BIOCHEMICAL PHARMACOLOGY 74 (2007) 545–556

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The effect of the lipophilic cation lucigenin on mitochondria depends on the site of its reduction

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ARTICLE INFO

Article history:
Received 24 February 2007
Accepted 16 May 2007

Keywords:
Cationic dyes
Permeability transition pore
Outer mitochondrial membrane
NAD(P)H-dependent oxidoreductases
Xenobiotics
Reactive oxygen species

ABSTRACT

The role of NAD(P)H-dependent oxidoreductases of the outer mitochondrial membrane (OMM) in the activation of lipophilic cationic dyes is poorly understood. In the present study we compared the rates of production of reactive oxygen species (ROS) and mitochondriotoxic effects of the redox-cycling lipophilic cationic dye lucigenin upon its activation by the respiratory chain and NAD(P)H-dependent oxidoreductases of the OMM. We found that, only in the presence of external NADH and NADPH, which are unable to penetrate the inner membrane, lucigenin stimulated a massive superoxide production and a fast permeabilization of mitochondrial membranes. The permeabilization was biphasic. The first, cyclosporin A-insensitive and Ca^{2+}-independent phase was characterized by increased permeability of the inner mitochondrial membrane to solutes with molecular masses of $\leq$200 Da. The second phase was sensitive to the antagonists of the permeability transition pore (mPTP) and was characterized by permeability similar to that of mPTP ($\leq$1500 Da). A massive cytochrome *c* release was observed even at the first phase of permeability when the second phase was inhibited by mPTP antagonists. Whatever the site of lucigenin activation, antioxidants and scavengers of ROS that strongly decrease the ROS level were unable to delay or prevent the permeabilization of membranes, which casts doubt on the involvement of ROS in the regulation of permeability by redox-cycling lipophilic cations. Our results strongly support the idea that the NAD(P)H-dependent reductases of xenobiotics of the OMM can mediate the toxicity of cationic dyes.

1. Introduction

The cytotoxicity of lipophilic cationic dyes is due to their rapid accumulation in mitochondria followed by bio- or photo-activation and disturbance of mitochondrial functions [1–5]. Since lipophilic cationic dyes preferentially accumulate in the mitochondria of cancer cells owing to their higher mitochondrial transmembrane potential ($\Delta\Psi_m$), they are used as

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Abbreviations: $\Delta\Psi_m$, inner membrane transmembrane potential; BA, bongkrekic acid; BHT, 2,6-di-*tert*-butyl-4-methylphenol; CsA, cyclosporin A; DBA, dimethylbiacridene; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; IMM, inner mitochondrial membrane; MCLA, 3,7-dihydro-2-methyl-6-(4-methoxyphenyl)imidazo[1,2-a]pyrazine-3-one; MDCL, MCLA-derived chemiluminescence; mPTP, mitochondrial permeability transition pore; OMM, outer mitochondrial membrane; PEG, polyethylene glycol; PMC, 2,2,5,7,8-pentamethyl-6-chromanol; RLM, rat liver mitochondria; ROS, reactive oxygen species; SOD, superoxide dismutase; TEMPO, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl; TPP^+, tetraphenylphosphonium

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doi:10.1016/j.bcp.2007.05.012

