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Detection of Superoxide and Peroxynitrite in Model Systems and Mitochondria by the Luminol Analogue L-012

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In the present study we investigated the specificity and sensitivity of the chemiluminescence (CL) dye and luminol analogue 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H) dione (L-012) to detect reactive oxygen species (ROS) such as superoxide, peroxynitrite and hydrogen peroxide in cell free systems as well as in isolated mitochondria. The results obtained by L-012 were compared with other CL substances such as luminol, lucigenin, coelenterazine and the fluorescence dye dihydroethidine. The results indicate that the L-012-derived chemiluminescence induced by superoxide from hypoxanthine/xanthine oxidase (HX/XO) or by 3-morpholino sydnonimine (SIN-1)-derived peroxynitrite largely depends on the incubation time. Irrespective of the experimental conditions, L-012-derived CL in response to HX/XO and SIN-1 was 10–100 fold higher than with other CL dyes tested. In a cell-free system, authentic peroxynitrite yielded a higher L-012-enhanced CL signal than authentic superoxide and the superoxide-induced signal in cell-free as well as isolated mitochondria increased in the presence of equimolar concentrations of nitrogen monoxide (NO). The superoxide signal/background ratio detected by L-012-enhanced CL in isolated mitochondria with blocked respiration was 7 fold higher than that obtained by the superoxide sensitive fluorescence dye dihydroethidine. We conclude that L-012-derived CL may provide a sensitive and reliable tool to detect superoxide and peroxynitrite formation in mitochondrial suspensions.

Keywords: L-012; Chemiluminescence; Superoxide; Peroxynitrite; Mitochondria; NO-donors

Abbreviations: CL, chemiluminescence; DEA/NO, diethylamine NONOate; DHE, dihydroethidine; DMSO, dimethylsulfoxide; DPI, diphenyleneiodonium chloride; DTPA, diethylenetriaminepentaacetic acid; HX, hypoxanthine; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; MOPS, 3-morpholinopropanesulfonic acid; PBS, phosphate buffered saline; PEG-SOD, polyethylene glycolated superoxide dismutase; PTIO, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide; ROS, reactive oxygen species; SIN-1, 3-morpholino sydnonimine; SMP, submitochondrial particles; SNP, sodium nitroprusside; SPE/NO, spermine NONOate; Tris, tris(hydroxymethyl)aminomethane; XO, xanthine oxidase.

INTRODUCTION

The last decades have expanded the knowledge on the role of reactive oxygen species (ROS) in organisms. Many studies have revealed that ROS participate in signaling pathways.[1] Examples include the stimulatory effect of the endothelium-derived relaxing factor nitrogen monoxide (NO) on the soluble guanylyl cyclase[2] and the oxidative activation/inactivation of key enzymes by the superoxide anion or related species,[3] including reversible processes classified by the term "redox regulation".[4,5] Radical intermediates also play an essential role in the biosynthesis of prostaglandins and in the lipoxygenase pathway.[6] Under physiological conditions, production of ROS is well controlled within the organism, but under certain circumstances the redox equilibrium is shifted towards oxidative stress. Enzymatic sources accounting for this phenomenon include the vascular NAD(P)H oxidase,[7,8] the xanthine oxidase (XO),[9] nitric oxide synthase isoforms in uncoupled conditions[10,11] and a disturbed respiratory chain in mitochondria.[12] Despite the evidence that ROS play an important role in vascular disease (for review see[13]), there is continued discussion about the specificity, sensitivity

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and validation of methods used to quantify them in intact tissue. Especially the discrimination between superoxide and its diffusion-controlled reaction product with NO, peroxynitrite,[14] is of special interest and requires optimized detection methods as well as appropriate controls.

Methods used include chemiluminescence techniques (CL),[15] fluorescence-based assays,[16] UV/Vis and electron paramagnetic resonance (EPR) spectroscopy. The frequently used cytochrome c assay may lead to false-positive results in the presence of both reactive species due to the oxidation of superoxide-reduced ferrous cytochrome c by peroxynitrite.[17] Recently, the novel luminol analogue L-012 was introduced and several groups demonstrated that this CL-substance is able to specifically detect superoxide in vascular[18] and inflammatory cells with high sensitivity.[19,20]

In the present study, we investigated the specificity as well as the sensitivity of L-012 to detect peroxynitrite and superoxide. To discriminate between peroxynitrite- and superoxide-derived signals, constant fluxes of superoxide from the hypoxanthine (HX)/XO system were employed and exposed to increasing fluxes of NO derived from various donors. Since previous observations point to a crucial role of the mitochondrial electron transport system as a significant source of ROS in the development of oxidative stress in aging and ischemia/reperfusion models[12,21] as well as in hypercholesterolemia,[22] L-012 was tested for its ability to detect superoxide generated within isolated mitochondria or submitochondrial particles (SMP). To this end, several inhibitors of mitochondrial respiration were used to stimulate the formation of superoxide and these fluxes were matched by NO fluxes from various donors to test whether L-012 is able to detect peroxynitrite formation within mitochondria.

MATERIALS AND METHODS

Materials

Authentic peroxynitrite was purchased as an alkaline stock solution from Calbiochem and the concentration was determined using an extinction coefficient of 1670/M/cm at 302 nm. L-012 was purchased from Wako Pure Chemical Industries (Japan) and argon as well as NO (technical grade) from Air Products GmbH (Hattingen, Germany). DHE was obtained from Molecular Probes Inc. (Leiden, Netherlands) and MnTMPyP, SPE/NO, DEA/NO, SIN-1 and PTIO from Calbiochem (La Jolla, USA). Lucigenin, coelenterazine, luminol, antimycin A, myxothiazol, rotenone, potassium cyanide, sodium nitroprusside, PEG-SOD, PEG-catalase, DPI, DTPA, uric acid, succinate and a suspension of XO (grade 1) from buttermilk were all obtained from Sigma. PBS buffer was from Dulbecco and did not contain bicarbonate, Mg^{2+} and Ca^{2+}. All other chemicals were of high purity grade and purchased from Sigma, Alexis or Fluka.

