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Biochemical Characterization of Lucigenin (Bis-*N*-methylacridinium) as a Chemiluminescent Probe for Detecting Intramitochondrial Superoxide Anion Radical Production

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Direct detection of intramitochondrial superoxide anion radical ($O_2^{-\cdot}$) production is of critical importance for investigating the pathophysiological consequences resulting from altered cellular reactive oxygen homeostasis. The purpose of this study with isolated mitochondria was to characterize the biochemical basis for lucigenin as a chemiluminescent probe to detect intramitochondrial $O_2^{-\cdot}$ production. Incubation of isolated mitochondria with lucigenin at non-redox cycling concentration produced lucigenin-derived chemiluminescence (LDCL), which was increased markedly by mitochondrial substrates, pyruvate/malate or succinate. The LDCL was reduced greatly by the membrane permeable superoxide dismutase (SOD) mimetics, 2,2,6,6-tetramethylpiperidine-*N*-oxyl and Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin, but not by Cu,Zn-SOD. With an ion-pair HPLC method, a concentration-dependent accumulation of lucigenin was detected within mitochondria. The accumulation of lucigenin by mitochondria was reduced markedly in the presence of carbonyl cyanide p-(trifluoromethoxy)phenyhyldrazone, an uncoupler known to dissipate the mitochondrial membrane potential. With submitochondrial particles, we observed that both complexes I and III of the mitochondrial electron transport chain appear to be able to catalyze the one electron reduction of lucigenin, a critical step involved in LDCL. After incubation of mitochondria with lucigenin at non-redox cycling concentrations, formation of *N*-methylacridone, the proposed end product of the reaction pathway leading to LDCL, within the mitochondrial fraction was also detected. In addition, a significant linear correlation was observed between the LDCL and either the lucigenin accumulation or the *N*-methylacridone formation within the mitochondria. Taken together, our results conclusively demonstrate that when properly used LDCL can reliably detect intramitochondrial $O_2^{-\cdot}$ production. © 1999 Academic Press

Key Words: **lucigenin; chemiluminescence; superoxide; mitochondria.**

Because of its sensitivity, lucigenin (bis-*N*-methylacridinium)-derived chemiluminescence (LDCL) has frequently been used in the specific detection of superoxide anion radical ($O_2^{-\cdot}$) production by various enzymatic and cellular systems (1–11). For lucigenin to specifically detect biological $O_2^{-\cdot}$ production, three key steps must take place. These include the one electron reduction of lucigenin to its cation radical; the reaction of the lucigenin cation radical with biological $O_2^{-\cdot}$ to yield lucigenin dioxetane; and the decomposition of the lucigenin dioxetane to form an electronically excited *N*-methylacridone, which upon relaxation to the ground state emits a photon (3).

Although there are many potential cellular sources of reactive oxygen species (ROS) production, mitochondrial respiration usually consumes about 90% of the O_2 utilized by cells and is generally considered the major source of cellular ROS (12–14). As such, mitochondria-derived ROS have frequently been implicated in a number of diseases and disorders, including aging (15), neurodegenerative diseases (16, 17), cancers (14, 18), ischemia/reperfusion injury (19), and cytotoxicities induced by both xenobiotics and endobiotics (14, 20). Thus, the detection and measurement of mitochondria-

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Abbreviations used: LDCL, lucigenin-derived chemiluminescence; SOD, superoxide dismutase; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin, METC, mitochondrial electron transport chain; AA, antimycin A; MYX, myxothiazol; FCCP, carbonyl cyanide p-(trifluoromethoxy-)phenylhydrazone; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate buffered saline; SMP, submitochondrial particles; ROS, reactive oxygen species; KCN, potassium cyanide.

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generated ROS are of critical importance for investigating the pathophysiological consequences of mitochondria-derived ROS. Toward this goal, recent studies in this laboratory have demonstrated that a significant LDCL response can be observed with unstimulated rat alveolar macrophages through a mechanism not accounted for by the activity of the plasma membrane-associated NADPH oxidase, as measured by cytochrome c reduction (21). Further studies using confocal microscopy demonstrated that in rat alveolar macrophages lucigenin exhibited a localization similar to that of rhodamine 123, a fluorescent indicator of mitochondrial membrane potential (7). By examining the effects of various mitochondrial inhibitors, it was concluded that the LDCL appears to reflect mitochondrial $O_2^{-\cdot}$ formation in rat alveolar macrophages (7). However, regarding the use of LDCL for reliably detecting mitochondrial $O_2^{-\cdot}$ production, several critical points remain to be experimentally addressed. These include whether: LDCL can be elicited by isolated intact mitochondria; the mitochondrial inner membrane is permeable to lucigenin; the mitochondrial electron transport chain (METC) is able to catalyze the first electron reduction of lucigenin; and the formation of *N*-methylacridone, the proposed end product of the reaction pathway leading to LDCL (3), occurs within the mitochondria. In this study with isolated intact mitochondria, we have investigated these important aspects to further characterize lucigenin as a probe for detecting intramitochondrial $O_2^{-\cdot}$ production. Our results demonstrate that lucigenin can get into and accumulate in mitochondria, and then be reduced to its cation radical by the METC. The lucigenin cation radical can then react with the METC-derived $O_2^{-\cdot}$, leading to LDCL and the production of *N*-methylacridone.

