Superoxide anion formation from lucigenin: an electron spin resonance spin-trapping study

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Abstract Lucigenin (LC^{2+}) is frequently used as a superoxide probe. To detect superoxide, lucigenin must be reduced to the lucigenin cation radical ($LC^{\bullet+}$). We show, using the phosphorylated spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), that lucigenin stimulates NADPH-dependent superoxide production by endothelial nitric oxide synthase (eNOS). The formation of the DEPMPO-superoxide adduct is calcium/calmodulin independent. DEPMPO-superoxide adduct formation is inhibited by diphenyleneiodonium and is abolished by superoxide dismutase. It is likely that eNOS/NADPH can reduce lucigenin to $LC^{\bullet+}$ which reduces oxygen to superoxide. Consequently, lucigenin cannot be used to measure superoxide formation.

Key words: Electron spin resonance; Spin-trapping; Lucigenin; Nitric oxide synthase; Superoxide

1. Introduction

Lucigenin (bis-*N*-methylacridinium nitrate, Fig. 1) is an acridine-based probe that has been used extensively to detect superoxide [1,2]. The reaction between superoxide and lucigenin has been shown to generate chemiluminescence in enzymatic and cellular systems. For example, lucigenin has been used to detect superoxide production from NADPH-oxidase in phagocytic cells, NADH-dehydrogenase in mitochondria, NADPH-cytochrome P_{450} reductase in microsomes, and from xanthine oxidase and NAD(P)H oxidases in endothelial and vascular smooth muscle cells [2–11]. The proposed mechanism for the evolution of chemiluminescence involves the one-electron reduction of lucigenin to form the lucigenin cation radical ($LC^{\bullet+}$, Fig. 1). This radical reacts with superoxide to form a dioxethane intermediate which decays by a light-emitting process (Eqs. 1 and 2).

$$LC^{2+} + e^- \rightarrow LC^{\bullet+} \quad (1)$$

$$LC^{\bullet+} + O_2^{\bullet-} \rightarrow \text{Products} + \text{Light} \quad (2)$$

Evidence suggests that superoxide cannot reduce lucigenin to the cation radical [12]. Consequently, superoxide alone will not stimulate chemiluminescence from lucigenin and an additional reducing agent is required. In several systems, including xanthine/xanthine oxidase and NAD(P)H/NAD(P)H-oxidase, it is assumed that the enzyme catalyzes the reduction of lucigenin to the cation radical intermediate. However, in most systems the mechanism of lucigenin reduction is unknown.

Electron transfer between reduced flavoproteins and xenobiotics is a well known phenomenon [13]. For example, viologens such as paraquat (PQ^{2+}, Fig. 1) can be reduced to the corresponding cation radicals by flavoproteins in the presence of NAD(P)H. The paraquat cation radical ($PQ^{\bullet+}$, Fig. 1) is an unstable species and undergoes futile redox-cycling, reducing oxygen to superoxide (Eq. 3). In this way paraquat provides a kinetic mechanism for the one-electron reduction of oxygen by NAD(P)H.

$$PQ^{2+} \xrightarrow{e^-} PQ^{\bullet+} \xrightarrow{O_2} PQ^{2+} + O_2^{\bullet-} \quad (3)$$

The close structural similarity between lucigenin and paraquat (Fig. 1) suggests that the lucigenin cation radial should also be unstable in the presence of oxygen and that lucigenin may act as a redox-cycling agent that generates superoxide anion radical. To investigate whether reduction of lucigenin can also give rise to superoxide formation we have used the electron spin resonance (ESR) spin-trapping technique incorporating the spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO, Fig. 1). The superoxide adduct of DEPMPO is fifteen times more stable than that of the more commonly used spin trap 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO). Moreover, unlike the DMPO-superoxide adduct, the DEPMPO-superoxide adduct does not spontaneously decay to the DEPMPO-hydroxyl radical adduct. Consequently, the steady state level of the DEPMPO-superoxide adduct is higher than the DMPO-superoxide adduct at the same rate of superoxide anion generation [14].

In this study we have examined the production of superoxide by endothelial nitric oxide synthase (eNOS)-dependent reduction of lucigenin. eNOS consists of a flavin-containing reductase domain and a heme-containing oxidase domain [15,16]. NADPH reduces the flavin component of the reductase domain but electron transfer to heme will not occur unless calcium/calmodulin is present. In the presence of calcium/calmodulin, the reductase and oxidase domains align allowing electron transfer from the flavins to the heme [16]. In the presence of arginine (the physiological substrate for eNOS) electrons flow to the heme moiety of the oxidase domain to activate oxygen which is used oxidize arginine to citrulline and nitric oxide. However, in the absence of arginine, low levels of superoxide are generated by dissociation of the heme-peroxo complex (Vásquez-Vivar, Hogg, Martasek, Kalyanaraman and Pritchard Jr., unpublished results).