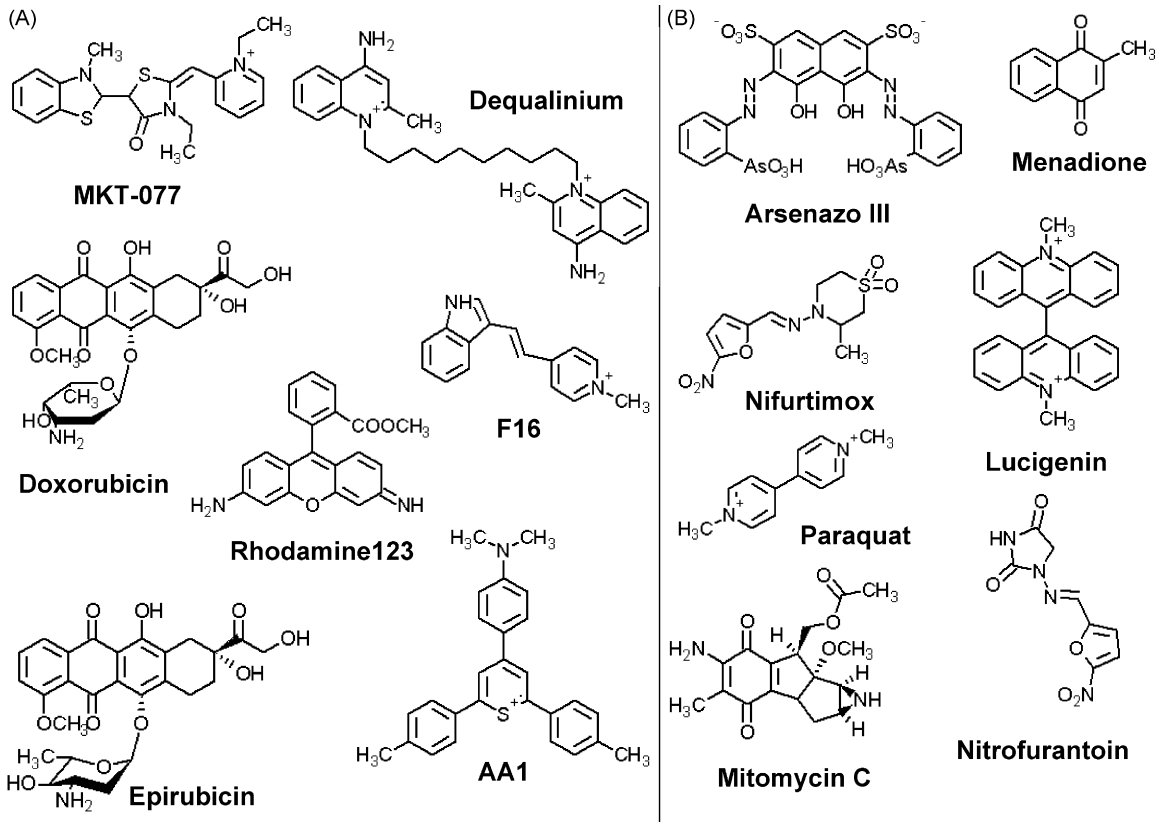

Fig. 1 – Chemical structures of cationic dyes applied as antitumor drugs (A) and of heterocyclic compounds known to be reduced by NAD(P)H-dependent oxidoreductases of the OMM (B).

relatively selective anticancer drugs [1,5–9]. The mechanisms of the mitochondrial damage by lipophilic cations are often poorly understood. In some detailed studies, the damaging effect was shown to be related to the overproduction of ROS due to the autoxidation of radical forms of dyes, the opening of the mPTP, and the release of cytochrome *c* [3,4,9,10].

Presently, only a small number of the cationic dyes known to accumulate in energized mitochondria and mediate the destruction of tumor cells was approved for clinical application (Fig. 1A) [2,6–12]. Moreover, some of these cationic drugs were found to cause severe side effects [8,10–12]. The slow progress in developing new effective antitumor drugs may be due partially to the shortcomings of the experimental approaches to the selection and characterization of cationic dyes, which are mainly limited to two experimental models: cells in culture and isolated mitochondria (or even submitochondrial particles). In cell culture models, the influence of the compound under study on cell death, caspase activation and the expression of pro-/antiapoptotic proteins is usually assessed [6,9]. In studies on isolated mitochondria in the presence of respiratory substrates, the main emphasis is made on the effects of dyes on respiration rates, $\Delta\Psi_m$, ATPase/ATP synthase activity, ROS production, mPTP induction, and cytochrome *c* release, i.e., those mitochondrial functions that are directly or indirectly related to the activities and integrity of the inner mitochondrial membrane (IMM) [2–5,9].