MATERIALS AND METHODS

Materials. Lucigenin, *N*-methylacridone, succinic acid (sodium salt), pyruvic acid (sodium salt), maleic acid (disodium salt), 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), NADH, rotenone, antimycin A (AA), myxothiazol (MYX), carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone (FCCP), superoxide dismutase (SOD) from bovine erythrocytes, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) was a product of Cayman Chemical Co. (Ann Arbor, MI). Fetal bovine serum (FBS) was obtained from Biowhittaker (Walkersville, MD). RPMI 1640, phosphate-buffered saline (PBS), penicillin and streptomycin were products of Gibco (Grand Island, NY). Tissue culture flasks were purchased from Corning Incorporated (Corning, NY).

Culture and differentiation of ML-1 cells to monocytes/macrophages. Human monoblastic ML-1 cells were obtained from Dr. Ruth W. Craig, Dartmouth Medical School, NH. The cells were cultured at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with penicillin 50 (units/ml) and streptomycin (50 μg/ml) and 7.5% FBS in 150 cm^2 tissue culture flasks. The procedure used for differentiation of ML-1 cells to monocytes/macrophages was as described previously (11).

Isolation of mitochondria from monocytes/macrophages. Mitochondria were isolated from freshly harvested monocytes/macrophages according to the method of Rickwood *et al.* (22) with minor modifications as described previously (11). Mitochondrial protein was measured with Bio-Rad protein assay dye based on the method of Bradford (23) with BSA as the standard.

Preparation of submitochondrial particles (SMP). SMP were prepared according to the method of Pedersen *et al.* (24). Briefly, about 20 mg freshly isolated mitochondria were suspended in 1 ml sucrose buffer (0.25 M sucrose, 10 mM Hepes, 1 mM EGTA, and 0.5% BSA, pH 7.4). The sample was then sonicated on ice for 2 min at 15-second intervals, and centrifuged at 10,000 *g* for 10 min to remove the unbroken mitochondria. The supernatant was further centrifuged at 105,000 *g* for 60 min and then the resulting SMP pellet was washed once with sucrose buffer and resuspended in the same buffer. SMP protein was measured as described above.

Chemiluminescent measurement of $O_2^{-\cdot}$ production by intact mitochondria and SMP. LDCL was monitored with a Berthod Biolumat LB9505 at 37°C. For measurement of the LDCL elicited with intact mitochondria, the reaction mixture contained 0.5 mg protein of mitochondria in 1 ml air-saturated respiration buffer in the presence or absence of exogenous substrates or other reagents. The respiration buffer contained 70 mM sucrose, 220 mM mannitol, 2 mM Hepes, 2.5 mM KH_2PO_4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, and 0.1% BSA, pH 7.4. The mitochondrial substrates used included 6 mM pyruvate/malate or 6 mM succinate. The LDCL response was initiated by adding various concentrations of lucigenin, and was continuously monitored for 60 min. For measurement of the LDCL elicited with SMP, the reaction mixture contained 25–200 μg protein of SMP in 1 ml respiration buffer in the presence of 100 μM NADH. The LDCL response was initiated by adding 5 μM lucigenin, and was continuously monitored for 15 min. Data from LDCL experiments are expressed as the integrated area under the curve.

Quantitation of SMP-derived $O_2^{-\cdot}$ formation by epinephrine oxidation assay. The $O_2^{-\cdot}$ production by SMP was measured as SOD-inhibitable oxidation of epinephrine to adrenochrome as described by Boveris (25). Briefly, the reaction mixture contained 1 mM epinephrine, 100 μM NADH and 25–200 μg protein of SMP in 1 ml respiration buffer in the presence or absence of 300 units/ml Cu,Zn-SOD. After 15 min incubation at 37°C, the reaction was stopped by placing the tubes on ice, and the adrenochrome formation was immediately measured at 480 nm. Data are expressed as nmol adrenochrome formed.

Measurement of O_2 consumption. The rate of O_2 consumption was measured polarographically with a Clark-type oxygen electrode (YSI-53, Yellow Springs, OH) at 37°C in 2.5 ml reaction mixture. The reaction mixture contained 0.5 mg protein/ml of intact mitochondria or 0.25 mg protein/ml of SMP in the presence or absence of exogenous substrates or other agents in respiration buffer. The final concentration of NADH or succinate was 6 mM.

