\pm$ 4 ^a
+ 3 mM cysteine	395 $\pm$ 3
+ 1 mM urate	397 $\pm$ 4
+ 2.3 units/ml SOD	378 $\pm$ 4 ^a
+ 23.0 units/ml SOD	386 $\pm$ 3 ^{a,b}

Figure 6 Effect of antioxidants on luminol chemiluminescence

Assay conditions were: 100 μ M ONOO⁻, 400 μ M luminol with no additions (●), 100 mM DMSO (○), 100 mM mannitol (□), 3 mM cysteine (■), 1 mM urate (▲) and 0.375 μ M SOD (△) in 50 mM bicarbonate, pH 10.5, at 25 °C. Where indicated, results are means $\pm$ S.E.M.

duration by about 10–20%, whereas nitrogen saturation only slightly inhibited chemiluminescence (Figure 5, inset). Similar extents of SOD inhibition of ONOO⁻-induced luminol chemiluminescence occurred in oxygen, air or nitrogen-saturated conditions.

Effect of antioxidants

Uric acid (1 mM) and cysteine (3.0 mM) completely inhibited both luminol chemiluminescence and luminol oxidation (Figure 6, Table 2). The $\cdot$ OH scavengers mannitol (100 mM) and DMSO (100 mM), and the disulphide cystine (3.0 mM), did not affect luminol chemiluminescence and luminol oxidation (Table 2).

Secondary products from ONOO⁻-reaction with luminol

After luminol reacted with ONOO⁻, a pronounced 425 nm yellow absorbance was found in samples which progressively disap-

Figure 7 Absorption spectra of luminol after oxidation by ONOO^-

Luminol ($400 \mu\text{M}$) reacted with $100 \mu\text{M}$ ONOO^- in 50 mM bicarbonate, pH 10.5 at 25°C . Spectra were recorded immediately before (----) and 3 min after (—) ONOO^- addition. A decrease in A_{350} and the appearance of a peak in the 425 nm region was observed. Whereas A_{350} did not change with time, the 425 nm absorbance was transient and decayed (inset).

peared over 15–25 min at both pH 10.5 and 7.5 in either 50 mM bicarbonate or phosphate buffers (Figure 7 and inset). This 425 nm absorbance was pH insensitive. Addition of uric acid (1 mM) and cysteine (3 mM) but not DMSO (100 mM) or mannitol (100 mM) to ONOO^- -luminol reactions inhibited the appearance of the 425 nm absorbance. Luminol reaction with tetranitromethane ($100 \mu\text{M}$) or much higher concentrations of nitronium tetrafluoroborate (5 mM) resulted in a stable yellow product with a broad absorbance at $400\text{--}500 \text{ nm}$ rather than the sharper 425 nm peak after luminol reaction with ONOO^- . Oxidation of luminol with horseradish peroxidase plus H_2O_2 did not generate this 425 nm -absorbing species (results not shown).

DISCUSSION

Peroxynitrite-induced luminol chemiluminescence was greatly enhanced by HCO_3^- (Figure 1). Spectral analysis of luminol chemiluminescence (Figure 1) indicated that excited aminophthalate was the emitting species [33]. Since formation of aminophthalate depends on decomposition of luminol endoperoxide, ONOO^- reaction with luminol must yield this unstable intermediate.

Peroxynitrite is an effective one-electron oxidant ($E'_{(\text{ONOO}^-/\text{NO}_2)} = +1.4 \text{ V}$ [34]), thus oxidation of luminol by ONOO^- is thermodynamically feasible. The first oxidation by ONOO^- or its conjugate acid would lead to the formation of luminol radical ($\text{L}^\bullet$) as follows:

If NO_2 is in close proximity to luminol radical, an immediate second oxidation will take place to yield a diazaquinone (L), with the overall process a two-electron oxidation according to:

Alternatively, luminol radical can be oxidized to the diazaquinone by direct reaction with a second molecule of ONOO^- . If the diazaquinone reacts with H_2O_2 , the luminol endoperoxide intermediate is formed [21]. A diazaquinone- H_2O_2 reaction was not responsible for ONOO^- -induced chemiluminescence, since

there was no direct source of H_2O_2 in our reaction mixtures. Exogenous addition of H_2O_2 had no significant effect on light yield as well. Formation of a nitrated luminol endoperoxide by ONOO^- adduct formation with the diazaquinone, followed by internal rearrangement, also does not account for light emission because nitrophthalate is not chemiluminescent (G. Merenyi and J. Lind, personal communication).

