Validation of Lucigenin (Bis-N-methylacridinium) as a Chemilumigenc Probe for Detecting Superoxide Anion Radical Production by Enzymatic and Cellular Systems*

(Received for publication, September 29, 1997, and in revised form, November 14, 1997)

Yunbo Li, Hong Zhu, Periannan Kuppusamy‡, Valerie Roubaud‡, Jay L. Zweier‡, and Michael A. Trush§

From the Division of Toxicological Sciences, Department of Environmental Health Sciences, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205 and the Molecular and Cellular Biophysics Laboratories, Department of Medicine, Division of Cardiology and the Electron Paramagnetic Resonance Center, The Johns Hopkins School of Medicine, Baltimore, Maryland 21224

Lucigenin is most noted for its wide use as a chemiluminescent detector of superoxide anion radical (O$_2^-$) production by biological systems. However, its validity as a O$_2^-$-detecting probe has recently been questioned in view of its ability to undergo redox cycling in several in vitro enzymatic systems, which produce little or no O$_2^-$. Whether and to what extent lucigenin redox cycling occurs in systems that produce significant amounts of O$_2$ has not been carefully investigated. We examined and correlated three end points, including sensitive measurement of lucigenin-derived chemiluminescence (LDCL), O$_2$ consumption by oxygen polarography, and O$_2$ production by 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide spin trapping to characterize the potential of lucigenin to undergo redox cycling and as such to act as an additional source of O$_2$ in various enzymatic and cellular systems. Marked LDCL was elicited at lucigenin concentrations ranging from 1 to 5 μM in all of the O$_2$-generating systems examined, including xanthine oxidase (XO)/xanthine, lipoamide dehydrogenase/NADH, isolated mitochondria, mitochondria in intact cells, and phagocytic NADPH oxidase. These concentrations of lucigenin were far below those that stimulated additional O$_2$ consumption or O$_2$ production in the above systems. Moreover, a significant linear correlation between LDCL and superoxide dismutase-inhibitable cytochrome c reduction was observed in the XO/xanthine and phagocytic NADPH oxidase systems. In contrast to the above O$_2^-$-generating systems, no LDCL was observed at non-redox cycling concentrations of lucigenin in the glucose oxidase/glucose and XO/NADH systems, which do not produce a significant amount of O$_2^-$. Thus, LDCL still appears to be a valid probe for detecting O$_2^-$ production by enzymatic and cellular sources.

The detection and measurement of fluxes of O$_2^-$ within cells are of critical importance for investigating the physiological and pathological roles of O$_2^-$. Because of its sensitivity lucigenin-derived chemiluminescence (LDCL)$^1$ has been frequently used in the specific detection of O$_2^-$ production by both in vitro enzymatic systems and intact cells. For example, LDCL has been used to detect O$_2^-$ production by xanthine oxidase (XO) plus xanthine or hypoxanthine, NADPH-cytochrome P450 reductase in microsomes, NADPH oxidase in phagocytic cells, and a possible diphenyleneiodonium-sensitive NAD/P/H oxidase in endothelial, fibroblastic, and vascular smooth muscle cells (1–8). Our recent studies have also demonstrated that LDCL can be used to monitor mitochondrial O$_2^-$ production in intact cells (9–11).

As illustrated in Fig. 1, to detect O$_2^-$, lucigenin must first be reduced by one electron to produce the lucigenin cation radical and O$_2^-$. The lucigenin cation radical then reacts with the biologically derived O$_2^-$ to yield an unstable dioxetane intermediate. The lucigenin dioxetane decomposes to produce two molecules of N-methylacridone, one of which is in an electronically excited state, which upon relaxation to the ground state emits a photon (3, 12). Through sensitive measurement of the photon emission, the biological production of O$_2^-$ can be monitored. However, the validity of lucigenin as a chemilumogenic probe for detecting biological O$_2^-$ has recently been questioned based on the observation that in several in vitro enzymatic systems lucigenin may itself act as a source of O$_2^-$ via autoxidation of the lucigenin cation radical (13, 14). These include glucose oxidase (GO)/glucose at pH 9.5, XO/NADH, and endothelial nitric oxide synthase/NADPH systems that either do not produce O$_2^-$ or their ability to reduce O$_2$ to O$_2^-$ is very limited (13, 14). Because of the opposite charge of the lucigenin cation radical and O$_2^-$ the lucigenin cation radical may have a much higher affinity for O$_2^-$/O$_2$ than for O$_2$. As such, in cellular systems that produce significant amounts of O$_2^-$ under physiological conditions, the propensity of lucigenin to undergo redox cycling may be very limited. In this study, we examined and correlated three end points, including sensitive measurement of LDCL, O$_2$ consumption by oxygen polarography, and O$_2$ production by 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) spin trapping. Using these end points, we