Incubation of mitochondria with lucigenin, and extraction of lucigenin and N-methylacridone from mitochondria. Mitochondria (0.5 mg protein) were incubated with 6 mM succinate and various concentrations of lucigenin in 1 ml air-saturated respiration buffer at 37°C for 60 min unless indicated otherwise. After incubation, the sample was centrifuged at 10,000 *g* for 10 min. The resulting mitochondrial pellet was washed once with PBS. 100 μl cold methanol was then added to the mitochondrial pellet, followed by sonication and centrifugation at 13,000 *g* for 10 min. The supernatant was collected. The above step was repeated four times. The resulting supernatants were pooled and desiccated completely under nitrogen blowing. For HPLC analysis of lucigenin and *N*-methylacridone, the desiccated sample was dissolved in 200 μl methanol. The concentration of lucigenin or *N*-methylacridone within mitochondrial fractions was calculated according to standard curves derived for lucigenin and *N*-methylacridone.

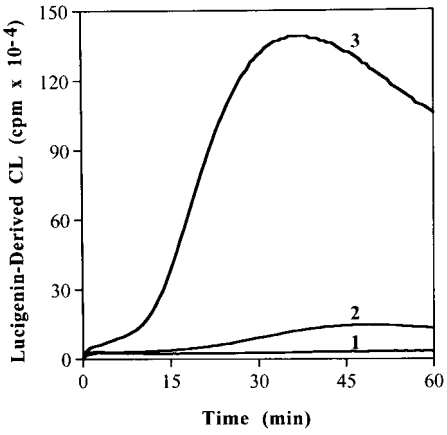

FIG. 1. Profiles of LDCL response elicited with isolated mitochondria. Mitochondria (0.5 mg/ml) were incubated in the presence or absence of 6 mM pyruvate/malate or 6 mM succinate at 37°C (see Materials and Methods). The LDCL response was initiated by adding 5 μM lucigenin. Curve 1, mitochondria + lucigenin; curve 2, mitochondria + pyruvate/malate + lucigenin; curve 3, mitochondria + succinate + lucigenin.

Ion-pair HPLC analysis of lucigenin and N-methylacridone. Analysis of lucigenin and *N*-methylacridone by HPLC was performed with a liquid chromatograph system consisting of a Waters U6K injector, two Waters Model 501 solvent delivery systems (Waters Associates, Milford, MA) controlled with a NEC Powermate 2 computer running a Waters Maxima 820 software program. Absorbance was monitored at 260 nm using a Waters Model 490 multiwavelength detector. Separations performed with a Zorbax (Dupont, Wilmington, Del) reverse phase C18 column (5 μm, 4.6 $\times$ 250 mm) were carried out at ambient temperature with a flow rate of 1 ml/min using a methanol-water gradient system. For ion pairing with the positively charged lucigenin molecule, the water portion of the methanol-water gradient system contained 60 mM perchloric acid at pH 4. Initial solvent conditions were 40% methanol with a linear gradient to 55% methanol in 10 min, after which conditions remained isocratic at 55% methanol for another 20 min.

RESULTS

LDCL elicited with isolated mitochondria. As shown in Fig. 1, incubation of mitochondria with 5 μM lucigenin elicited a CL response above the background of the luminometer (Curve 1). Addition of pyruvate/malate or succinate enhanced dramatically the LDCL (Curves 2 and 3). The LDCL response with the succinate-driven mitochondria was about 10 times as high as that of the pyruvate/malate-supported mitochondria (Fig. 1). Previously, mitochondria supported by succinate have also been shown to produce more H_2O_2 than those supported by pyruvate/malate (26, 27). The LDCL elicited with either pyruvate/malate- or succinate-driven mitochondria was correlated linearly with the concentration of lucigenin added (1–20 μM) (Fig. 2A and B). As shown in Fig. 2C, with succinate-supported mitochondria lucigenin at these concentrations ($\leq$20 μM) didn't undergo redox cycling as evidenced by the failure to stimulate potassium cyanide (KCN)-resistant O_2 consumption. However, lucigenin at 50 and 100 μM did stimulate KCN-resistant O_2 utilization (Fig. 2C). This indicates that lucigenin at these concentrations (50 and 100 μM) undergoes redox cycling, resulting in additional $O_2^{-\cdot}$ production. Therefore, to specifically detect the METC-derived $O_2^{-\cdot}$ in our isolated mitochondrial system, only non-redox cycling concentrations of lucigenin (20 μM or less) were used. Appreciation of this property of lucigenin is an important consideration when lucigenin is being used to specifically detect biological $O_2^{-\cdot}$ production. This has recently been elucidated through extensively assessing the concentration-dependent redox cycling of lucigenin in a wide spectrum of biological systems (11). In data not shown, lucigenin at concentrations $\leq$20 μM did not significantly affect mitochondrial O_2 utilization in the absence of KCN. This suggests that lucigenin at concentrations $\leq$20 μM did not disturb the normal mito-