We show here that eNOS-dependent superoxide production is greatly enhanced by lucigenin and is calcium/calmodulin

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independent. We propose that superoxide production occurs as a result of the reduction of oxygen by the lucigenin cation radical. Consequently any system that reduces lucigenin to the lucigenin cation radical can lead to the formation of superoxide. As the formation of the lucigenin cation radical is a prerequisite for superoxide detection [12], lucigenin is an unreliable probe for superoxide anion production in biological systems.

2. Materials and methods

2.1. Materials

Lucigenin was obtained from Aldrich Chemical Co. (Milwaukee, WI), NADPH and L-arginine were obtained from Sigma Chemical Co. (St. Louis, MO), diphenyleneiodonium chloride was obtained from Calbiochem (San Diego, CA) and L-[^{14}C]arginine was obtained from Dupont NEN (Boston, MA). DEPMPO was a gift from Oxis International Inc. (Portland, OR). eNOS was purified as previously described [15] but including an additional HPLC purification step (Superose 6 HR 10/30 column, Pharmacia Biotech AB, Uppsala, Sweden). The top fractions of a peak corresponding to the eNOS dimer were collected and concentrated. The preparation had an absorbance ratio (280 nm/407 nm) of 2.3–2.4 and was >95% pure based on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2.2. ESR measurements

ESR spectra were recorded at room temperature on a Varian E-109 spectrometer at 9.5 GHz employing 100 kHz field modulation. A typical incubation mixture for ESR analysis consisted of eNOS (20 pmol), lucigenin (20 μM), NADPH (0.1 mM) diethylenetriaminepentaacetic acid (DTPA, 0.1 mM) and DEPMPO (50 mM) in HEPES buffer (50 mM, pH 7.4) in a total volume of 0.2 ml. Reactions were initiated by the addition of enzyme and quickly transferred to a quartz flat cell for ESR analysis. Computer-based simulations of ESR spectra were performed using software written by D. Duling [17].

2.3. Biochemical assays

eNOS activity was determined by monitoring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as previously described [18]. Briefly, reaction mixtures containing HEPES (50 mM, pH 7.4), DTPA (0.1 mM), calcium chloride (0.4 mM), calmodulin (10 μg/ml), tetrahydrobiopterin (10 μM), flavin adenine dinucleotide (1 μM), flavin mononucleotide (1 μM), bovine serum albumin (100 μg/ml), L-[^{14}C]arginine (100 μM, 0.83 μCi), NADPH (0.5 mM) and glutathione (0.1 mM) were incubated with eNOS (18 pmol), and formation of L-[^{14}C]citrulline was measured by liquid scintillation counting [18].

NADPH oxidation was monitored spectrophotometrically (λ_{max} = 340 nm, ε = 6.22 mM^{-1} cm^{-1}). Reaction mixtures contained HEPES (50 mM, pH 7.4), DTPA (0.1 mM), lucigenin (20 μM), calcium chloride (0.1 mM), calmodulin (18 μg/ml) and eNOS (20 pmol).

3. Results

3.1. Activity of purified bovine eNOS

The activity of eNOS was monitored by following the formation of L-[^{14}C]citrulline from L-[^{14}C]arginine. The specific activity of the enzyme was 394 nmol citrulline/min/mg protein at 25°C.

3.2. eNOS-mediated NADPH oxidation in the absence and presence of lucigenin

When NADPH was added to an incubation containing either lucigenin or eNOS, negligible consumption of NADPH was observed (Fig. 2A,B). However, the combination of lucigenin and eNOS caused a complete oxidation of NADPH within 25 min (Fig. 2C). When lucigenin was added to eNOS in the presence of calcium/calmodulin a similar rate of NADPH oxidation was observed (data not shown). These results suggest that eNOS catalyzes lucigenin-dependent NADPH oxidation. Diphenyleneiodonium, a specific flavoprotein reductase inhibitor, completely inhibited lucigenin-stimulated oxidation of NADPH by eNOS (Fig. 2D). This indicates that lucigenin enhances NADPH oxidation by accepting electrons from the reductase domain of eNOS.