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*This work was supported by National Institutes of Health Grants ES02769, ES03819, and ES08078 (to M. A. T.) and HL38324 and HL52315 (to J. L. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Rm. 7032, Division of Toxicological Sciences, Dept. of Environmental Health Sciences, The Johns Hopkins School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, MD 21205. Tel.: 410-955-4712; Fax: 410-955-0116; E-mail: mtrush@jhsph.edu.

1 The abbreviations used are: LDCL, lucigenin-derived chemiluminescence; XO, xanthine oxidase; GO, glucose oxidase; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) spin trapping. Using these end points, we
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have characterized the potential of lucigenin to undergo redox cycling and as such to act as an additional source of O₂ in systems that generate O₂, including XO/xanthine, lipoamide dehydrogenase (LADH)/NADH, isolated mitochondria, mitochondria in intact cells, and phagocytic NADPH oxidase, as well as in systems that produce little or no O₂ including GO/glucose and XO/NADH. Our results demonstrate that in the O₂-producing systems examined, significant LDCL was always elicited at lucigenin concentrations far below those that stimulated additional O₂ utilization or O₂ formation via the redox cycling of the lucigenin molecule.

EXPERIMENTAL PROCEDURES

Materials—Lucigenin, XO from buttermilk, xanthine, LADH (type III) from porcine heart, NADH, glucose, superoxide dismutase (SOD), diethylenetriaminepentaacetic acid (DTPA), succinate, rotenone, myxothiazol, cytochrome c, RPMI 1640, penicillin/streptomycin, and bovine serum albumin were from Sigma. Glucose oxidase (grade I) was from Boehringer Mannheim. 12-O-Tetradecanoylphorbol-13-acetate (TPA)
was from LC laboratories (Woburn, MA). Fetal bovine serum was from Biossentials (Walkersville, MD). Dulbecco’s phosphate-buffered saline (PBS, pH 7.4) was from Life Technologies, Inc. Tissue culture flasks were from Corning Costar Co. (Cambridge, MA). DEPMPO was synthesized and prepared as reported (15).

**Measurement of LDCL—**The lucigenin chemiluminescence (LDCL) was measured using a Berthold LB9505 luminometer at 37 °C. For enzymatic systems, the reaction mixtures contained lucigenin (0.5 µM), LADH/NADH, and the substrate and lucigenin concentrations were as in panel A, except with 0.5 µM lucigenin and cytochrome c reduced.

**Measurement of O2 Consumption—**The O2 consumption was measured polarographically with a Clark-type oxygen electrode (YSI-53, Yellow Springs, OH) at 37 °C in 2.5 ml of reaction mixture as described previously (20). The buffers and the concentrations of the enzymes/substrates, cells, and mitochondria were identical to those used for measurement of LDCL as described above.

**Detection of O2 by Ferricytochrome c Reduction**—The generation of O2 was measured indirectly by the reduction of ferricytochrome at 550 nm as described previously (21). Non-O2-dependent reduction of cytochrome c was measured as described above.
chrome c was corrected for by deducting all activity not inhibited by SOD. The buffers and the concentrations of the enzymes/substrates and cells were identical to these used for measurement of LDCL as described above.

**RESULTS**

**LDCL, O$_2$ Consumption, and DEPMPO-OOH Spin Adduct Formation by the XO/Xanthine and LADH/NADH Systems**—Both the XO/xanthine and the LADH/NADH systems consume O$_2$ and produce O$_2$. as detected by SOD-inhibitable cytochrome c reduction and DEPMPO spin trapping (Table I, Figs. 2 and 3). DEPMPO reacts with O$_2$. to form a relatively stable DEPMPO-OOH adduct (half-life $\tau = 15$ min) with characteristic hyperfine splittings that give rise to 12 resolved peaks (15, 23). The hyperfine splitting constants of the DEPMPO spin adduct formed in the XO/xanthine and LADH/NADH systems (Figs. 2 and 3) matched directly with experimental data to extract the spectral parameters.