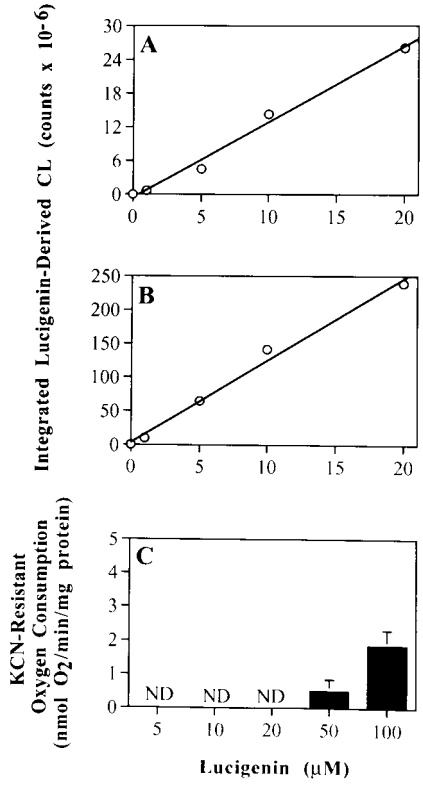

FIG. 2. Concentration-dependent LDCL elicited with pyruvate/malate (panel A)- or succinate (panel B)-supported mitochondria, and the KCN-resistant O_2 consumption induced by lucigenin with succinate-supported mitochondria (panel C). Mitochondria (0.5 mg/ml) were incubated in the presence or absence of 6 mM pyruvate/malate or 6 mM succinate at 37°C (see Materials and Methods). The LDCL response was initiated by adding the indicated concentrations of lucigenin. KCN-resistant O_2 consumption was measured at 37°C after incubation of the KCN (0.2 mM)-treated succinate-supplemented mitochondria with the indicated concentrations of lucigenin (panel C). Values in panels A and B represent means from three experiments; values in panel C represent mean $\pm$ S.E. from at least three experiments. *ND,* not detectable.

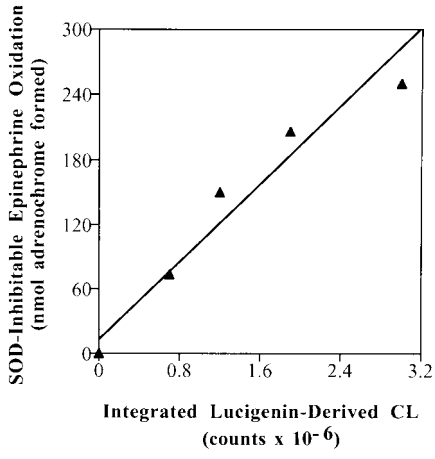

FIG. 3. Correlation between LDCL and SOD-inhibitable epinephrine oxidation in NADH-supported SMP. LDCL at 5 μM lucigenin and SOD-inhibitable epinephrine oxidation were measured at 37°C for 15 min after incubation of 100 μM NADH with various amounts of SMP (25, 50, 100 and 200 μg protein/ml) (see Materials and Methods). Values represent the mean from triplicate measurements.

chondrial electron flow. However, we did observe a decrease in mitochondrial aconitase activity at the redox cycling concentrations of 50 and 100 μM lucigenin but not at 5 μM lucigenin, which is the typical concentration that we generally use to assess intramitochondrial $O_2^{-\cdot}$ generation.

We have previously demonstrated that in both xanthine/xanthine oxidase and phagocytic NADPH oxidase systems, LDCL correlates linearly with SOD-inhibitable cytochrome c reduction in detecting the $O_2^{-\cdot}$ generation by these two systems (11). To further link the amount of LDCL to that of METC-derived $O_2^{-\cdot}$ production, we next examined the correlation between the LDCL and the SOD-inhibitable epinephrine oxidation by mitochondria. SOD-inhibitable epinephrine oxidation assay has been widely used to quantitate $O_2^{-\cdot}$ production by SMP (25). Because methods such as epinephrine oxidation assay can not be used to directly assess intramitochondrial $O_2^{-\cdot}$ production with intact mitochondria, NADH-supported SMP were used to demonstrate the above correlation. As shown in Fig. 3, when the amount of SMP was varied, a significant linear correlation (r = 0.97) between LDCL and SOD-inhibitable epinephrine oxidation by NADH-supported SMP was observed.

Effects of Cu,Zn-SOD and SOD-mimetics on LDCL. To assess the involvement of $O_2^{-\cdot}$ in the LDCL with intact mitochondria, we examined the effects of Cu,Zn-SOD and the membrane permeable SOD mimetics, TEMPO and MnTMPyP (28, 29). As shown, the LDCL elicited with the succinate-supplemented mitochondria was not affected significantly by Cu,Zn-SOD, but was reduced greatly in the presence of TEMPO or Mn(III)TMPyP (Fig. 4). In data not shown, inhibition of LDCL by TEMPO and MnTMPyP was concentration dependent with MnTMPyP being a more potent inhibitor. In addition, neither TEMPO nor MnTMPyP at the concentrations used significantly affected mitochondrial respiration as assessed by O_2 consumption (data not shown).

