

Pitfalls in the Use of Common Luminescent Probes for Oxidative and Nitrosative Stress

Peter Wardman,^{1,2} Mark J. Burkitt,¹ Kantilal B. Patel,¹ Andrew Lawrence,¹
Clare M. Jones,¹ Steven A. Everett,¹ and Borivoj Vojnovic¹

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Lucigenin (LC^{2+} , bis-*N*-methylacridinium) and 2',7'-dichlorofluorescein (DCFH₂) are widely used as chemiluminescent or fluorescent probes for cellular oxidative stress, to reflect levels of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide, respectively. We report mechanistic studies that add to the growing evidence for the unsuitability of either probe except in very well-defined circumstances. The ability for lucigenin to generate superoxide via reduction of LC^{2+} to $LC^{\cdot+}$ and redox cycling with oxygen depends on the reduction potential of the $LC^{2+}/LC^{\cdot+}$ couple. Redox equilibrium between $LC^{\cdot+}$ and the redox indicator benzyl viologen is established in microseconds after generation of the radicals by pulse radiolysis and indicated $E(LC^{2+}/LC^{\cdot+}) \sim -0.28$ V vs. NHE. Reaction of $LC^{\cdot+}$ with O_2 to generate $O_2^{\cdot-}$ was also observed directly similarly, occurring in milliseconds, with a rate constant $k \sim 3 \times 10^6 M^{-1} s^{-1}$. Quinones act as redox mediators in $LC^{\cdot+}/O_2$ redox cycling. Oxidation of DCFH₂ to fluorescent DCF is not achieved by $O_2^{\cdot-}$ or H_2O_2 , but NO_2^{\cdot} reacts rapidly: $k \sim 1 \times 10^7 M^{-1} s^{-1}$. Oxidation by H_2O_2 requires a catalyst: cytochrome *c* (released into the cytosol in apoptosis) is very effective (even 10 nM). Fluorescence reflects catalyst level as much as $O_2^{\cdot-}$ production.

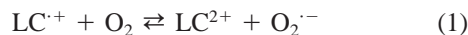
KEY WORDS: Oxidative stress; lucigenin; dichlorofluorescein; superoxide; hydrogen peroxide.

INTRODUCTION

The diverse roles of free radicals in biological processes are of intense current interest. Within this field, cellular oxidative stress has been linked to numerous pathological conditions [1]: the search term "oxidative stress" retrieves currently about 16,000 articles in the PubMed (MEDLINE) database. In addition, the explosion of interest in nitric oxide as a physiologically important molecule [2] has introduced the related term "nitrosative stress." Both stress responses involve excess production of free radicals: superoxide ($O_2^{\cdot-}$, a precursor to hydrogen peroxide), and nitric oxide, which also can act together,

yielding peroxynitrite and hence powerful free radical oxidants, nitrogen dioxide (NO_2^{\cdot}), hydroxyl ($\cdot OH$), and carbonate ($CO_3^{\cdot-}$) radicals [3].

Despite wide interest, the identification of specific probes for oxidative and nitrosative stress is proving difficult. Lucigenin (LC^{2+} , bis-*N*-methylacridinium) is widely used as a chemiluminescent probe for superoxide radicals. The mechanism of lucigenin chemiluminescence probably involves addition of $O_2^{\cdot-}$ to the radical-cation $LC^{\cdot+}$ and production of *N*-methylacridone in an excited state via a dioxetane [4]. However, the production of $O_2^{\cdot-}$ by "redox cycling" the radical [Eq. (1)] has been demonstrated [5,6]:



The position and kinetics of equilibrium have not been measured. One report indicates an equilibrium constant $K_1 = 10^{-6}$ [7], whereas electrochemical studies [8] sug-

¹ Gray Cancer Institute, PO Box 100, Mount Vernon Hospital, Northwood, Middx HA6 2JR, U.K.

² To whom correspondence should be addressed. E-mail: Wardman@gc.ac.uk

gest $K_1 \sim 0.3$. Lucigenin is an analogue of methyl viologen, and the redox and kinetic properties of viologen radicals can be easily characterized by pulse radiolysis [9]. We have used this technique to define Eq. (1).

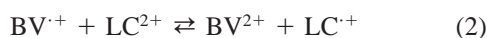
The most common luminescent probe for hydrogen peroxide is 2',7'-dichlorofluorescein (DCFH₂), oxidized to the fluorescent product DCF. However, a catalyst is required [10]. We report the activity of cytochrome *c* in this role, relevant to the putative link between oxidative stress and apoptosis. A preliminary report has been published [11].