This experimental strategy does not allow one to assess the contribution of NAD(P)H-dependent oxidoreductases of the OMM to the activation of cationic dyes. However, NADH- and NADPH-dependent reductases of xenobiotics in the OMM are able to activate both cationic and anionic dyes (Fig. 1B) [13–20] and to produce ROS at very high rates in the presence of appropriate redox-cycling acceptors [13–18]. Since the cytosolic NADPH/$NADP^+$ ratio is high and the NADH/NAD^+ ratio can increase in some states (e.g., ischemia), oxidoreductases of the OMM may operate under physiological and pathophysiological conditions. The cytotoxicity of some of redox-cycling compounds (e.g., paraquate, mitomycin C, doxorubicin) may be mediated by these systems [17–19]. Therefore, the contribution of NAD(P)H-dependent oxidoreductases of the OMM to the mitochondrial damage caused by cationic dyes deserves careful study.

The lipophilic cationic dye and selective superoxide probe lucigenin has previously been shown to activate ROS production in various biological systems, including the respiratory chain of mitochondria [21,22]. In mitochondria ROS are generated due to the autoxidation of the lucigenin cation radical produced by the respiratory chain. Energized isolated mitochondria accumulate substantial amounts of lucigenin [23]; however, its mitochondriotoxic and cytotoxic effects were not described.

In the present study, we compared the rates and mechanisms of lucigenin-derived ROS production and permeabilization of mitochondrial membranes when lucigenin was activated in different sites of mitochondria, by the respiratory chain (IMM) and NAD(P)H-dependent reductases of xenobiotics (OMM). We

demonstrated that the ROS production and permeabilization of membranes were much faster when lucigenin was activated in the OMM than upon its reduction by the respiratory chain. Moreover, the mechanisms of membrane permeabilization were different. This must be taken into account in the evaluation of lipophilic cationic dyes as potential antitumor drugs. Whatever the site of lucigenin activation, antioxidants and ROS scavengers that decreased the ROS level were unable to delay or prevent the permeabilization of membranes. These data call into question the involvement of ROS in the regulation of permeability transition by redox-cycling lipophilic cations.

2. Materials and methods

2.1. Materials

Reagents were obtained from the Sigma Chemical Company, St. Louis, OR. Cyclosporin A (CsA) was a gift of the Sandoz Company (Basel, CH). Polyethylene glycols (PEGs) of different molecular weights were a kind gift of Dr. G.D. Mironova (Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences).

2.2. Isolation and purification of mitochondria

Rat liver mitochondria (RLM) were isolated from adult male Wistar rats according to a standard differential centrifugation procedure [24]. The homogenization medium contained 220 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH adjusted to 7.4 with Trizma Base), 1 mM EGTA, and 0.05% BSA. Routinely, isolated RLM were washed three times. The medium for two last washings was devoid of EGTA and BSA. Then the RLM fraction was analyzed for the presence of glucose-6-phosphatase activity [25]. The final pellets were resuspended in the same medium to yield 80–120 mg protein/ml. In preliminary studies, the comparison of preparations washed one, two, three, four, and six times revealed that after three washings, the influence of contaminating microsomes on NAD(P)H oxidation, ROS production, and membrane permeabilization became negligible. The traces of glucose-6-phosphatase activity were hardly detectable starting after 4–5 washings. Thus, in our studies we used a fraction of heavy mitochondria with insignificant microsomal contamination. All measurements were performed at 30 °C in standard KCl-based medium (125 mM KCl, 10 mM HEPES, 2 mM KH_2PO_4) supplemented, unless otherwise indicated, with 5 mM glutamate and 5 mM malate. Other experimental details are given in the figures. Mitochondrial protein was assayed by the Biuret method using BSA as a standard [26].