Ion-pair HPLC detection of lucigenin and N-methylacridone. Previous studies with confocal microscopy indicate that lucigenin accumulates in mitochondria of alveolar macrophages (7). To determine the accumulation of lucigenin and the presence of its $O_2^{-\cdot}$-dependent breakdown product, *N*-methylacridone in isolated intact mitochondria, an ion-pair HPLC system was developed. Figure 5 illustrates the HPLC profile and the standard curves of authentic standards of lucigenin and *N*-methylacridone. The peak retention time for lucigenin and *N*-methylacridone was 8.3 and 21.9 min, respectively. Two absorbencies with the above peak retention times were detected in extracts of succinate-supported mitochondria following incubation with lucigenin (chromatograph not shown). Co-chromatography studies further indicated the occurrence of lucigenin and its metabolite, *N*-methylacridone in the mitochondrial extracts.

Accumulation of lucigenin by mitochondria and the role of mitochondrial membrane potential in lucigenin accumulation. As shown in Fig. 6A, lucigenin accumulation within the mitochondrial fraction was proportional to the concentration of lucigenin added to the mitochondrial suspension. Significant accumulation of lucigenin was detected within the mitochondrial fraction after incubation with 1 μM lucigenin (Fig. 6A). Moreover, a significant linear correlation resulted between the concentration of lucigenin added to the mitochondrial suspension and the mitochondrial accumu-

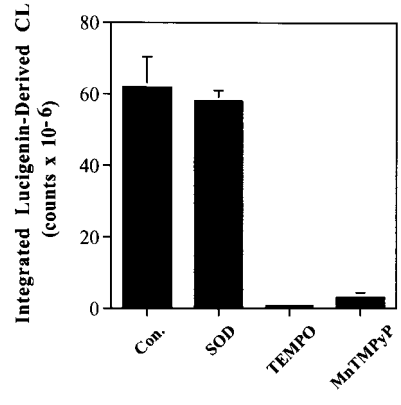

FIG. 4. Effects of Cu,ZnSOD and the SOD mimetics, TEMPO and MnTMPyP on the LDCL elicited with succinate-supported mitochondria. Mitochondria (0.5 mg/ml) were incubated with 6 mM succinate in the presence or absence of Cu,ZnSOD (500 units/ml), TEMPO (3 mM) or MnTMPyP (5 μM) at 37°C (see Materials and Methods). The LDCL response was initiated by adding 5 μM lucigenin. Values represent mean ± S.E. from three experiments.

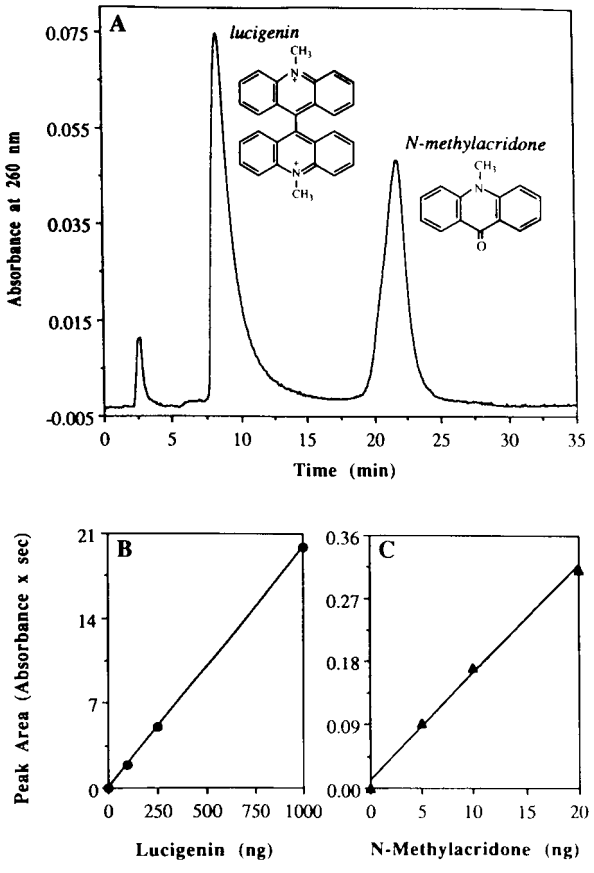

FIG. 5. HPLC profile (panel A) and standard curves (panels B and C) of the authentic standards of lucigenin and *N*-methylacridone. Separation was performed based on ion-pair HPLC (see Materials and Methods).

lation of lucigenin (r = 0.99). It should be noted that since lucigenin accumulates in the mitochondria, the concentration of lucigenin within the mitochondria is likely to be higher than that added to the mitochondrial preparation. Despite this accumulation, it is our experience that addition of lucigenin at concentrations $\leq$20 μM does not result in an increase in KCN-resistant O_2 consumption (11, Fig. 2). Moreover, with intact ML-1-derived monocytes/macrophages we did not observe a stimulation of KCN-resistant O_2 consumption by up to 50 μM lucigenin (11). Similar to the studies presented here, we can detect lucigenin in the mitochondria of these cells by HPLC (data not shown).