**Fig. 5.** LDCL (panel A) and the effects of lucigenin on O$_2$. consumption (panel B) and O$_2$. production (panel C) in the GO plus glucose system. Measurement of LDCL and O$_2$. consumption and the DEPMPO spin trapping detection of O$_2$. were as described under “Experimental Procedures.” In panel A, LDCL data represent the integrated area under the curve over a period of 15 min. In panel C, a is GO/glucose plus 10 mM DEPMPO; b is as in a but with 5 $\mu$m lucigenin; c is as in a but with 50 $\mu$m lucigenin. The DEPMPO-hydroxyl adduct (DEPMPO-OH) has the following parameters: $a_N = 14.05$ G; $a_p = 47.29$ G; $a_H^b = 13.40$ G; $a_H^g = 0.6 (\times 3)$ G. The spin adduct was not SOD-inhibitable. Values in panels A and B represent the mean ± S.E. from at least three experiments. ESR spectra represent the averaged signal of 10 scans of 30 s with receiver gain being $1 \times 10^9$.

**Fig. 6.** LDCL (panel A) and the effects of lucigenin on O$_2$. consumption (panel B) and O$_2$. production (panel C) in the XO plus NADH system. Measurement of LDCL and O$_2$. consumption, and the DEPMPO spin trapping detection of O$_2$. were as described under “Experimental Procedures.” In panel A, LDCL data represent the integrated area under the curve over a period of 15 min. In panel C, a is XO/NADH plus 10 mM DEPMPO; b is as in a but with 5 $\mu$m lucigenin; c is as in a but with 50 $\mu$m lucigenin. Spectrum c corresponds to the addition of two spin adducts, DEPMPO-OOH and DEPMPO-OH. Values in panels A and B represent the mean ± S.E. from at least three experiments. ESR spectra represent the averaged signal of 10 scans of 30 s with receiver gain being $1 \times 10^9$. *, significantly different from 0 $\mu$m lucigenin.

**Statistical Analysis**—Student’s $t$ test was used. Statistical significance was considered at $p < 0.05$. 

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**Fig. 7.** LDCL (panel A) and KCN-resistant O₂ consumption (panel B) in isolated mitochondria driven by succinate. LDCL was monitored continuously for 60 min after incubation of mitochondria with the indicated concentrations of lucigenin in the presence or absence of 0.2 mM KCN. KCN-resistant O₂ consumption was measured after incubation of the mitochondria with either the indicated concentrations of lucigenin or 5 μM BPQ. Values in panel A represent the average from two experiments with range less than 10% of the average. Values in panel B represent the mean ± S.E. from at least three experiments. *, significantly different from 20 μM lucigenin; #, significantly different from 50 and 100 μM lucigenin. ND, not detectable.

and 3) are similar to the reported values for DEPMPO-OOH (15, 23). As shown in Figs. 2 and 3, marked LDCL was also elicited in both the XO/xanthine and LADH/NADH systems. With the XO/xanthine system, the LDCL response reached a plateau at concentrations of lucigenin above 20 μM. No stimulation of either additional O₂ consumption or DEPMPO-OOH formation was detected at up to 100 μM lucigenin with the XO/xanthine system (Fig. 2). Moreover, when the concentration of XO was varied, a significant linear correlation (r = 0.98) between the LDCL and SOD-inhibitable cytochrome c reduction by the XO/xanthine system was observed (Fig. 4). With the LADH/NADH system, varying the lucigenin concentration resulted in a biphasic LDCL response with the second phase occurring between 20 and 50 μM lucigenin (Fig. 3). No stimulation of additional O₂ consumption was observed in the presence of a lucigenin concentration up to 20 μM. However, both O₂ consumption (Fig. 3B) and DEPMPO-OOH formation (Fig. 3C) were elevated by ~30% in the presence of 50 μM lucigenin. 100 μM lucigenin stimulated further O₂ consumption (Fig. 3B).

Based on the above results, lucigenin does not appear to redox cycle with the XO/xanthine system, although it does with the LADH/NADH system at concentrations of 50 and 100 μM.

**LDCL, O₂ Consumption, and DEPMPO-OOH Spin Adduct Formation in the GO/glucose and XO/NADH Systems—**Neither GO/glucose nor the XO/NADH system produces a significant amount of O₂ as detected by SOD-inhibitable cytochrome c reduction and DEPMPO spin trapping (Table I, Figs. 5 and 6). Neither a significant LDCL nor stimulation of additional O₂ consumption was detected in the GO/glucose system at a concentration of lucigenin up to 100 μM (Fig. 5). A weak DEPMPO-hydroxyl (DEPMPO-OOH) signal was observed at 50 μM lucigenin (Fig. 5C). However, the formation of this spin adduct was not inhibited by SOD (data not shown), suggesting that O₂ was not produced. The XO/NADH system was previously shown to catalyze the one electron reduction of lucigenin (13). We examined whether redox cycling of lucigenin by this system could lead to LDCL. As shown in Fig. 6, significant LDCL was detected in the presence of 20 and 50 μM but not 5 μM lucigenin. Lucigenin at 20 and 50 μM but not 5 μM also stimulated additional O₂ consumption (Fig. 6B). A DEPMPO-OOH adduct was also detected at 50 μM lucigenin (Fig. 6C).