Determination of the Octanol/Water Partition Coefficients for CL Dyes

The absorption (using λ_{max}) of L-012, luminol, lucigenin (each 100 μM) and coelenterazine (50 μM) in 1 ml PBS at room temperature was determined with a Beckman DU 640 spectrophotometer. After repeated Vortex mixing with an equal volume of octanol the absorption of the CL dyes in the aqueous fraction was remeasured again. The partition coefficients were calculated as a ratio of the concentration of the CL dye in the organic and aqueous phase.

Sensitivity and Selectivity of Different CL Dyes

L-012 (100 μM), luminol (100 μM), lucigenin (100 μM) or coelenterazine (100 μM) were incubated for 10 min in PBS buffer containing DTPA (100 μM) and HX (1 mM) at room temperature. Thereafter, the basal (background) signal was determined in a chemiluminometer (Lumat LB9507, Berthold Techn., Bad Wildbad, Germany) at intervals of 60 s for 10 min. This procedure was repeated for all CL dyes in the presence of either XO (10 mU/ml), SIN-1 (1 mM) or H_2O_2 (1 mM). The final volume of all samples was 0.5 ml and the results were expressed as counts/min at 10 min. In the similar experiments the incubation time and concentrations of XO, SIN-1 as well as CL dyes were varied. DTPA was added to suppress Fenton reactions which could originate from free metals in the XO preparations.

Detection of Authentic and *In Situ* Generated Superoxide and Peroxynitrite

A saturated solution of KO_2 in water-free DMSO (5 mM at room temperature based on the absorption at 260 nm) served as the superoxide source. A saturated stock of NO in argon-purged, oxygen-free water (2 mM at 20°C) served as the NO source. NO (2.5–250 μM) was added to the L-012 (100 μM) containing PBS solution and the sample was immediately positioned in the chemiluminometer. Measurements were started by the addition of KO_2 in DMSO through the counter's injection unit, yielding a final superoxide concentration of approximately 50 μM. The CL was counted at intervals of 3 s and the value after 15 s was used for further analysis. At higher concentrations of KO_2 the initial CL signal within the first 3 s exceeded the detection limit of the chemiluminometer and then rapidly declined due to the fast decomposition of superoxide.

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An alkaline stock of authentic peroxynitrite was diluted to final concentrations of 0.2–200 μM in 5 mM NaOH. Aliquots of 25 μl of these solutions were added to a L-012 (100 μM) containing 0.5 M potassium phosphate buffer, pH 7.4 and simultaneously the measurements were started. The addition of the alkaline stock solutions caused no detectable shift in pH and yielded final peroxynitrite concentrations of 0.01–10 μM. The effect of excess NO (80 μM) on the peroxynitrite-induced CL signal was tested. As a control, 10 μM decomposed peroxynitrite (30 min at room temperature in PBS) were added to test the effect of contaminants such as H_2O_2. The CL was counted at intervals of 3 s and the value after 25 s was used for further analysis. At higher concentrations of Peroxynitrite the initial CL signal within the first 3 s exceeded the detection limit of the chemiluminometer and then rapidly declined due to the fast decomposition of Peroxynitrite.

The L-012-derived CL was also measured in PBS containing DTPA (50 μM) upon addition of XO (2.5 mU/ml)/HX (1 mM) and/or DEA/NO (1–10,000 nM) at room temperature at intervals of 30 s. To some samples PTIO (0.01–1 mM), sodium bicarbonate (0.1–10 mM), uric acid (10 μM) or SOD (500 U/ml) were added. The counts were recorded after 5 min. Similar experiments were performed with SNP (1–1000 μM) or SPE/NO (10–10,000 nM) as alternative NO-donors.

The experiments in the XO (2.5 mU/ml)/HX (1 mM) system with varying concentrations of SPE/NO (0.01–100 μM) were repeated in the presence of either lucigenin, luminol (both 100 μM) or coelenterazine (1 μM) in PBS containing DTPA (50 μM).

Detection of Superoxide and Peroxynitrite Formation in Isolated Rat Heart Mitochondria

Rat heart mitochondria were prepared according to a modified method of Raha *et al.*[23] Hearts from untreated Wistar rats were homogenized in HEPES buffer (composition in mM: 50 HEPES, 70 sucrose, 220 mannitol, 1 EGTA and 0.033 BSA) and subjected to centrifugation steps at 4°C of 1500*g* for 10 min and 2000*g* for 5 min (the pellets were discarded). The resulting supernatant was centrifuged at 16,000*g* for 20 min, and the pellet resuspended in 1 ml of Tris buffer (composition in mM: 10 Tris, 340 sucrose, 100 KCl and 1 EDTA). The last centrifugation step was repeated and the pellet was finally resuspended in 1 ml of Tris buffer. For preparation of SMP, the mitochondrial suspension was sonicated, centrifuged at 50,000*g* for 30 min at room temperature and the pellet was resuspended in buffer at pH 7.4 (10 mM KH_2PO_4, 100 mM KCl, 250 mM sucrose). The mitochondrial suspensions (total protein 3–10 mg/ml) were kept on ice.

Mitochondrial and SMP suspensions were diluted to a final protein concentration of 0.1 mg/ml in 0.5 ml of PBS buffer containing L-012 (100 μM). ROS production was detected after stimulation with succinate (4 mM final concentration) in the presence or absence of antimycin A (20 μg/ml), myxothiazol (20 μM), KCN (10 mM), rotenone (100 μM) or DPI (100 μM). The CL was registered at intervals of 30 s over 5 min with a chemiluminometer and the signal was expressed as counts/min at 5 min.