The inhibitory effects of SOD and free Cu^{2+} on chemiluminescence imply the participation of $\text{O}_2^{\bullet-}$ in chemiexcitation. This concept is reinforced by the observation that Cu/EDTA , which lacks SOD activity [35], did not inhibit chemiluminescence. Different mechanisms could operate to generate $\text{O}_2^{\bullet-}$ during ONOO^- reaction with luminol. $\text{O}_2^{\bullet-}$ could have been produced by a luminol radical-dependent univalent reduction of molecular oxygen [21]. This mechanism was probably not the main source of $\text{O}_2^{\bullet-}$, since saturation with oxygen or nitrogen had a marginal effect on chemiluminescence yield (Figure 5, inset). Moreover, SOD inhibited light emission in both aerobic and anaerobic conditions. The direct formation of $\text{O}_2^{\bullet-}$ (plus NO) from ONOO^- did not occur for thermodynamic and kinetic considerations. The ΔG° for formation of ONOO^- from $\text{O}_2^{\bullet-}$ and NO is about -92 kJ/mol (-22 kcal/mol), thus $K_{\text{eq}} = 5 \times 10^{15} \text{ M}^{-1}$ at 25°C [25]. With the k_2 for formation of ONOO^- being at least $3.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ [8], it can be estimated that the reverse reaction (formation of $\text{O}_2^{\bullet-}$ from ONOO^-) will proceed slowly at 10^{-8} s^{-1} . Thus $\text{O}_2^{\bullet-}$ must be generated after direct reaction of ONOO^- with luminol and by a bicarbonate-stimulated mechanism (Figures 1 and 4). When bicarbonate was absent, luminol could still be oxidized by ONOO^- (Table 1), but the presence of bicarbonate led the oxidation process to a light-emitting pathway, presumably by favouring $\text{O}_2^{\bullet-}$ production, thus supporting a greater chemiluminescence quantum yield. In the presence of high bicarbonate concentrations, the rate of light decay was independent of both ONOO^- and luminol concentration and was pseudo-first-order with respect to bicarbonate. Thus, the formation of the key oxidant might precede the reaction of ONOO^- with luminol. A mechanism consistent with these observations can involve a peroxynitrite-bicarbonate intermediate:

With phosphate-buffered systems, a similar but minor reaction could be responsible for some light generation, with the principal light emission being due to the reduction of ONOO^- to NO_2 and H_2O by luminol. The hallmark of eqns. (4)–(6) is that the reduction of ONOO^- can yield $\text{O}_2^{\bullet-}$, with the efficiency of this reaction increasing after ONOO^- reaction with bicarbonate.

Other mechanisms might also participate in ONOO^- -dependent bicarbonate-mediated luminol-oxidation reactions. For example, formation of a bicarbonate radical from ONCO^- reaction may occur, with the $E'_{(\text{HCO}_3^-/\text{HCO}_3^{\bullet-})}$ being $+1.5 \text{ V}$ [36]. $\text{ONOO}^-/\text{NO}_2$ and $\text{HCO}_3^-/\text{HCO}_3^{\bullet-}$ couples have similar one-electron standard redox potentials. Thus, under conditions where ONOO^- reacts with excess HCO_3^- , one-electron oxidation of bicarbonate by ONOO^- is thermodynamically favourable according to:

Bicarbonate radical is known to oxidize luminol [37] and other aromatic and heterocyclic molecules with second-order rate constants ranging from 5×10^5 to $5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ [38].

Alternatively, ONOO^- may peroxidize bicarbonate to peroxybicarbonate [39], another strong oxidizing species. Thus, there are several potential mechanisms by which bicarbonate could influence the overall reactivity of ONOO^- in both model and biological conditions. Interestingly, bicarbonate is expected to accumulate in tissues undergoing ischaemia-reperfusion, a condition for which peroxynitrite is proposed to contribute significantly to net oxidant stress [11,14,15,28].