**Detection of Mitochondrial O₂ Production by LDCL with Isolated Mitochondria and Intact Monocytes/Macrophages—**The mitochondrial electron transport system is known to be able to reduce O₂ to O₂⁻ univalently (24–26). As shown in Fig. 7, with succinate-driven isolated mitochondria a linear relationship existed between LDCL and the concentration of lucigenin up to 20 μM. To test whether LDCL was derived from the mitochondrial electron transport chain, the effects of several inhibitors known to affect mitochondrial respiration were determined. The LDCL was elevated markedly in the presence of KCN (Fig. 7) and was abolished completely by 10 μM rotenone/myxothiazol (data not shown). KCN is a mitochondrial cytochrome oxidase inhibitor that causes electrons to build up leading to enhanced production of O₂⁻ (10). Rotenone and myxothiazol are specific inhibitors of mitochondrial NADH-coenzyme Q reductase and coenzyme Q-cytochrome c reductase, respectively (27, 28). To examine whether lucigenin undergoes redox cycling while being used to detect mitochondrial O₂⁻, KCN-resistant O₂ consumption was determined in the presence of various concentrations of lucigenin. KCN was used to inhibit the O₂ utilization by mitochondrial respiration so that the O₂ consumption caused by the redox cycling of lucigenin could be detected. No
stimulation of KCN-resistant O$_2$ consumption was observed at a concentration of lucigenin up to 20 μM. 50 μM and 100 μM lucigenin slightly stimulated the KCN-resistant O$_2$ consumption (Fig. 7). In contrast, the presence of 5 μM benzo(a)pyrene-1,6-quinone (BPQ) resulted in a marked KCN-resistant O$_2$ consumption (Fig. 7). BPQ has been shown to redox cycle in mitochondria (29). Detection of mitochondrial O$_{2}^\cdot$ production by LDCL in unstimulated intact monocyteic cells has been demonstrated previously (9–11, 16, 17). Shown in Fig. 8 are representative LDCL responses observed with 5 μM lucigenin in unstimulated monocytes/macrophages in the presence or absence of KCN or rotenone/myxothiazol. As shown, LDCL was stimulated markedly by KCN and was abolished completely by rotenone/myxothiazol (Fig. 8 and Table II), indicating that LDCL in the unstimulated monocytes/macrophages was derived totally from mitochondrial respiration. With the intact cells no stimulation of KCN-resistant O$_2$ consumption was detected in the presence of up to 50 μM lucigenin (Table II). In contrast, incubation of cells with 5 μM BPQ resulted in a marked stimulation of KCN-resistant O$_2$ consumption (Table II).

**LDCL, O$_2$ Consumption, and DEPMPO-OOH Spin Adduct Formation by the TPA-stimulated Phagocytic NADPH Oxidase System**—LDCL has frequently been used to detect the O$_2$ production by phagocytic NADPH oxidase (2–4). Undifferentiated monoblastic ML-1 cells lack a functional NADPH oxidase activity, whereas differentiation of ML-1 cells to monocytes/macrophages results in the expression of membrane NADPH oxidase and the maturation of mitochondrial respiration (16, 17). Because monocytes/macrophages exhibit such a strong mitochondrial respiration and LDCL due to the mitochondrial electron transport chain (16, 17, Figs. 7 and 8), we have observed that it is difficult to assess the contribution of NADPH oxidase-derived O$_2$ to LDCL (11, 30). As such, 10 μM rotenone and myxothiazol were added to the monocytes/macrophages to block mitochondrial respiration and its accompanying O$_2$ production. As shown in Fig. 9, under these experimental conditions, LDCL as well as SOD-inhibitable cytochrome c reduction, O$_2$ consumption, and DEPMPO spin trapping all equally reported a TPA-stimulated O$_2\cdot$-producing activity by NADPH oxidase in the monocytes/macrophages but not in the undifferentiated ML-1 cells. In data not shown, no LDCL was detected in TPA-stimulated undifferentiated ML-1 cells even at 100 μM lucigenin. Fig. 10A shows the relationship between the lucigenin concentration and the LDCL elicited after TPA activation of NADPH oxidase in the monocytes/macrophages. Lucigenin at up to 50 μM did not stimulate any additional O$_2$ utilization or DEPMPO-OOH formation (Fig. 10, B and C). In fact, the DEPMPO-OOH formation was slightly reduced in the presence of 50 μM lucigenin, which may result from the competition by