In another set of experiments, the L-012-derived CL was measured in succinate/antimycin A stimulated mitochondrial suspensions as described above but in the presence of either DEA/NO (0.01–100 μM) or SNP (1–5000 μM). In addition, the effect of PTIO (0.1 or 1 mM), sodium bicarbonate (0.1–10 mM) or SOD (10–1000 U/ml) on the CL signal was tested. Similar experiments were performed in mitochondrial suspensions stimulated with KCN (10 mM) in the presence or absence of SNP (1–1000 μM) using lucigenin (100 μM) as the CL probe.

Detection of Superoxide Formation in Isolated Rat Heart Mitochondria by DHE-derived Fluorescence

Suspensions of mitochondria (0.2 mg/ml) in PBS were treated with DHE (1 μM) and stimulated with succinate (5 mM) and/or antimycin A (20 μg/ml). The fluorescence was read with a plate reader (Twinkle LB 970, Berthold Techn.) over 11 min at 37°C with excitation and emission wavelengths of 520 and 590 nm, respectively.[24] Each well contained a final volume of 200 μl.

The formation of ROS in mitochondria was also visualized by fluorescence microscopy. The mitochondrial suspensions (1 mg/ml) in MOPS buffer were incubated at 37°C for 30 min in the presence of DHE (5 μM) and Mito Tracker Green FM (100 nM, Molecular Probes). The samples were either stimulated with succinate (4 mM) and myxothiazol (20 μM) or remained untreated. After washing with Tris-buffer, fixation with paraformaldehyde (4%, 10 min at room temperature) and additional washing steps, the samples were transferred to microscope slides. Finally, images of the mitochondrial samples were recorded by fluorescence microscopy (Leica DML, magnification 1000-fold).

RESULTS

Partition Coefficients of Different CL Dyes

The permeability of a CL dye for phospholipid membranes determines whether it detects extracellular or intracellular reactive species or both. Figure 1 shows the structures of the CL dyes. The partition coefficients were determined for

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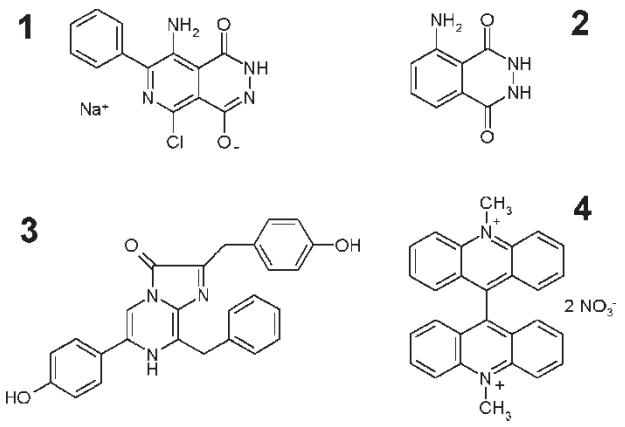

FIGURE 1 Structures of the chemiluminescence dyes L-012 (1), luminol (2), coelenterazine (3) and lucigenin (4).

octanol/water and are 0.11 ± 0.01 for lucigenin, 13.17 ± 0.84 for coelenterazine, 1.13 ± 0.08 for luminol and 2.43 ± 0.73 for L-012 in PBS buffer.

Sensitivity and Selectivity of Different CL Dyes

The CL-dyes L-012, luminol, lucigenin and coelenterazine were used to compare for their sensitivity and specificity to detect superoxide (formed by the HX/XO system), reactive species such as peroxynitrite (derived from SIN-1) and authentic H_2O_2 (Table I). The incubation time was crucial for the SIN-1 CL reactivity. Irrespective of the chosen experimental conditions, L-012 yielded 100 fold higher CL-signals than other CL substances in response to acute challenges with superoxide and peroxynitrite.

Detection of Superoxide and Peroxynitrite by L-012 and other CL Dyes

To verify that L-012 detects peroxynitrite, the authentic compound was used from an alkaline stock. The results depicted in Table II show that authentic peroxynitrite (0.01–10 μM) increases the L-012-derived CL signal in a dose-dependent manner. Peroxynitrite (10 μM) yielded a 20 fold higher CL signal than that obtained with KO_2 (50 μM). Decomposed peroxynitrite yielded the lowest CL signal and excess NO decreased the peroxynitrite-derived CL.

KO_2 and NO were used, to study the specificity of L-012 for the detection of superoxide and peroxynitrite, respectively, without relying on the activity of an enzymatic system such as HX/XO or a peroxynitrite donor such as SIN-1. As shown in Table II, the CL signal intensified with increasing amounts of NO, compatible with the formation of peroxynitrite. As expected, excess NO quenched the signal in accordance with its peroxynitrite scavenging activity in aerated solutions.

Similar observations were made when superoxide produced by the HX/XO system was exposed to increasing amounts of NO derived from various NO-donors. The L-012 CL signal reached a maximum at a concentration of 100 nM DEA/NO (Table III). A further increase in the concentration of DEA/NO resulted in a decline of the L-012-derived CL signal reaching background values at a concentration of 1–10 μM DEA/NO. DEA/NO (0.1–10 μM) in the absence of superoxide did not modify the CL signal at all. When SPE/NO was used, the maximum CL signal was reached at the concentration of 1 μM (Table III) followed by a decline in the presence of excess NO. Comparable results were obtained when SNP was used (not shown).