To examine a role for the mitochondrial membrane potential in lucigenin accumulation, the uncoupler FCCP was used to dissipate the membrane potential (30). As shown in Fig. 6B, when mitochondria were incubated with 5 μM lucigenin in the presence of 1 μM FCCP, the amount of mitochondrial lucigenin accumulation was greatly reduced. The reduction of mitochondrial lucigenin accumulation was observed at both 15 and 60 min after incubation with lucigenin in the presence of FCCP (Fig. 6B).

One electron reduction of lucigenin by METC. It has been shown that at high concentrations lucigenin is able to undergo redox cycling in several systems, including isolated mitochondria (11, 31, Fig. 2C). The redox cycling of lucigenin causes additional consumption of O_2. Using SMP, we took advantage of this property of lucigenin to determine the site(s) of the METC involved in the one electron reduction of lucigenin. As shown with NADH-supported SMP, lucigenin at 50 μM increased the O_2 consumption in the presence of rotenone (Fig. 7A), a complex I inhibitor that blocks electron flow from NADH dehydrogenase to ubiquinone (32). With SMP energized by succinate, lucigenin at 150 μM also caused additional O_2 consumption (Fig. 7B). This stimulation of O_2 consumption could be blocked completely by the complex III inhibitors, AA plus MYX (32), no matter whether they were added prior to or after the lucigenin (Fig. 7B and C).

Formation of N-methylacridone within mitochondria. *N*-methylacridone is the proposed end product of the reaction pathway leading to LDCL (3). We there-

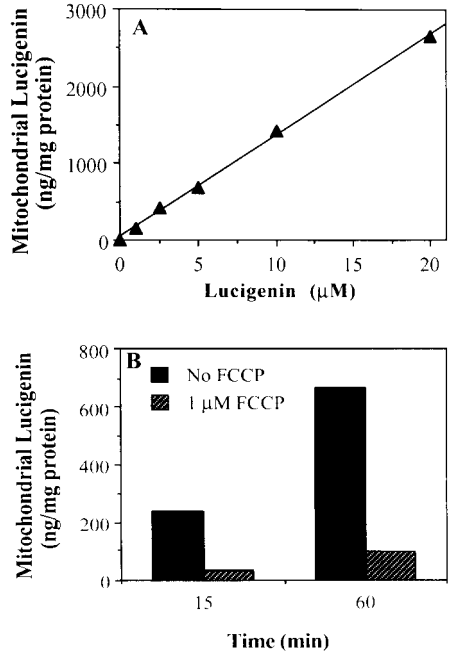

FIG. 6. Accumulation of lucigenin by mitochondria (panel A) and the effect of FCCP on mitochondrial lucigenin accumulation (panel B). In panel A, succinate-supported mitochondria (0.5 mg/ml) were incubated with the indicated concentrations of lucigenin at 37°C for 60 min, and then the accumulation of lucigenin within the mitochondrial fraction was analyzed (see Materials and Methods). The concentration of lucigenin within the mitochondrial fraction was calculated according to the standard curve derived for lucigenin (Fig. 5B). In panel B, succinate-supported mitochondria (0.5 mg/ml) were incubated with 5 μM lucigenin in the presence or absence of 1 μM FCCP at 37°C for the indicated times, and then the accumulation of lucigenin within the mitochondrial fraction was measured. In both panels A and B, values represent means from two experiments.