2.3. Measurements of oxygen consumption rate and $\Delta\Psi_m$ in isolated RLM

The mitochondrial respiration was detected by polarography using an oxygen Clark-type electrode and a computerized recording system Record 4 (Russia). $\Delta\Psi_m$ was assessed with the use of a tetraphenylphosphonium (TPP^+)-selective electrode according to the equation $\Delta\Psi_m = \lg([TPP^+]_{in}/[TPP^+]_{out})$ [27].

2.4. Measurement of ROS production

ROS production was measured using two highly selective and sensitive chemiluminescent superoxide probes—MCLA [28] and lucigenin [23] (the latter was also used for inducing ROS generation to prevent the interference with other probes). Chemiluminescence was detected with a Victor3^{TM} multiplate reader (Perkin Elmer, USA). The low-amplitude luminescence of lucigenin in RLM without exogenous NAD(P)H was detected using a Lucifer 2 M luminometer (Nika, Russia). ROS production was also indirectly detected as the cyanide-resistant respiration in RLM by polarography.

2.5. Recording the permeabilization of mitochondrial membranes

The permeabilization of the IMM of RLM due to mPTP opening (CsA-sensitive or -insensitive) causes the mitochondrial swelling and dissipation of $\Delta\Psi_m$. Swelling was recorded as a decrease in A_{540}. In several cases, RLM were incubated in media of different composition: NaCl-based medium (125 mM NaCl, 10 mM HEPES, 2 mM NaH_2PO_4), and sucrose/mannitol-based medium (60 mM sucrose, 200 mM mannitol, 10 mM HEPES, 2 mM NaH_2PO_4 or KH_2PO_4).

2.6. Measurement of NAD(P)H oxidation and lucigenin reduction

The course of NAD(P)H oxidation was followed at 340 nm in 2-ml thermostated cuvettes using a Uvikon 923 spectrophotometer (Kontron Instruments, USA). The extinction coefficient (ε) for NADPH and NADH at 339 and 340 nm, respectively, was 6.22 M^{-1} cm^{-1}. The reduction of lucigenin was followed from a decrease in A_{367}. The extinction coefficient for lucigenin was taken to be 3.3×10^4 M^{-1} cm^{-1} at 367 nm.

2.7. Determination of solute size exclusion properties of lucigenin-dependent pores

The size of pores induced by lucigenin plus NADH or Ca^{2+} (mPTP) was assessed as described in [29]. Briefly, RLM (1 mg/ml) were exposed to Ca^{2+} or lucigenin plus NADH in sucrose/mannitol media, with 40% of the osmotic pressure being due to PEGs of different molecular masses. The maximal amplitude of swelling was then recorded.

2.8. Measurement of cytochrome c release from RLM

RLM (2 mg/ml in 6-ml chamber) were incubated in standard medium supplemented with respiratory substrates with continuous control of respiration and $\Delta\Psi_m$ and parallel control of swelling. Swelling and a fall in $\Delta\Psi_m$ were initiated by the addition of indicated amounts of Ca^{2+}, alamethicin, or lucigenin plus NAD(P)H in the presence or absence of mPTP antagonists. After a 10-min incubation with stirring, RLM were sedimented by high-speed centrifugation, and the differential absorption spectra of the supernatant (reduced with dithionite/oxidized) were recorded in broad cuvettes (l = 3 cm). The extinction coefficient for cytochrome *c* absorption ($A_{550} - A_{540}$ nm) was taken to be 19.1 mM^{-1} cm^{-1}.

2.9. Statistical procedures

The data shown represent the means ± standard error of means (S.E.M.) or are the means of at least three experiments. Statistical probability (*P*) values were derived by the Student's *t*-test, unless otherwise indicated.

3. Results

3.1. Activation of lucigenin in the respiratory chain

Fig. 2 shows that, when activated by the respiratory chain, lucigenin even at high, ''redox-cycling'' concentrations (Fig. 2, insert) [21–23] caused a relatively slight increase in the basal level of mitochondrial superoxide anion, which was measured as MCLA-derived chemiluminescence (MDCL) (trace 2).