The addition of the non-specific NO-scavenger PTIO (10 μM) to the HX/XO system alone had no significant effect on L-012-enhanced CL, whereas the CL signal obtained in the presence of HX/XO and SPE/NO (1 μM) was suppressed by approximately 80%. Bicarbonate (10 mM) did not modify the CL signal evoked by superoxide, whereas in the presence of peroxynitrite, the L-012-derived CL

TABLE I Comparison of different chemiluminescence (CL) dyes with respect to sensitivity and specificity for superoxide, SIN-1-derived reactive species and hydrogen peroxide

	CL (counts/min)			
Condition	Luminol	Lucigenin	L-012	Coelenterazine
Basal*	528 ± 11	626 ± 11	47,265 ± 3861	493 ± 25
+XO	22,158 ± 1610	22,043 ± 901	5441,856 ± 463,942	1131 ± 9
+SIN-1	2369 ± 350	838 ± 29	642,851 ± 43964	1576 ± 132
+H_2O_2	2395 ± 25	1828 ± 77	131,027 ± 3874	635 ± 4
Basal†	352 ± 9	344 ± 26	36,119 ± 10,596	65,588 ± 872
+XO	84,463 ± 1328	91,923 ± 5463	27843,333 ± 1673,363	149,816 ± 4229
+SIN-1	26,844 ± 629	585 ± 54	81216,667 ± 4324,135	52,901 ± 913
+H_2O_2	679 ± 16	2897 ± 209	63,495 ± 15,515	75,034 ± 3226

*L-012 (100 μM), luminol (100 μM), lucigenin (100 μM) or coelenterazine (1 μM) were incubated for 5 min in PBS containing DTPA (100 μM) at room temperature in the presence of either XO (2.5 mU/ml)/HX (1 mM), SIN-1 (100 μM) or hydrogen peroxide (1 mM). The data is expressed as counts/min at the 5 min time point and is the mean ± SEM of 3–4 independent experiments. †L-012 (100 μM), luminol (100 μM), lucigenin (100 μM) or coelenterazine (100 μM) were incubated for 10 min in PBS buffer containing DTPA (100 μM) and HX (1 mM) at room temperature in the presence of either XO (10 mU/ml), SIN-1 (1 mM) or hydrogen peroxide (1 mM). The data is expressed as counts/min at the 10 min time point and is the mean ± SEM of three independent experiments.

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TABLE II Effect of NO on potassium superoxide- or peroxynitrite-induced L-012-derived CL

	DMSO	KO_2	KO_2 + 2.5 μM NO	KO_2 + 12.5 μM NO	KO_2 + 25 μM NO	KO_2 + 250 μM NO
CL × 10^6 (counts/min)*	0.19 ± 0.11	0.49 ± 0.12	1.75 ± 0.25	3.05 ± 0.45	6.05 ± 0.95	1.80 ± 0.14
	Decomposed $ONOO^-$	0.01 μM $ONOO^-$	0.1 μM $ONOO^-$	1 μM $ONOO^-$	10 μM $ONOO^-$	10 μM $ONOO^-$ + 80 μM NO
CL × 10^6 (counts/min)†	0.10 ± 0.03	0.36 ± 0.02	1.69 ± 0.23	9.08 ± 0.25	10.49 ± 0.23	1.11 ± 0.14

*L-012 (100 μM)-derived CL was measured upon rapid addition of KO_2 (50 μM in DMSO) to PBS with or without NO (2.5–250 μM). DMSO was added as a blank to exclude non-specific effects of the solvent. The data is expressed as counts/3 s at the 15 s time point × 10^6 and is the mean ± SEM of three independent experiments. †L-012 (100 μM)-derived CL was measured upon rapid addition of $ONOO^-$ to PBS with or without NO. Decomposed $ONOO^-$ (10 μM) was added as a blank to exclude non-specific effects of the solvent and contaminants. The data is expressed as counts/3 s at the 25 s time point × 10^6 and is the mean ± SEM of 3–6 independent experiments.

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increased by about 20%. Cu, Zn-SOD (500 U/ml) effectively suppressed both signals. Similar results were obtained with the HX/XO system in the presence of DEA/NO.

Similar experiments were performed using the chemiluminescent substance lucigenin. The CL signal evoked by the HX/XO reaction declined dose dependently by adding NO, pointing to a high specificity of lucigenin for the detection of superoxide and the lack of detection of peroxynitrite (not shown). In contrast, under similar experimental conditions, luminol- as well as coelenterazine-derived CL signals reached a maximum in the presence of superoxide formed by the HX/XO reaction and SPE/NO (1 μM) (not shown). The increase in the CL intensity, however, was only 50–100% for luminol and coelenterazine, respectively, whereas a striking 8-fold increase was observed when using L-012 as a chemiluminescence probe (see Table III).

Detection of Superoxide and Peroxynitrite Formation in Isolated Rat Heart Mitochondria by L-012-derived CL

Next it was tested whether the luminol analogue L-012 can also be used to detect ROS in isolated rat heart mitochondria. As shown in Table IV, superoxide was detected in response to stimulation of mitochondrial respiration by succinate. When the respiratory chain was blocked by inhibitors of complex III such as antimycin A or myxothiazol, the L-012-enhanced CL signal significantly increased in the presence of succinate whereas without succinate, no significant change was observed. Upon addition of the complex IV inhibitor KCN, the CL signal intensified. When SMP were used instead of intact mitochondria, the signal in response to myxothiazol increased approximately 10-fold. The addition of the complex I inhibitors rotenone and DPI (inhibitor of flavin-dependent oxidoreductases) to mitochondria did not significantly alter the L-012-derived CL. The CL signal was completely suppressed by the addition of Cu, Zn-SOD and PEG-SOD. Upon mitochondrial stimulation with an ethanolic solution of antimycin A lucigenin enhanced CL was not modified, but showed a significant increase in CL upon stimulation with KCN (not shown).