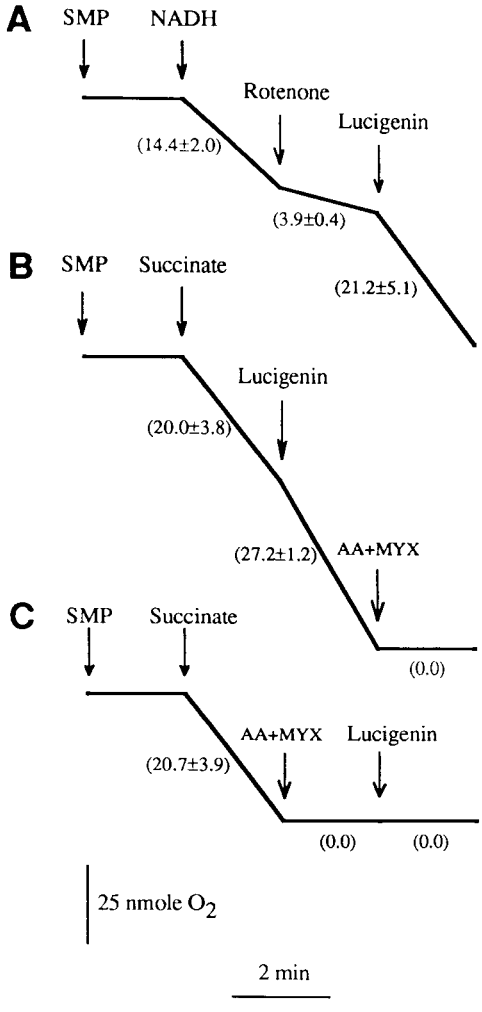

FIG. 7. Stimulation of O_2 consumption by lucigenin with SMP. O_2 consumption was monitored after incubation of SMP (0.25 mg/ml) with the indicated chemicals at 37°C (see Materials and Methods). The concentration of NADH or succinate was 6 mM; of rotenone, AA or MYX was 10 μM; of lucigenin was 50 μM (panel A) and 150 μM (panels B and C). The values under the curves represent mean ± S.E. from at least three experiments, with the unit being nmol O_2/min/mg protein of SMP.

fore examined whether *N*-methylacridone could be formed within the mitochondria following incubation with lucigenin. As shown in Fig. 8 after incubation with lucigenin at concentrations between 5 and 20 μM, *N*-methylacridone was detected within the mitochondria. Moreover, the formation of *N*-methylacridone within the mitochondrial fraction correlated linearly with the concentration of lucigenin added to the mitochondrial suspension (r = 0.98) (Fig. 8).

Correlation between LDCL and mitochondrial lucigenin accumulation or N-methylacridone formation. To further validate lucigenin as a probe for detecting intramitochondrial $O_2^{-\cdot}$, we next determined the correlation between LDCL and mitochondrial lucigenin accumulation or *N*-methylacridone formation. As shown in

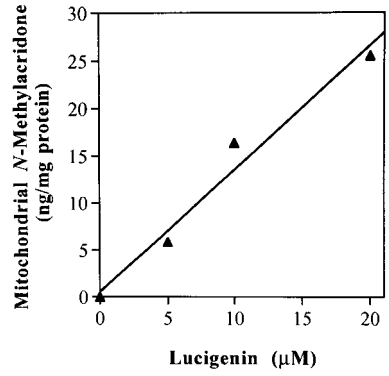

FIG. 8. Formation of *N*-methylacridone within mitochondria following incubation with lucigenin. Succinate-supported mitochondria (0.5 mg/ml) were incubated with the indicated concentrations of lucigenin at 37°C for 60 min, and then the formation of *N*-methylacridone within the mitochondrial fraction was analyzed (see Materials and Methods). The concentration of *N*-methylacridone within the mitochondrial fraction was calculated according to the standard curve derived for *N*-methylacridone (Fig. 5C). Values represent means from two experiments.

Fig. 9, a significant linear relationship (r = 0.99) was observed between LDCL and either lucigenin accumulation or *N*-methylacridone formation within the mitochondria. It should be noted that the concentrations of lucigenin used to determine these correlations are below those that we found to redox cycle in mitochondria (Fig. 2C).

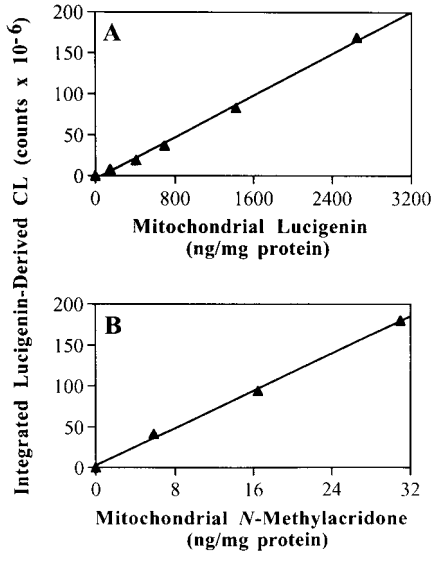

FIG. 9. Correlation between LDCL and mitochondrial lucigenin accumulation (panel A) or *N*-methylacridone formation (panel B). LDCL was measured with succinate-supported mitochondria (0.5 mg/ml) in the presence of 1, 2.5, 5, 10 and 20 μM of lucigenin at 37°C for 60 min. After this 60 min of incubation, the lucigenin accumulation and *N*-methylacridone formation within the mitochondrial fraction were analyzed (see Materials and Methods). Values represent means from two experiments.

DISCUSSION

The results of this study demonstrate that LDCL can be elicited with isolated intact mitochondria, which strongly supports our previous conclusion that LDCL appears to reflect mitochondrial $O_2^{-\cdot}$ formation in intact cells (7). To determine the role of $O_2^{-\cdot}$ in the production of the LDCL, we examined the effects of SOD and the membrane permeable SOD mimetics, TEMPO and MnTMPyP. The nearly complete inhibition of LDCL by TEMPO and Mn(III)TMPyP, but not by Cu,Zn-SOD (Fig. 4) suggests that LDCL detects intramitochondrial $O_2^{-\cdot}$ production exclusively. This observation also indicates that lucigenin may be able to penetrate the inner membrane of mitochondria. Indeed, lucigenin was detected in mitochondrial extracts following incubation of mitochondria with lucigenin (Fig. 6), proving that the mitochondrial inner membrane is permeable to lucigenin.