Smaller yields of ONOO^- -induced luminol chemiluminescence were found at lower pH (Figure 3). This could be due to the fact that only the decomposition of luminol monoanion (apparent $pK_a = 8.2$) leads to the light-emitting route [21]. In addition, proton-catalysed decomposition of ONOO^- becomes a more efficient competing reaction by decreasing pH [11]. Consistent with this concept, peroxynitrite oxidized luminol more efficiently at pH 10.5 than at 7.5 (Table 2) and there was a shorter half-life of luminol chemiluminescence at lower pH (Figure 3). Luminol-oxidation studies were conducted at alkaline pH in order to define mechanisms of luminol reaction with ONOO^- and limit the proton-catalysed decomposition of ONOO^- , so that information could be more precisely obtained about the kinetics and mechanism of luminol oxidation. There were no differences in the mechanisms of luminol oxidation at physiological versus alkaline pH, thus all results relate to biological events.

Cysteine and uric acid inhibited luminol chemiluminescence. Cysteine reacts with ONOO^- with an apparent second-order rate constant of $30\text{--}40\text{ M}^{-1}\cdot\text{s}^{-1}$ at pH 10.5 [14]. Cysteine can therefore inhibit luminol chemiluminescence by both direct reaction with ONOO^- and by reacting with bicarbonate-derived oxidizing species [38]. Cystine, which does not react at significant rates with ONOO^- [14], did not have any effect on luminol chemiluminescence or oxidation. Uric acid inhibits xanthine/xanthine oxidase and H_2O_2 /cytochrome *c*-induced luminol chemiluminescence by scavenging $\cdot\text{OH}$ and iron complexes having $\cdot\text{OH}$ -like reactivity [20,24]. However, the $\cdot\text{OH}$ radical scavengers mannitol and DMSO did not affect light emission, ruling out a primary role for $\cdot\text{OH}$ in chemiexcitation. We propose that uric acid reacted directly with ONOO^- and/or bicarbonate-derived oxidizing species, for which we have supporting preliminary h.p.l.c.-based product analyses. Uric acid and cysteine might also directly reduce luminol radical to its ground state, preventing chemiexcitation [20]. Scavenging of reactive species rather than quenching of excited species (resulting in non-radiative pathways of relaxation) was the mechanism of cysteine and urate inhibition of luminol chemiluminescence, because luminol oxidation was prevented by these compounds (Table 2).

Whereas urate and cysteine inhibited both luminol chemiexcitation and oxidation to similar extents, SOD significantly inhibited only chemiluminescence. SOD activity, ten times greater (23.0 units/ml) than that required to almost completely inhibit light emission (2.3 units/ml , Figure 5), only partially inhibited luminol oxidation (Table 2). SOD effects on light yields are explained by the scavenging of $\text{O}_2^{\cdot-}$, whereas the influence on oxidative yields occurring with higher SOD activities were probably due to a direct reaction between ONOO^- and the enzyme. Indeed, Cu/Zn SOD catalyses decomposition of ONOO^- to a species with a reactivity similar to nitronium ion [16]. Thus SOD can directly inhibit ONOO^- -mediated luminol-oxidation reactions by also scavenging ONOO^- . Since nitronium ion could then attack luminol, we added nitronium tetrafluoroborate to luminol and saw that it did not yield chemiluminescence.

Unlike ONOO^- , its biological precursors $\text{O}_2^{\cdot-}$ and $\cdot\text{NO}$ did not stimulate chemiexcitation of luminol. We observed no direct oxidation of luminol or light emission with up to 2.5 mM

potassium superoxide and $200\text{ }\mu\text{M}$ $\cdot\text{NO}$. This concurs with previous observations of poor reactivity between $\text{O}_2^{\cdot-}$ and luminol [38]. When $\text{O}_2^{\cdot-}$ participates in luminol chemiluminescence reactions, it does so by reacting with luminol radical rather than luminol. Luminol radical can be formed through a one-electron oxidation of luminol by $\cdot\text{OH}$, ferryl ion (FeO^{2+}) or by other oxidants of similar reactivity [18,20,39]. When $\text{O}_2^{\cdot-}$ is formed after reaction of ONOO^- with HCO_3^- , it is possible that H_2O_2 (formed after dismutation of $\text{O}_2^{\cdot-}$) can contribute to chemiluminescence by reacting with the diazaquinone formed after reaction with a second molecule of ONOO^- (eqns. (2) and (3)).