Similar experiments were performed with the superoxide sensitive fluorescence probe DHE. As shown in Table V, the addition of succinate to isolated mitochondria increased markedly the DHE-derived fluorescence and was further intensified by adding antimycin A. In addition, we can also demonstrate that DHE allows localization of mitochondria within intact cells or even tissue as exemplified by DHE/mito-tracker co-staining and microscopy in a suspension of isolated mitochondria (Fig. 2). Here, incubation with myxothiazol led to an increase in DHE-derived fluorescence within mitochondria as detected by co-staining with the fluorescence of the mito-tracker.

We were also able to detect peroxynitrite formation in mitochondrial suspensions by using L-012-enhanced CL upon stimulation with antimycin A in the presence of increasing amounts of NO derived from DEA/NO (Table VI). A CL maximum was reached at a DEA/NO concentration of 1.25 μM while higher DEA/NO concentrations resulted in a decline of the signal. In the presence of DEA/NO without antimycin A stimulation, the signal did not exceed the background counts. Similar results were obtained when SNP was used as a source of NO but the maximum CL signal was reached at a SNP concentration of 100 μM. The addition of PTIO (1 mM) or bicarbonate (1 mM) to the antimycin A-stimulated mitochondria caused no significant change in CL, whereas PTIO almost completely suppressed the signal in the antimycin A/DEA/NO containing preparations and bicarbonate (1 mM) caused a slight increase in L-012-derived CL (Table VI). When superoxide formation in KCN-stimulated mitochondria was detected by lucigenin-enhanced CL, the addition of SNP (1–100 μM) caused a marked decrease in the signal (data not shown).

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TABLE III Effects of increasing concentrations of NO-donors on the HX/XO-induced L-012 CL signal

XO + DEA/NO (nM)	0	1	10	25	50	100	200	500	1000	10,000	w/o XO + 10,000
CL × 10^6 (counts/min)*	4.7 ± 0.8	6.0 ± 0.6	8.7 ± 1.0	11.3 ± 1.6	14.1 ± 2.1	16.1 ± 1.0	11.6 ± 2.7	0.90 ± 0.34	0.13 ± 0.08	< 0.002	< 0.001
XO + SPE/NO (nM)	0	10	100	500	900	1000	1100	1500	2000	3000	10,000
CL × 10^6 (counts/min)†	2.7 ± 0.5	4.2 ± 0.0	5.4 ± 0.6	13.7 ± 0.2	15.9 ± 0.2	21.0 ± 1.8	14.6 ± 0.9	7.0 ± 0.5	4.0 ± 0.3	2.8 ± 0.4	< 0.003

*L-012 (100 μM)-derived CL in the presence of XO (2.5 mU/ml)/HX (1 mM) and/or NO from DEA/NO (1–10,000 nM) in PBS containing DTPA (50 μM) at room temperature. †L-012 (100 μM)-derived CL in the presence of XO (2.5 mU/ml)/HX (1 mM) and/or NO from SPE/NO (0.01–100 μM) in PBS containing DTPA (50 μM) at room temperature. The data is expressed as counts/min at the 5 min time point and is the mean ± SEM of 3–5 independent experiments.

DISCUSSION

The results of the present study show that the chemiluminescence probe L-012 when used in cell-free systems preferentially detects peroxynitrite compared to superoxide. No significant signals were observed in response to H_2O_2 challenges. Moreover, experiments with isolated mitochondria from the heart indicate that this luminol analogue can be used for the detection of superoxide and peroxynitrite in mitochondrial samples providing a better signal to noise ratio than other CL dyes tested such as coelenterazine, luminol and lucigenin or the fluorescence dye DHE.

Intra- vs. Extracellular Detection of ROS by CL Dyes

Another interesting aspect is whether the CL substances are able to detect ROS formed intra- or extracellular. According to the structures and partition coefficients of the CL dyes, it may be suggested that e.g. coelenterazine permeates membranes and detects intracellular ROS. In contrast, lucigenin would be considered to be rather membrane impermeable and therefore, to detect primarily extracellular ROS. Luminol and L-012 showed intermediate values, suggesting that they may detect intra- and extracellular ROS. The almost complete suppression, however, of the mitochondrial L-012-derived CL signal in particular by conventional SOD which can not penetrate membranes can be considered as a strong argument against an intracellular/intramitochondrial detection of ROS by L-012 in accordance with data recently presented by Imada *et al.*[25]

Specificity and Sensitivity of L-012

Previous reports have suggested a higher specificity of L-012 for superoxide than for peroxynitrite derived from SIN-1.[18] As demonstrated by Sohn *et al.* xanthine (100 μM)/XO (1 mU/ml) yielded a 10 fold higher L-012-derived CL signal than SIN-1 (10 μM) but the signal was only recorded for the first 120 s of the reaction. Our results, however, indicate that this kind of observation strictly depends on the chosen experimental conditions. Especially the incubation time proved to be an essential parameter for SIN-1 reactivity and at least 10 min are required until the system has equilibrated and yields constant fluxes of peroxynitrite and related species. The results obtained with L-012 and luminol clearly indicate that the experimental setup is a crucial point for the fluxes of superoxide and peroxynitrite produced from HX/XO and SIN-1.

The use of SIN-1 as a peroxynitrite donor and the use of the superoxide generating system HX/XO

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TABLE IV Detection of superoxide in isolated rat heart mitochondria or SMP upon inhibition of electron transport by CL

	CL (counts/min)			
Condition*	Control	+ Antimycin A	+ Myxothiazol	+ KCN
Mito	2090 ± 21	31,011 ± 1326	18,727 ± 1365	7640 ± 40
SMP	3401 ± 230	28,999 ± 3177	210,996 ± 14,564	4455 ± 414

*The L-012 (100 μM)-derived CL in mitochondria or SMP (both 0.1 mg/ml) was determined upon treatment with either antimycin A (20 μg/ml), myxothiazol (20 μM) or KCN (10 mM) in PBS containing succinate (4 mM). The data is expressed as counts/min at the 5 min time point and is the mean ± SEM of three independent experiments.