![Fig. 9. Detection of TPA-stimulated NADPH oxidase activity in the undifferentiated ML-1 cells and the monocytes/macrophages differentiated from ML-1 cells. The O$_2\cdot$-producing activity of the TPA (30 ng/ml)-stimulated NADPH oxidase was assessed by LDCL at 5 μM lucigenin (panel A), SOD-inhibitable cytochrome c reduction (panel B), O$_2$ consumption (panel C), and DEPMPO spin trapping (panel D), as described under “Experimental Procedures.” In panels A, B, and C measurements were for 30 min. Values represent the mean ± S.E. from at least three experiments. In panel D the ESR spectra represent the averaged signal of 10 scans of 30 s with receiver gain being 1 x 10$^5$. ND, not detectable.](image)

**Table II**

| Lucigenin (μM) | Integrated CL (x10$^{-6}$) | % Stimulation by KCN | ROT/MYX | % Inhibition by ROT/MYX | KCN-resistant O$_2$ consumption (nmol O$_2$/min/10$^6$ cells) |
|---------------|-----------------------------|-----------------------|---------|------------------------|---------------------------------|
|               | Control                     | KCN                   | RO/MYX  |                        |                                 |
| Lucigenin (μM)|                             |                       |         |                        |                                 |
| 1             | 12.1 ± 1.8                  | 22.2 ± 2.9*           | 83.5    | 0.2 ± 0.1*             | 98.3                            |
| 5             | 43.6 ± 5.7                  | 110.1 ± 3.8*          | 152.5   | 1.6 ± 1.2*             | 96.3                            |
| 50            | 470.0 ± 23.1                | 1169.0 ± 46.7*        | 145.7   | 5.5 ± 0.6*             | 98.8                            |
| BPQ (μM)      |                             |                       |         |                        |                                 |
| 5             |                             |                       |         |                        | 6.2 ± 0.6                       |
the lucigenin cation radical for $O_2^\cdot$. When the monocytes/macrophages were stimulated with various concentrations of TPA (1.9–15 ng/ml), a significant linear relationship was observed between LDCL and SOD-inhibitable cytochrome $c$ reduction ($r = 0.99$) or $O_2$ consumption ($r = 0.99$) by the respiratory burst (Fig. 11).