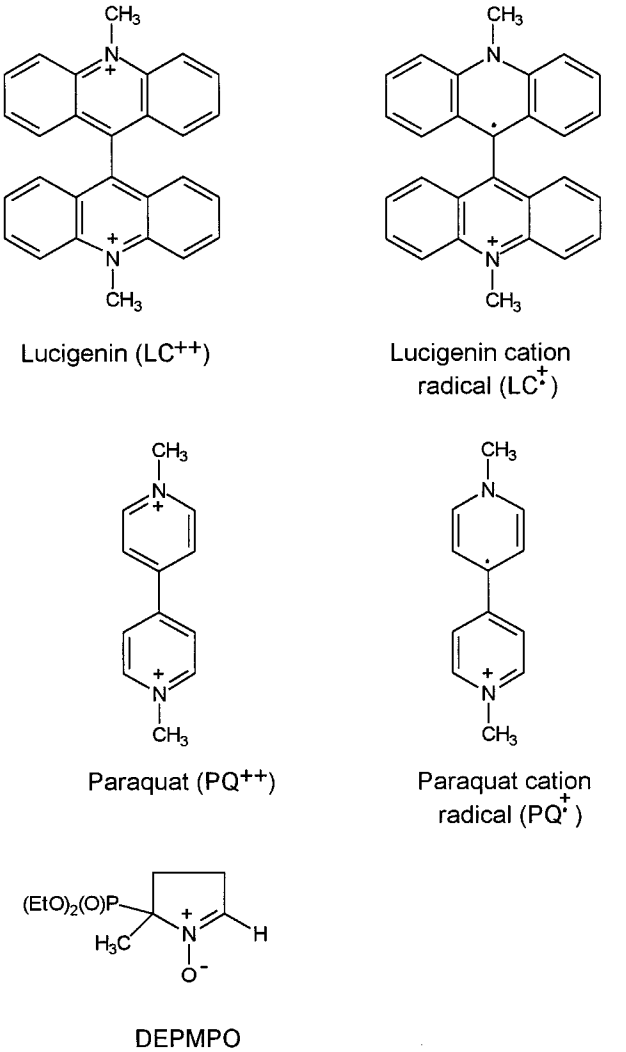

Fig. 1. Chemical structures.

3.3. Spin-trapping of superoxide anion from the reduction of lucigenin

Addition of NADPH to an incubation mixture containing eNOS, and the spin trap, DEPMPO, in HEPES buffer did not produce detectable levels of the DEPMPO-superoxide adduct (Fig. 3A). However, addition of lucigenin to the same incubation mixture produced an intense ESR signal corresponding to that of the DEPMPO-superoxide adduct (Fig. 3B). Fig. 3B shows a computer simulation of the two major diastereoisomers (63% of isomer I and 37% of isomer II) of the DEPMPO-superoxide adduct whose hyperfine coupling constants are (in Gauss: a^N = 13.1, a_β^H = 10.7, a^P = 49.2 for isomer I and a^N = 13.1, a_β^H = 11.6, a^P = 50.9 for isomer II) in accordance with previously reported values [14]. An identical ESR spectrum to that shown in Fig. 3B was observed if eNOS was incubated with NADPH, DEPMPO and lucigenin in the presence of calcium/calmodulin (data not shown). The

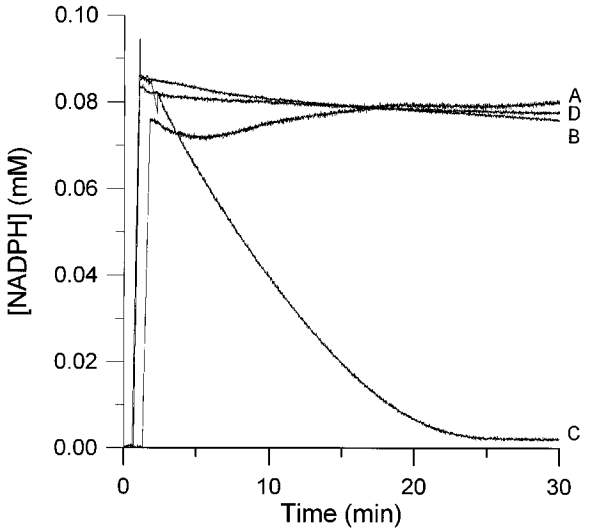

Fig. 2. NADPH consumption by eNOS in the presence and absence of lucigenin. NADPH was incubated in HEPES (50 mM, pH 7.4) containing DTPA (0.1 mM) at 25°C in the presence of (A) lucigenin (20 μM), (B) eNOS (20 pmol), (C) lucigenin (20 μM) and eNOS (20 pmol), and (D) lucigenin (20 μM), eNOS (20 pmol) and diphenyleneiodonium (100 μM). NADPH consumption was monitored at 340 nm. Data are representative of at least two independent experiments.