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may be subject to many artifacts including changes of ROS production due to oxygen depletion, a direct interaction of L-012 with the molybdenium active site of the XO or a loss of the activity of the oxidase during the reaction. Moreover, incubation time as well as the chosen concentration of HX may markedly influence the L-012-enhanced CL signal as illustrated in Table I. Artifacts may also arise from reactions of the chosen buffer with peroxynitrite, e.g. the reaction with HEPES to produce superoxide,[26] all of which may interfere with peroxynitrite detection by CL and fluorescence dyes.

In contrast to L-012, the CL substance lucigenin showed significant increases in the CL signal solely, when superoxide is formed by the HX/XO reaction independently of the applied conditions. Coelenterazine showed comparable selectivity/sensitivity for superoxide and SIN-1-derived reactive species but not for H_2O_2. Importantly, the use of coelenterazine at high but also at low concentrations resulted in the worst background to stimulated signal ratio all of which may limit its usefulness for the detection of ROS in cell-free systems but also in intact vascular tissue.

Detection of Authentic or Co-generated Peroxynitrite by L-012-derived CL

The preferred detection of peroxynitrite by L-012 was proven by the use of authentic peroxynitrite, which at 100 nM yielded a significantly higher CL signal than KO_2 (50 μM) (see Table II).

Experiments with fixed XO concentrations and therefore, constant superoxide fluxes and varying concentrations of NO-donors such as SPE/NO, DEA/NO and SNP revealed that there is a maximum in L-012-derived CL in accordance with maximal peroxynitrite formation at equimolar fluxes of NO and superoxide. Excess NO scavenged peroxynitrite and accordingly the CL signal declined dose dependently. At higher concentrations of NO the signal was completely suppressed. Experiments performed with NO donors such as DEA/NO and SPE/NO led to similar results demonstrating that SPE/NO required 10-fold higher concentrations to cause maximal L-012-enhanced CL. This is compatible with a roughly 10 fold faster decomposition rate of DEA/NO, yielding 10-fold more NO in the same time interval.

Luminol showed comparable results in the HX/XO/SPE/NO system but the ratio of the peroxynitrite-derived and the superoxide-derived CL signal was lower than that obtained with L-012. Lucigenin showed a decrease upon addition of the NO-donor in agreement with its high selectivity for superoxide and the known reaction mechanism involving a dioxetan intermediate that leads to the CL signal.[27] Using the same conditions, coelenterazine only showed a slight increase in the CL signal, peaking at the same concentration of SPE/NO as in the luminol and L-012 experiments (not shown). When the CL signal was maximally stimulated by peroxynitrite, the NO-scavenger PTIO sharply decreased the signal, whereas bicarbonate slightly increased the signal. These observations indicate a preferred detection of peroxynitrite by L-012, since scavenging of NO by PTIO decreases the yield of peroxynitrite and thereby decreases the CL signal.

The increase in peroxynitrite induced L-012 CL by bicarbonate may be explained by bicarbonate-derived CO_2 which increases the formation of peroxynitrite-derived $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals and/or other reactive species via the 1-carboxylato-2-nitrosodioxidan ($ONOOCO_2^-$) intermediate.[28,29] These results are also in accordance with previous findings on peroxynitrite formation in the HX/XO system in the presence of increasing concentrations of NO, using different methodologies including dihydrorhodamine fluorescence,[16] thiol oxidation in alcohol dehydrogenase[30] and tyrosine nitration.[31]

TABLE V Detection of superoxide in isolated rat heart mitochondria by DHE-derived fluorescence

	Fluorescence [RLU]*		
PBS	Mito	+ Succinate	+ Succinate/Antimycin A
48 ± 5	263 ± 17	393 ± 17	863 ± 51

*The DHE (1 μM)-derived fluorescence in mitochondrial suspensions (0.2 mg/ml) was quantified in the presence or absence of succinate (4 mM)/antimycin A (20 μg/ml). The data is expressed as relative light units (RLU) at the 11 min time point and is the mean ± SEM of six independent experiments.