**DISCUSSION**

Recently, the use of LDCL for detecting $O_2^\cdot$ in biological systems has been questioned (13, 14, 31). To validate lucigenin as a $O_2^\cdot$-detecting probe, in this study we have characterized the potential of lucigenin to undergo redox cycling in systems that produce significant amounts of $O_2^\cdot$ as well as in systems that produce little or no $O_2^\cdot$. LDCL was observed in the $O_2^\cdot$-producing XO/xanthine system more than 3 decades ago (1). The univalent reduction of lucigenin by XO has also been shown to precede its reaction with $O_2^\cdot$ (1). The complete inhibition of the LDCL by SOD but not by catalase in the XO/xanthine system at physiological pH indicates the specific involvement of $O_2^\cdot$ in the reaction pathway leading to LDCL (Fig. 1). The failure of lucigenin at up to 100 $\mu M$ to stimulate additional $O_2$ consumption and DEPMPO-OOH adduct formation in the XO/xanthine system indicates that lucigenin at these concentrations does not undergo redox cycling in this $O_2^\cdot$-generating system. The validity of using LDCL for detecting $O_2^\cdot$ production by the XO/xanthine system was strengthened further by the significant linear correlation between the LDCL and the SOD-inhibitable cytochrome $c$ reduction (Fig. 4), a standard assay for measuring $O_2^\cdot$ production (32). Stimulation of additional $O_2$ consumption and DEPMPO-OOH adduct formation by lucigenin at 50 $\mu M$ and above in the LADH/NADH system suggests that lucigenin is more likely to undergo redox cycling in this system than in the XO/xanthine system. Based on cytochrome $c$ reduction and $O_2$ consumption, the LADH/NADH system was less efficient than the XO/xanthine system with regard to $O_2^\cdot$ production (Table I). This may account, at least in part, for the redox cycling of lucigenin at high concentrations in the LADH/NADH system. LDCL and SOD-inhibitable cytochrome $c$ reduction were also observed previously in the LADH plus NADH system (33). There is no $O_2^\cdot$ production by the GO/glucose system. However, a significant LDCL response has recently been...
shown to be elicited by the GO/glucose system at pH 9.5 (13). The $H_2O_2$ produced by the GO/glucose at pH 9.5 was thought to reduce lucigenin to its cation radical, followed by autoxidation of the lucigenin cation radical, leading to an LDCL response (13). Data presented in Fig. 5 however, clearly demonstrated that this does not happen at a physiological pH. Because GO/glucose ordinarily catalyzes the two electron reduction of $O_2$ to $H_2O_2$, this enzymatic system is unlikely to be able to reduce lucigenin univalently to its cation radical at physiological pH. In contrast to XO/xanthine, XO plus NADH did not produce a significant amount of $O_2^·$ as determined by SOD-inhibitable cytochrome $c$ reduction and DEPMPO spin trapping (Table I and Fig. 6). In the presence of 5 µM lucigenin, a strong LDCL response was elicited from the XO/xanthine system, whereas no significant LDCL was observed with the XO/NADH system (Fig. 6). On the other hand, the significant LDCL response and the stimulation of additional $O_2$ utilization and DEPMPO-OOH adduct formation observed at 20 and 50 µM lucigenin in the XO/NADH (Fig. 6) suggest that lucigenin undergoes redox cycling in this enzymatic system. The univalent reduction of lucigenin by the XO/NADH system has been demonstrated previously (13). It is likely that the lucigenin cation radical formed in the XO/NADH system in the absence of enzymatic $O_2^·$ autoxidizes and in so doing consumes $O_2$, producing $O_2^·$.

It has been long known that the mitochondrial electron transport chain is able to univalently reduce $O_2$ to $O_2^·$ (24–26) and is a major source of cellular reactive oxygen species (34, 35). The selective accumulation of the positively charged lucigenin molecule by mitochondria in cells makes lucigenin an ideal probe for detecting $O_2^·$ derived from mitochondrial respiration (10). The stimulation by KCN and complete inhibition by rotenone/myxothiazol of LDCL (Fig. 8 and Table II) suggest that redox cycling of lucigenin occurs at high concentrations in the isolated mitochondria. However, comparison of the KCN-resistant $O_2$ consumption induced by lucigenin and BPQ in both intact cells and isolated mitochondria indicates that lucigenin is not as good a redox cycling chemical as BPQ.

Another major application of LDCL with cellular systems has been to measure $O_2^·$ production by phagocytic cells after activation of their membrane NADPH oxidase by soluble and particulate stimuli (2–4). When mitochondrial respiration and $O_2^·$ formation were inhibited in the monocytes/macrophages by KCN and rotenone/myxothiazol of LDCL (Fig. 9 and Table II) suggests that redox cycling of lucigenin occurs at high concentrations in the isolated mitochondria. However, comparison of the KCN-resistant $O_2$ consumption induced by lucigenin and BPQ in both intact cells and isolated mitochondria indicates that lucigenin is not as good a redox cycling chemical as BPQ.

In summary, this study demonstrates that in the $O_2^·$-generating cellular system. As
depicted in Fig. 12, the relative rate of production of the lucigenin cation radical and $O_2^\cdot$ by biological one-electron reduction systems both appear to determine whether LDCL will reflect only biological $O_2^\cdot$ or $O_2^\cdot$ arising from both biological source and autooxidation of the lucigenin cation radical. In addition, the rate of production of the lucigenin cation radical is in turn determined by the lucigenin concentration used. As such, when careful measurement of $O_2$ consumption is used as a corollary approach to LDCL (Figs. 2, 3, 5–7, 10), a safe non-redox cycling concentration of lucigenin can be determined which sensitively and reliably detects $O_2^\cdot$ production by enzymatic and cellular systems. This safe non-redox cycling concentration of lucigenin may vary with different experimental systems and conditions. Accordingly, we recommend that whenever LDCL is used to detect $O_2^\cdot$ production by an enzymatic or cellular system under a particular experimental condition, a safe non-redox cycling concentration of lucigenin be determined through measurement of the stimulation of $O_2$ consumption by oxygen polarography or alternatively via detection of the stimulation of $O_2^\cdot$ formation by DEPMPO spin trapping techniques.

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