Table 1 summarizes the data on the effect of antioxidants and scavengers of ROS on the level of basal (MCLA) or lucigenin-derived (MCLA + lucigenin) superoxide anion in respiring RLM. The basal level of mitochondrial superoxide anion was equally decreased by scavengers that are able or unable to penetrate the membranes. Superoxide dismutase (SOD) (EC 1.15.1.1), alone or in combination with catalase (EC 1.11.1.6), and 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), its mimetic capable of penetrating the membranes,

Table 1 – Effect of antioxidants and scavengers of ROS on superoxide level in RLM

	MCLA-derived chemiluminescence (%)	
	MCLA	Lucigenin + MCLA
Control	100 (465 AU)	100 (675 AU)
SOD	51.4 ± 2.5	10.6 ± 6.0
Catalase	73.9 ± 5.5	109.7 ± 10.3
SOD + Catalase	36.3 ± 8.7	15.6 ± 2.5
TEMPO	57.6 ± 7.1	16.1 ± 4.2
BHT	86.9 ± 8.5	107.7 ± 6.5
α-Tocopherol	97.9 ± 8.2	116.5 ± 2.5
PMC	98.3 ± 7.8	87.9 ± 5.3
GSH	−29.1 ± 3.1	−1.7 ± 6.0

Chemiluminescence of MCLA was detected as in Fig. 2. 100% of chemiluminescence corresponds to the increment above the basal chemiluminescence of MCLA in RLM-free medium in arbitrary units (AU). The contribution of lucigenin-derived chemiluminescence to the total MCLA-dependent signal was less than 1%. Medium contained the following concentrations of antioxidants: SOD, 110 U/ml; catalase, 250 U/ml; TEMPO, 500 μM; BHT, 40 μM; α-tocopherol, 1 mM; PMC, 50 μM; GSH, 1 mM; α-lipoic acid, 500 μM (final concentrations). Values are the means ± S.E.M. of three experiments ($n = 15$). The inhibitory effects of SOD, SOD + catalase, catalase (MCLA), TEMPO, and GSH were significant ($P < 0.05$); for BHT (MCLA) and PMC (MCLA + Lucigenin) $P < 0.05$ (Wilcoxon test).

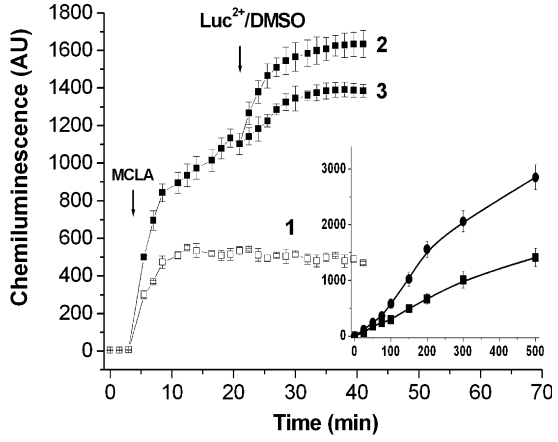

Fig. 2 – Stimulation of superoxide anion production in RLM by high concentrations of lucigenin. RLM (0.3 mg protein/ml) were incubated in standard KCl-based medium supplemented with 5 mM glutamate and 5 mM malate (traces 2 and 3). Where indicated, 20 μM MCLA and 250 μM lucigenin (Luc^{2+}) dissolved in DMSO (0.5%, v/v) (trace 2) or DMSO alone (0.5%, v/v) (trace 3) were added. Trace 1, medium without RLM. In the insert, the dependence of the maximal amplitude of the lucigenin-derived chemiluminescence on the lucigenin concentration is presented. RLM (1 mg protein/ml) were incubated in standard medium supplemented with 1 mM EGTA, 5 mM glutamate, and 5 mM malate (-■-) or 5 mM succinate and 1 μM rotenone (-●-) for 1 min before the addition of the indicated concentration of lucigenin. The ordinate shows maximal chemiluminescence (counts/s). The values are the means ± S.E.M. of five or three (the insert) experiments.