DEPMPO-superoxide adduct signal intensity was diminished if the solution was purged with nitrogen before analysis (Fig. 3C) and abolished by the addition of SOD (Fig. 3D). These results indicate that the DEPMPO-superoxide adduct is formed by trapping superoxide anion and that superoxide formation depends upon the presence of eNOS, NADPH and lucigenin.

In order to determine if the flavoprotein component of eNOS is involved in the lucigenin-dependent generation of superoxide, we performed spin-trapping experiments in the presence of diphenyleneiodonium, a flavoprotein reductase inhibitor. This compound inhibited the spectral intensity of the DEPMPO-superoxide adduct in a concentration-dependent manner (Fig. 4A–C), indicating that the reductase domain of eNOS reduces lucigenin.

4. Discussion

4.1. Lucigenin-dependent superoxide production

It has previously been reported that the interaction between superoxide and the lucigenin cation radical, but not lucigenin, gives rise to chemiluminescence [12]. Consequently, lucigenin must form a cation radical before it can react with superoxide to form light-emitting products (Eqs. 1 and 2). However, the reaction between the lucigenin cation radical and oxygen, to produce superoxide, has not been previously considered. Here we have demonstrated that lucigenin generates superoxide anion in the eNOS/NADPH system, under aerobic conditions.

Lucigenin is a water-soluble acridine-based compound that has a reduction potential of −0.4 V in DMSO and −0.3 V in water vs. Ag/AgCl electrode [19]. Analogous to paraquat, whose reduction potential at pH 7.0 is about −0.45 V, it is likely that lucigenin can be reduced by NADPH-cytochrome P_{450} reductase or the reductase domain of eNOS [20,21].

We show here that lucigenin stimulates eNOS-dependent NADPH consumption and superoxide generation. This occurs by a mechanism that depends entirely on the flavin component of the eNOS reductase domain. The most plausible explanation for lucigenin-stimulated superoxide production is that lucigenin acts as a redox-cycling compound in a manner similar to paraquat. By this mechanism, lucigenin is reduced to the cation radical ($LC^{\bullet+}$, Eq. 4) which reacts with oxygen to regenerate lucigenin and form superoxide (Eq. 5).

$$NADPH/eNOS + LC^{2+} \rightarrow LC^{\bullet+} \quad (4)$$

$$LC^{\bullet+} + O_2 \rightarrow LC^{2+} + O_2^{\bullet-} \quad (5)$$

In this way, the combination of eNOS and lucigenin catalyzes the NADPH-dependent reduction of oxygen to superoxide. The detailed mechanism of lucigenin-dependent superoxide production in this system is currently under investigation.

4.2. Lucigenin as a probe for superoxide

A considerable body of literature on vascular generation of superoxide has been obtained using the lucigenin chemiluminescence assay [3,9–11]. This assay is popular because it is assumed to measure intra- and extracellular superoxide generation with high sensitivity and specificity.

The data shown here indicate that lucigenin-derived chemiluminescence can not be attributed to endogenous superoxide production as lucigenin itself can generate superoxide in the presence of flavoprotein reductases or other enzymatic reduc-