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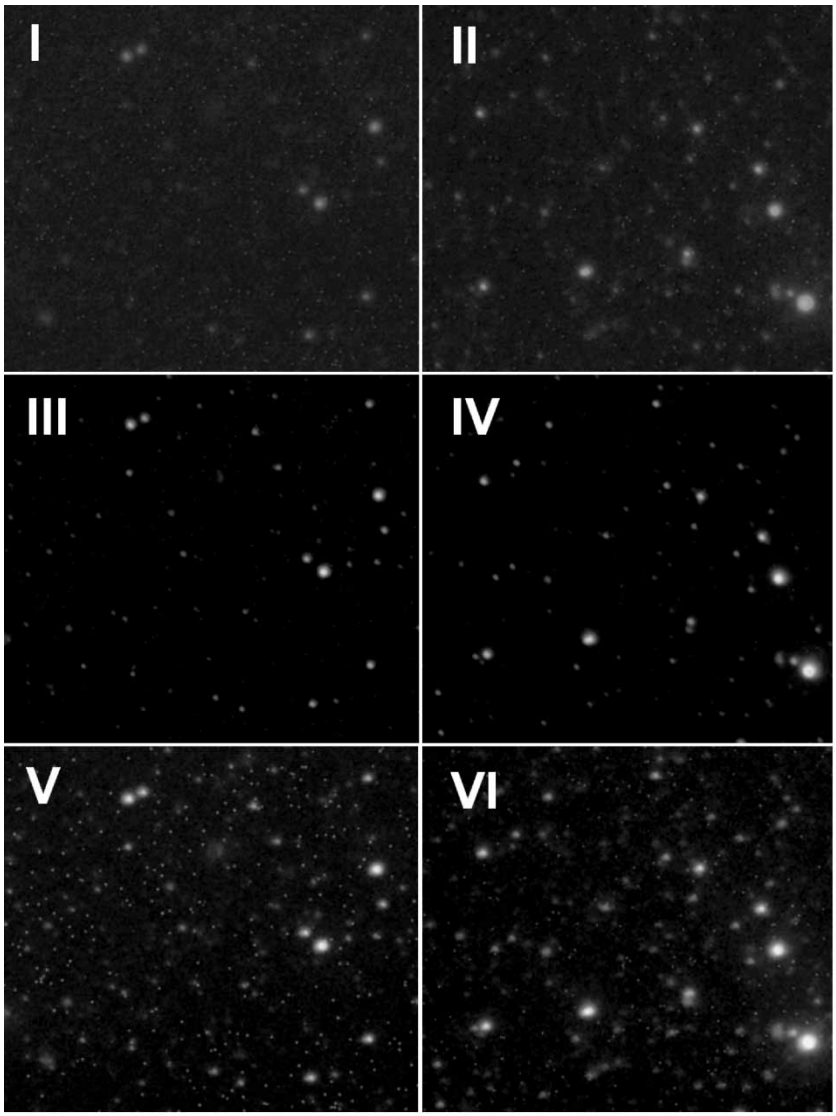

FIGURE 2 Detection of superoxide in isolated rat heart mitochondria by DHE fluorescence microscopy. Fluorescence microscope images were taken from mitochondria after incubation with DHE (10 μM) and mito-tracker (0.1 μM). Superoxide formation upon stimulation with succinate (4 mM) alone was detected by DHE-derived red fluorescence (I), mitochondria were envisaged by mito-tracker-derived green fluorescence (III) and the yellow colour in the computer-generated overlay shows the co-localization of both (V). Similar pictures were recorded from mitochondria upon stimulation with succinate (4 mM)/myxothiazol (20 μM) (II, IV and VI). The pictures are representative of at least three independent experiments.

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Previous reports indicate that NO at high concentrations and/or upon reaction with superoxide and subsequent formation of peroxynitrite may inhibit XO activity directly.[32,33] To avoid confounding changes in the activity of the XO itself we used short incubation periods (5 min) as well as an enzyme-independent superoxide forming system. For example when authentic KO_2 in DMSO was rapidly mixed with an aqueous solution of NO, the L-012-derived CL signal increased until equimolar concentrations of NO and $^{\bullet}O_2^-$ were reached. Like demonstrated before, excess NO markedly suppressed the CL signal. We also observed that the extent of maximal increases in CL was comparable to that observed in the HX/XO/SPE/NO system.

Detection of Superoxide in Mitochondria by L-012-derived CL

Different sites in mitochondria have been demonstrated to contribute significantly to superoxide formation. Superoxide may arise from mitochondrial complexes I, II, IV and in particular complex III seems to play an important role as a superoxide source in animal models such as hypoxia-reoxygenation (ischemia/reperfusion damage),[21], aging,[12] nitrate tolerance (unpublished observations) and

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TABLE VI Effects of increasing concentrations of DEA/NO on the antimycin A-induced L-012 CL signal in isolated rat heart mitochondria

Mitos + AA + DEA/NO (μM)	w/o AA + 0	0	0.01	0.05	0.1	0.5	0.75
CL × 10^6 (counts/min)*	0.004 ± 0.000	0.099 ± 0.003	0.79 ± 0.03	1.23 ± 0.01	2.17 ± 0.33	6.28 ± 0.14	9.28 ± 0.08
Mitos + AA + DEA/NO (μM)	1	1.25	2.5	5	10	100	w/o AA 1
CL × 10^6 (counts/min)*	10.62 ± 0.63	11.82 ± 0.42	5.23 ± 0.23	0.27 ± 0.10	0.036 ± 0.003	0.013 ± 0.001	0.003 ± 0.000
Mitos + AA	–	PTIO	HCO_3^-				
CL × 10^4 (counts/min)†	9.96 ± 0.29	12.20 ± 1.75	10.67 ± 0.85				
Mitos + AA + 1 μM DEA/NO	–	PTIO	HCO_3^-				
CL × 10^6 (counts/min)†	10.62 ± 0.63	0.43 ± 0.05	13.70 ± 0.45				

*L-012 (100 μM)-derived CL in mitochondrial suspensions (0.1 mg/ml) upon stimulation with antimycin A (20 μg/ml) in the presence of DEA/NO (0.01–100 μM) in PBS at room temperature. Control measurements were performed in the presence of DEA/NO (1 μM) but in the absence of antimycin A. † Effects of PTIO (1 mM) and bicarbonate (HCO_3^-, 1 mM) on the L-012 (100 μM)-derived CL in mitochondria were measured either upon stimulation with antimycin A (20 μg/ml) together with DEA/NO (1 μM) or with antimycin A alone. The data is expressed as counts/min at the 5 min time point and is the mean ± SEM of 3–6 independent experiments. AA, antimycin A.

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hypercholesterolemia.[22] In the present studies, mitochondrial superoxide production was stimulated by addition of succinate, a substrate for complex II and quantified using L-012 enhanced CL. As expected, inhibitors of complex I such as DPI and rotenone had no effect on the succinate-triggered superoxide formation suggesting that in this model the superoxide formation by complex I do not play a significant role. In contrast, inhibition with the blocker of complex IV, KCN, led to a marked increase in superoxide levels, compatible with a blockade of complex IV and leaking electron transport at this site. As previously reported, especially the Q (ubiquinone/ubiquinol)-cycle at complex III may increase formation of superoxide due to electron-transfer from semiubiquinone radicals to molecular oxygen.[23] The observed strong increase in L-012-derived CL upon inhibition of complex III by antimycin A is in accordance with the proposed inhibition of electron transport from cytochrome b to coenzyme Q, due to the accumulation of semiubiquinone radicals at the P-centre and extra-mitochondrial formation of superoxide.