decreased the level of natural mitochondrial ROS by 50–60% and the level of lucigenin-dependent ROS still more, by 85–90%. The inhibitors of free radical reactions and lipid peroxidation 2-,6-di-*tert*-butyl-4-methylphenol (BHT), α-tocopherol and its low-molecular-weight analogue 2,2,5,7,8-pentamethyl-6-chromanol (PMC), as well as catalase had a minor effect on the level of native or lucigenin-derived superoxide in mitochondria. GSH appeared to be a potent quencher of MDCL, since it inhibited both ROS-dependent and spontaneous chemiluminescence. These data show that, in the presence of lucigenin, a large portion of superoxide is generated in the outer compartments of RLM, and MDCL is more specific to superoxide than in the absence of lucigenin [28].

The relatively slight stimulation of superoxide production in RLM by lucigenin may be connected not only with a low rate of formation of autoxidizable cation radicals in the respiratory chain but also with the weak uncoupling effect of lucigenin (Fig. 3), which must suppress the natural mitochondrial ROS production [30]. It is seen from Fig. 3 that lucigenin caused a detectable decrease in $\Delta\Psi_m$ in a dose-dependent manner, which was associated with the acceleration of V_2 (resting) respiration on glutamate and malate. The respiratory control also slightly decreased in the presence of high concentrations of lucigenin (6.8 ± 1.6 without lucigenin versus 5.7 ± 1.45 ($n = 15$) in the presence of 400 μM lucigenin).

As shown, lucigenin stimulated a large-amplitude swelling of RLM preloaded with Ca^{2+} (Fig. 4A) and a drop in $\Delta\Psi_m$ (Fig. 4B) in a dose-dependent manner (traces 2 and 3). Without preloading (not shown) or in the presence of EGTA (trace 4), lucigenin even in high concentrations was unable to accelerate swelling and the dissipation of $\Delta\Psi_m$. The inhibitors of mPTP cyclosporin A and bongkrekic acid (BA) completely prevented Ca^{2+}-dependent and lucigenin-induced swelling

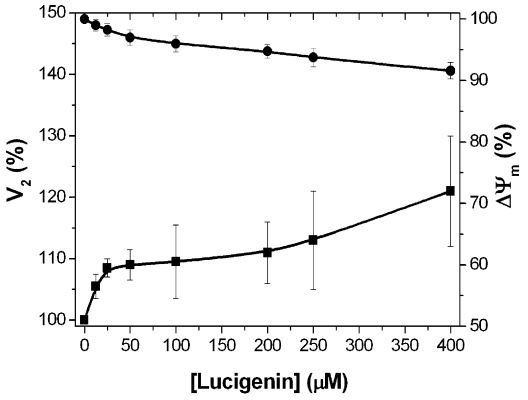

Fig. 3 – Effect of lucigenin on the rate of V_2 respiration and $\Delta\Psi_m$ in RLM. RLM (2 mg protein/ml) were incubated in standard medium supplemented with respiratory substrates and 2 μM TPP^+. (-■-): changes in V_2; (-●-): changes in $\Delta\Psi_m$. The values are the means ± S.E.M. (n = 3).