EXPERIMENTAL

Pulse radiolysis of aqueous solutions containing formate was used to generate superoxide, viologen, and lucigenin radicals [9] and solutions containing nitrite to generate NO₂[•] [12]. Superoxide was generated enzymatically using hypoxanthine and xanthine oxidase [11]. Fluorescence was measured using a Perkin-Elmer LS-50B fluorimeter.

RESULTS AND DISCUSSION

The single-electron reduction potential of lucigenin $E(\text{LC}^{2+}/\text{LC}^{\bullet+})$ was measured by characterizing the redox equilibrium [Eq. (2)] with the known redox indicator benzyl viologen (BV²⁺), which was established in microseconds [13]:



This indicated $E(\text{LC}^{2+}/\text{LC}^{\bullet+}) = -0.28$ V versus NHE, and hence K_1 is calculated to be of the order of 50 (because $E(\text{O}_2[1\text{ M}]/\text{O}_2^{\bullet-}) = -0.19$ V [9]). This value is significantly greater than both earlier claims [7,8]. That $K_1 \gg 1$ is not in doubt, however, because by generating LC^{•+} in solutions of varying [O₂], we observed directly reaction with O₂, Eq. (1) occurring in milliseconds to an equilibrium value. Representative transient absorptions are shown in Fig. 1. The exponential decay of LC^{•+} was linearly dependent on [O₂] and indicated $k_1 = 3 \times 10^6$ M⁻¹ s⁻¹.

This rate constant is rather lower than those of other viologen radicals in reacting with oxygen [14], as might be expected from the higher reduction potential, but it is still a very fast reaction. If $K_1 \sim 50$, then k_{-1} (rate constant for reaction of O₂^{•-} with LC^{•+}) is $\sim 6 \times 10^4$ M⁻¹ s⁻¹, a value $\sim 50,000$ -fold lower than that for reaction of O₂^{•-} with superoxide dismutase. Hence, unless intracellular levels of LC²⁺ approach ~ 0.1 M (far lower extracellu-

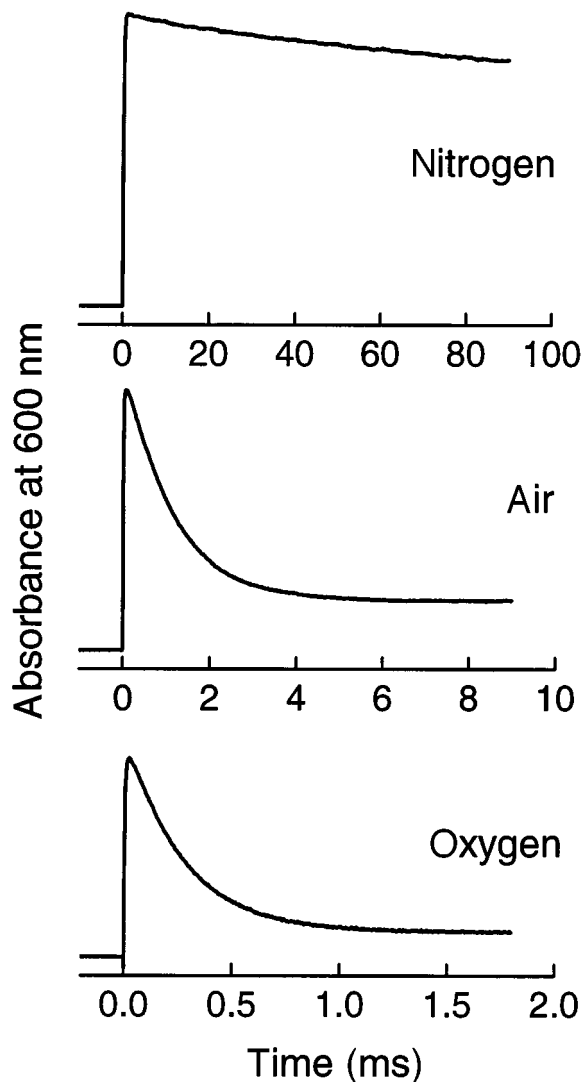


Fig. 1. Stability of the lucigenin radical-cation in nitrogen-, air- or oxygen-saturated aqueous solutions, observed by pulse radiolysis. Note the differing time scales.

lar concentrations are commonly used [15]), in the presence of normal levels of SOD, O₂^{•-} cannot be an important route of reduction of LC²⁺ to LC^{•+}—the presumed key intermediate in chemiluminescence. Rather, flavoprotein reductases are likely reductants forming LC^{•+}. There is then the complication of redox cycling, Eq. (1) occurring to generate O₂^{•-} in competition to LC^{•+} reacting with O₂^{•-} to form the dioxetane at a rate not yet characterized.