In contrast, experiments with the inhibitor of electron transport from coenzyme Q to the FeSIII protein (cytochrome c pathway) at complex III, myxothiazol, show that the increases in L-012 enhanced CL are significantly less than in experiments with antimycin A. There are several reasons to explain these findings. L-012 detects preferentially extra-cellular ROS[34] and in addition intra-mitochondrially formed superoxide may be scavenged by Mn-SOD. This assumption is supported by the approximately 10-fold increase in L-012-derived CL, which was observed upon treatment of SMP with myxothiazol, while antimycin A-induced CL signal remained unaltered. This observation may be explained by a direct access of L-012 to myxothiazol-derived superoxide in SMP and/or by an elimination of Mn-SOD by additional centrifugation steps. Accordingly, the addition of Cu, Zn-SOD or PEG-SOD decreased the L-012-derived CL signal obtained by stimulation of mitochondria with succinate/antimycin A or with succinate/myxothiazol-stimulated SMP.

Compared to the fluorescence dye, DHE, L-012-derived CL provided a 7-fold higher ratio of succinate/antimycin A- to succinate-stimulated signal and also the ratio of succinate-stimulated to unstimulated signal in mitochondria was 2-fold higher with the CL dye. When coelenterazine (2.5 μM) was used to quantify superoxide formation in mitochondria, a ratio of succinate/antimycin A- to succinate-stimulated signal of approximately three was found[23] whereas that detected with L-012 was about 15. In SMP preparations, coelenterazine showed a ratio of stimulated to background signal of four whereas that observed with L-012 was 10.

According to these data, we conclude that L-012 provides a better signal to noise ratio than coelenterazine and DHE for the detection of superoxide in intact mitochondria and in SMP. Although DHE fluorescence is less sensitive, it remains a powerful tool for the detection of superoxide in biological samples due to its high specificity for superoxide and the informations provided on the subcellular localization of the superoxide source. As illustrated in Fig. 2, staining with DHE for superoxide in addition to mitochondria tracker clearly identified the mitochondria as a significant superoxide source. The computer-generated overlay

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of both fluorescent stainings demonstrates the co-localization of superoxide and mitochondria.

Detection of Peroxynitrite in Mitochondria by L-012

The experiments performed with isolated mitochondria are completely in agreement with the results obtained in our *in vitro* systems. When succinate/antimycin A-stimulated mitochondria were used as a superoxide source in the presence of increasing concentrations of NO-donors (DEA/NO and SNP), a maximal increase in L-012-derived CL was observed due to a preferred detection of peroxynitrite formed by equimolar fluxes of NO and superoxide as shown before. As illustrated in Table VI, the NO scavenger PTIO decreased the CL signal derived by antimycin A stimulation in the presence of DEA/NO (1 μM) to the extent of the NO-free system whereas bicarbonate increased the signal. This is compatible with an increased formation of $^{\bullet}NO_2$ and $^{\bullet}CO_3^-$ radicals and other reactive species from the $ONOOCO_2^-$ intermediate from peroxynitrite in the presence of CO_2 as already discussed. The CL signal of the NO-free system was not modified neither by PTIO nor by bicarbonate.

An interesting observation was the quite dramatic difference between the increase of the CL signal in the presence of maximally stimulating NO in antimycin A treated mitochondria and the HX/XO system *in vitro*. The maximal increase of the L-012-derived CL in the HX/XO system in the presence of SPE/NO (1 μM) was approximately 10 fold whereas in stimulated mitochondria in the presence of DEA/NO (1.25 μM) it was 120 fold. The maximal CL in mitochondria also required 10 fold higher concentrations of NO than the HX/XO system.

This observation may be explained by the fact that NO easily diffuses across membranes and thereby can reach the intra-mitochondrial space where it forms peroxynitrite which is not detected by the extracellular CL-dye L-012. Another reason may be that there are many competitive targets for NO in mitochondrial preparations (e.g. metal-proteins such as cytochrome c oxidase). The stronger increase of CL in the mitochondrial preparations may be explained by the inhibitory effects of NO and peroxynitrite on the mitochondrial electron transport. It has been reported that NO and peroxynitrite may inhibit complex I and II and in addition NO reacts very rapidly with cytochrome c oxidase thereby inhibiting mitochondrial complex IV.[35] These inhibitory effects on the respiratory chain could cause additional formation of superoxide and peroxynitrite, respectively resulting in a vicious cycle. In addition, peroxynitrite is known to inhibit mitochondrial Mn-SOD[36] which may also increase the steady-state concentrations of superoxide.

In conclusion, the results of the present studies indicate that the chemiluminescence dye L-012 provides a better sensitivity for the detection of ROS and reactive nitrogen oxide intermediates in mitochondria in comparison with coelenterazine and DHE with a higher selectivity for peroxynitrite than for superoxide. The use of the appropriate controls such as the NO scavenger PTIO as well as the enhancer of peroxynitrite reactivity bicarbonate may allow to distinguish between superoxide and peroxynitrite, respectively. When NO is derived from an enzymatic source such as the nitric oxide synthase, an appropriate control would include the inhibition of the enzyme by N^G-nitro-L-arginine. Especially in homogenates or in suspensions of mitochondria, the CL dye L-012 appears to provide a better detection limit with respect to the detection of superoxide and peroxynitrite than fluorescence assays using DHE or dihydrorhodamine.

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