Peroxyxynitrite is, in addition to being an oxidant, a potent nitrating species [16,40]. Luminol nitration occurring concomitantly with oxidation by ONOO^- is suggested by the observed transient 425 nm absorbance. This by-product was not formed when other oxidants such as H_2O_2 attacked luminol. The mechanism of luminol nitration by ONOO^- is expected to be different from other nitrating agents such as tetranitromethane and nitronium tetrafluoroborate. Nitration of aromatic molecules by ONOO^- occurs in concert with the $\cdot\text{OH}$ -like reaction of ONOO^- with double bonds [40]. This mechanism requires the formation of a complex with ONOOH , and attack of the peroxyxynitrous acid hydroxyl with the aromatic molecule to form an aromatic radical species which then combines with the remaining NO_2 of peroxyxynitrous acid to yield the nitrated product [16]. In agreement with the concept of a concerted nitration-oxidation reaction, we observed that uric acid and cysteine inhibited both luminol oxidation and the appearance of the 425 nm absorbance peak. The final nitrated adduct was probably a derivative of luminol oxidation products, since exposure of luminol to nitrating agents did not result in the characteristic 425 nm peak. The transient nature of the nitrated adduct was not related to chemiluminescence, since the 425 nm absorbance was longer lived than light emission (Figure 7).

Superoxide is frequently invoked as being responsible for biologically produced luminol chemiluminescence due to the inhibitory effects of SOD. Still, oxidants stronger than $\text{O}_2^{\cdot-}$ and even H_2O_2 may be needed to explain the observed light yields [25]. Since macrophages and neutrophils will simultaneously produce $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ [2] and we have recently observed the formation of ONOO^- by macrophages [13], it will be important to assess what proportion of luminol chemiluminescence induced by activated macrophages and neutrophils depends on ONOO^- . For example, $\cdot\text{NO}$ - and $\text{O}_2^{\cdot-}$ -derived products from macrophage-like liver Kupffer cells synergistically contribute to luminol chemiluminescence. This observation strongly supports the role of ONOO^- as a source of hepatic oxidant stress [7]. If ONOO^- is a critical mediator of cell-derived luminol chemiluminescence, the inhibitory effects of SOD would depend on at least three different mechanisms: (1) inhibition of ONOO^- formation, preventing the reaction of $\text{O}_2^{\cdot-}$ with $\cdot\text{NO}$, (2) inhibition of $\text{O}_2^{\cdot-}$ -mediated oxidation of luminol radical [eqn. (6)] and (3) catalysis of the direct decomposition of ONOO^- . Mechanisms (2) and (3) would complement mechanism (1) because SOD is unable to totally inhibit ONOO^- production by activated macrophages, since a fraction of ONOO^- was apparently formed in sites where SOD does not have access [11].

Cell and tissue toxicity from excess production of peroxynitrite may occur because of its strong oxidizing properties. In addition, our data suggest that ONOO^- will react with bicarbonate, forming secondary bicarbonate-derived oxidizing species. Extracellular and intravascular compartments can have up to 25 mM bicarbonate, inferring that products of the reaction of ONOO^- with bicarbonate will be an important potential mech-

anism of oxidant tissue injury. The formation of bicarbonate radicals greatly increases $O_2^{\cdot-}$ -induced lysis of erythrocytes [41], stimulates enzyme inactivation and enhances luminol chemiluminescence [37]. We envisage that, after $ONOO^-$ production *in vivo*, several competing pathways will contribute to $ONOO^-$ reactions and decomposition. Some of these pathways will include (a) proton-catalysed $ONOO^-$ decomposition to potent secondary oxidants, (b) direct reaction of $ONOO^-$ with biomolecules such as methionine [42] or thiols and (c) reaction of $ONOO^-$ with bicarbonate to form toxic secondary reactive species.

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