The effects of cytochrome *c* on the fluorescence signal generated from DCFH₂ in solutions containing hypoxanthine and xanthine oxidase to generate O₂^{•-} at variable, steady rates were investigated [11]. Fig. 2 shows that in the presence of 10 nM cytochrome *c*, fluorescence

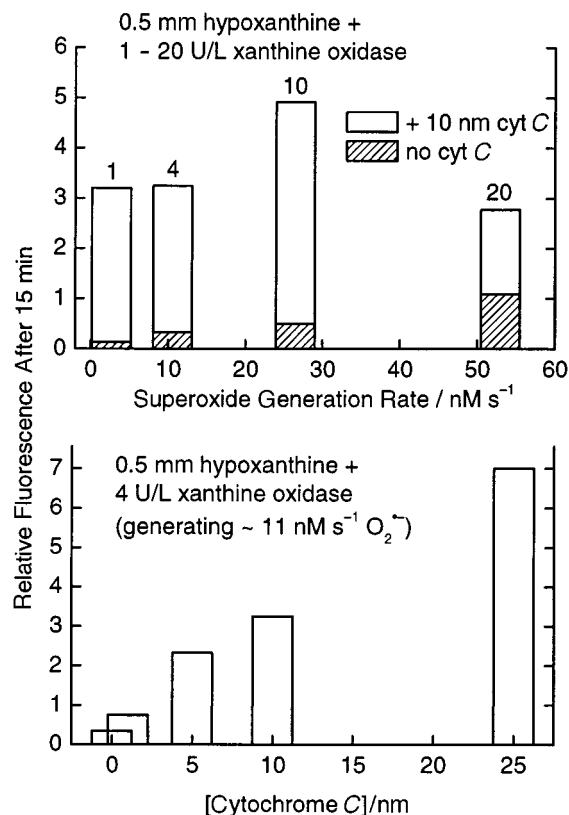


Fig. 2. Fluorescence signal intensities obtained from a solution of DCFH₂ (10 μM) with varying rates of superoxide generation with and without cytochrome *c* (10 nm), or at varying cytochrome *c* concentrations at a steady rate of radical generation.

hardly changed over a ~20-fold range of superoxide generation rate, whereas at a constant radical production rate, fluorescence was linearly dependent on cytochrome *c* over the range 1–25 nm. Clearly, DCF fluorescence in stressed cells containing DCFH₂ must often reflect the increase in cytosolic cytochrome *c* that occurs by transfer from the mitochondria in apoptotic cells [16,17].

Although even high concentrations of H₂O₂ do not oxidize DCFH₂ in the absence of a catalyst, radicals associated with nitrosative stress (NO₂[•], CO₃^{•-}), as well as [•]OH radicals of course, oxidize DCFH₂ very rapidly. Thus pulse radiolysis observations of the formation of DCF via the intermediate radical DCFH[•] from oxidation of DCFH₂ by NO₂[•] showed radical formation occurred with a rate constant *k*₃ of ~ 1 × 10⁷ M⁻¹ s⁻¹:



Glutathione thiol radicals (GS[•]) also oxidized DCFH₂ at a broadly similar rate, although the position of the equilibrium [Eq. (4)] and its perturbation via the coupled “redox switch” [Eq. (5)] which then leads on to

generate O₂^{•-} [Eq. (6)] [18] remains to be characterized and is the subject of current work.



CONCLUSIONS

Characterizing the rates of reactions of radical intermediates in the chemistry of luminescent probes for oxidative and nitrosative stress, using kinetic spectrophotometry after generation by pulse radiolysis or trapping radicals and observing formation using EPR spectroscopy [11,19] are essential tools if these probes are to be used with confidence. There is no question that lucigenin has the propensity to generate the species to be measured and that the oxidation of leuco dyes such as DCFH₂ cannot be used reliably unless it can be shown that catalysis is saturated, that thiol status is not changing, or that nitrosative stress can be decoupled from oxidative stress.

ACKNOWLEDGMENT

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