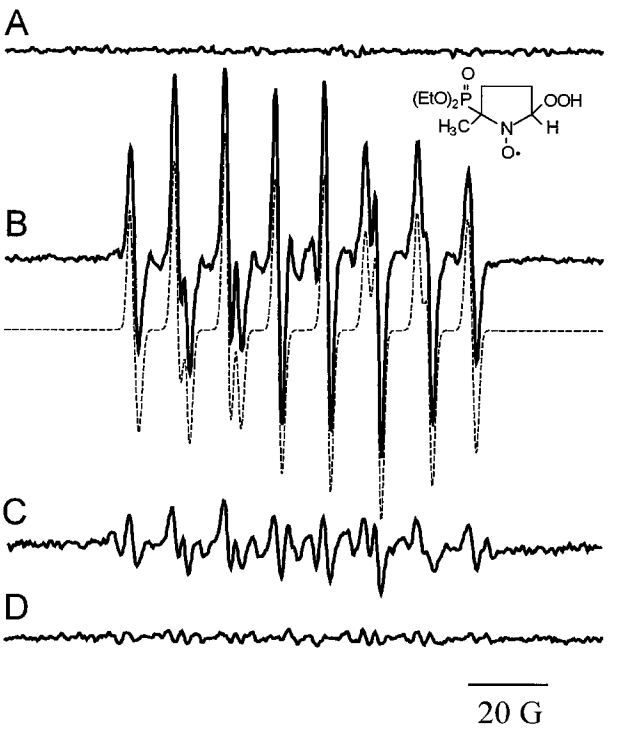

Fig. 3. Lucigenin-dependent superoxide formation by eNOS. eNOS (20 pmol), NADPH (0.1 mM) and DEPMPO (50 mM) in HEPES buffer (50 mM pH 7.4) containing DTPA (0.1 mM), were incubated at room temperature: (A) without lucigenin; (B) in the presence of lucigenin (20 μM); (C) as (B) after purging with nitrogen for 5 min; and (D) as (B) in the presence of SOD (10 μg/ml). Instrumental conditions: microwave power, 10 mW; modulation amplitude, 1 G; time constant, 0.128 s; scan rate, 1.67 G/s; and number of averaged scans, 5.

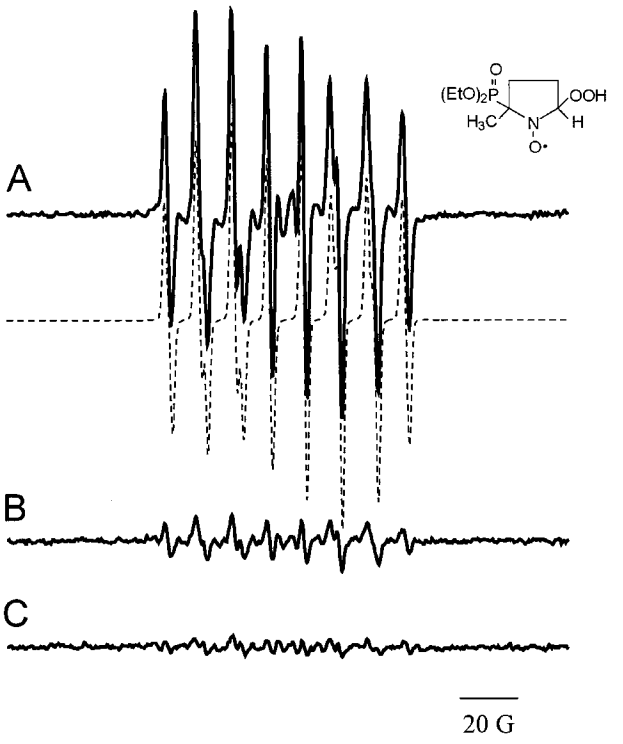

Fig. 4. Effect of diphenyleneiodonium on lucigenin-dependent superoxide formation by eNOS. Incubations of purified eNOS (20 pmol), NADPH (0.1 mM), lucigenin (20 μM) and DEPMPO (50 mM) in HEPES buffer (50 mM pH 7.4) containing DTPA (0.1 mM), were performed at room temperature: (A) without diphenyleneiodonium; (B) as (A) in the presence of diphenyleneiodonium (50 μM); (C) as (A) in the presence of diphenyleneiodonium (100 μM). Instrumental conditions: microwave power, 10 mW; modulation amplitude, 1 G; time constant, 0.128 s; scan rate, 1.67 G/s; and number of averaged scans, 5.

ing systems. Consequently lucigenin-dependent chemiluminescence is not a specific measure of superoxide.

4.3. Biological considerations

It is likely that redox-cycling xenobiotics such as quinones stimulate eNOS-dependent superoxide production by a similar mechanism to that observed with lucigenin (Eqs. 4 and 5). Redox metabolism of xenobiotics by eNOS will change the activity of eNOS from a nitric oxide synthase to a NADPH-oxidase, thus altering the balance between superoxide and nitric oxide in vascular cells. Changes in the rates of production of these two radicals have been shown to be an important component of vascular pathologies such as atherosclerosis [22] and can lead to increased formation of peroxynitrite, a potent biological oxidant [23].

Addition of lucigenin to a biological system may perturb the levels of nitric oxide and superoxide leading to increased peroxynitrite formation. The concomitant drain on NADPH levels will also render the cell more susceptible to increased oxidative stress.

4.4. Summary

The ESR spin-trapping data show unambiguously that lucigenin can mediate flavoprotein reductase-dependent superoxide production. Consequently, lucigenin can not be used as a probe to detect superoxide production in biological systems.

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