Rhodamine 123 is a cation that can accumulate in mitochondria as a result of the negative mitochondrial membrane potential (33). The structural similarity between the lucigenin molecule and rhodamine 123 suggests that lucigenin may be able to cross the mitochondrial inner membrane via an ionic attraction between the positively charged lucigenin molecule and the negatively charged mitochondrial inner membrane. To test this possibility, the mitochondrial membrane potential was destroyed by treatment with FCCP, an uncoupler of mitochondrial oxidative phosphorylation (30). The marked reduction of lucigenin accumulation within mitochondria in the presence of FCCP supports the above possibility. Thus, the mitochondrial membrane potential appears to be a major driving force, if not the only one, for lucigenin accumulation. As such, when LDCL is used to detect mitochondrial $O_2^{-\cdot}$ production, the status of the mitochondrial membrane potential becomes a critical factor. In this regard, a decrease in mitochondria-dependent LDCL may be more reflective of the loss of mitochondrial membrane potential rather than altered mitochondrial ROS generation. Accordingly, simultaneous detection of extramitochondrial H_2O_2 by luminol-amplified chemiluminescence can be used to correctly assess mitochondrial ROS generation when the mitochondrial membrane potential is significantly disrupted (34).

A critical process involved in the production of LDCL is the one electron reduction of lucigenin to its cation radical (3). To determine whether the METC is able to catalyze this step we took advantage of the fact that lucigenin at a concentration $\geq$50 μM can undergo redox cycling with isolated mitochondria (Fig. 2C). This occurs when the concentration of the lucigenin cation radical exceeds that of $O_2^{-\cdot}$ being produced by the biological source. When this happens the reaction between the lucigenin cation radical and molecular O_2 now leads to additional O_2 utilization (11). Therefore, stimulation of O_2 consumption by lucigenin redox cycling can be used to reflect the one electron reduction of lucigenin. The stimulation of rotenone-resistant O_2 consumption by lucigenin in NADH-supported SMP indicates that NADH-dehydrogenase is able to catalyze the one electron reduction of lucigenin. NADH-dehydrogenase has been shown to be capable of catalyzing the one electron reduction of a number of other chemicals, including paraquat, adriamycin, menadione and benzo(a)pyrene-derived quinones (14). Both AA and MYX are inhibitors of mitochondrial complex III (32). Previous studies reported that simultaneous treatment of mitochondria with AA and MYX resulted in a complete blockage of the electron flow through complex III (35). The complete inhibition of lucigenin-induced stimulation of O_2 consumption by AA plus MYX with succinate-supported mitochondria suggests that complex III is also able to catalyze the one electron reduction of lucigenin.

Formation of *N*-methylacridone within mitochondria demonstrates that the proposed reaction pathway leading to LDCL (1, 3) also occurs within isolated mitochondria. Based on the data presented in Figs. 6 and 8, the molar ratio of *N*-methylacridone formed to lucigenin accumulated within the mitochondria was calculated to be 0.019. This ratio is close to the one estimated by a previous study (36). In that study, based on a fluorospectrophotometric analysis of the xanthine oxidase plus hypoxanthine system, Totter reported that the averaged molar ratio of the presumed *N*-methylacridone to lucigenin was about 0.016 (36). These observations indicate that only a minor portion ($<$1%) of the lucigenin is finally converted to *N*-methylacridone in the above systems. Lastly, the validity of lucigenin as a detector of $O_2^{-\cdot}$ production within mitochondria was also strongly supported by the significant linear correlation between LDCL and either mitochondrial lucigenin accumulation or *N*-methylacridone formation (Fig. 9). LDCL thus appears to provide a qualitative rather than absolute measure of $O_2^{-\cdot}$ production. However, when a standard curve for LDCL versus $O_2^{-\cdot}$ is derived, LDCL may be used to precisely quantify biological $O_2^{-\cdot}$ as demonstrated in SMP (Fig. 3) and other $O_2^{-\cdot}$-generating systems (11).

In conclusion, this study demonstrates that the chemilumigenic probe lucigenin can get into and accumulate in mitochondria, and then be reduced to its cation radical by the METC. The lucigenin cation radical can then react with the METC-derived $O_2^{-\cdot}$, leading to LDCL and the production of *N*-methylacridone. The evidence that lucigenin undergoes these processes in isolated mitochondria further indicates that mitochondria may be a major contributor when LDCL is observed with intact cells or tissues. In view of the existence of other potential cellular sources of $O_2^{-\cdot}$ the mitochondria-derived $O_2^{-\cdot}$ as detected by LDCL can be

identified and distinguished from that generated by other sources through using specific mitochondrial inhibitors or by creating rho^{o} cells (7, 11, unpublished data).

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