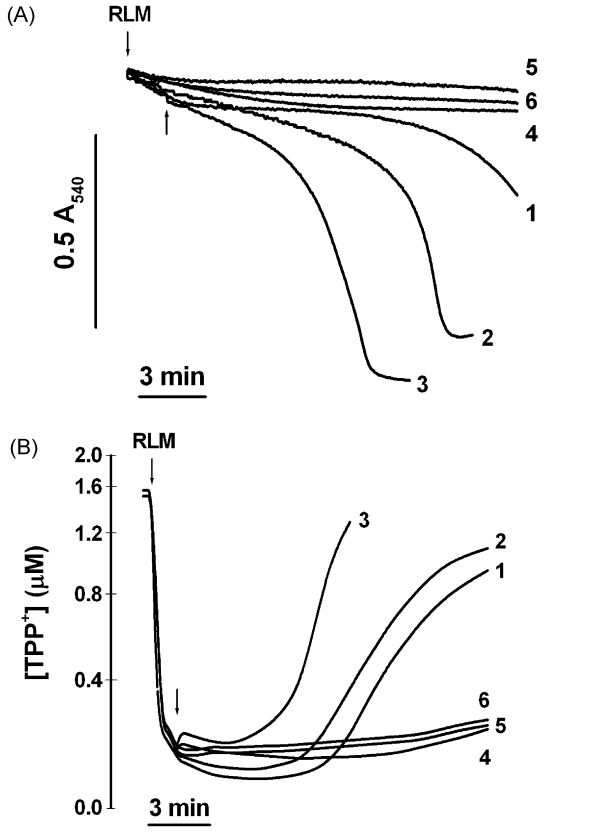

Fig. 4 – Stimulation of permeability transition pore opening in RLM by lucigenin. RLM (1 or 2 mg protein/ml (panels A and B, respectively)) were incubated in standard medium supplemented with $CaCl_2$ (20 nmol/mg protein), with addition of 1.6 μM TPP^+ (panel B) and, if noted, with other additions. The injection of lucigenin is indicated by arrow. Trace 1, RLM without additions; traces 2 and 3, swelling induced by 50 or 400 μM lucigenin, respectively; traces 4–6, swelling induced by 400 μM lucigenin in the presence of 1 mM EGTA, CsA (1 nmol/mg protein), and 10 μM BA, respectively. Panels A and B present mitochondrial swelling and changes in $\Delta\Psi_m$. The data given are the original traces of one parallel experiment of at least five identical.

and $\Delta\Psi_m$ dissipation (traces 5 and 6), indicating the mPTP induction by lucigenin.

We studied the influence of antioxidants and scavengers of ROS on the mPTP induction to determine whether lucigenin-dependent mPTP opening is mediated by the overproduction of ROS (Fig. 5). SOD (alone or with catalase), TEMPO and GSH had no protective effect on the lucigenin-induced mPTP opening (Fig. 5, traces 3–7). Only BHT and, to a smaller extent, PMC, which are known to be direct modulators of mPTP [31], delayed the lucigenin- and Ca^{2+}-dependent swelling of RLM. (All antioxidants were used at concentrations at which they did not influence the RLM swelling.) Thus, the reduction of the lipophilic cation lucigenin in the respiratory chain only slightly affected mitochondrial functions.

3.2. *Activation of lucigenin in the OMM*

Fig. 6 shows that the relatively weak lucigenin-dependent superoxide production in RLM was drastically stimulated after the activation of reductases of xenobiotics in the OMM by exogenous NADPH or NADH (Fig. 6A, traces 5 and 6). This stimulation was not affected by the presence of respiratory substrates (malate and glutamate) in the incubation medium (data not shown). In the absence of MCLA, the extremely weak lucigenin-derived chemiluminescence was also stimulated by one to two orders of magnitude by NADH or NADPH (traces 3 and 4). In the absence of lucigenin, NAD(P)H did not stimulate the MDCL (traces 2). NADH and, to a smaller extent, NADPH significantly accelerated the swelling of RLM in the presence of lucigenin (Fig. 6B traces 5 and 6, respectively). The swelling was accompanied by the dissipation of $\Delta\Psi_m$ and a loss of respiratory control (data not shown). The acceleration of NAD(P)H-dependent swelling was more pronounced at lucigenin concentrations higher than 50 μM (75–400 μM) and did not demand the preloading of RLM with Ca^{2+}.

The lucigenin- and NADH-dependent swelling of well-coupled RLM in KCl-based medium, as a rule, had two well distinguishable phases (Fig. 7A, trace 2), the first, a very fast ''low''- and a delayed ''high''-amplitude swelling (the absorbance changes associated with each phase of swelling varied considerably). The first phase of swelling was independent of the operation of K_{ATP} channels, since the channel inhibitor glibenclamide did not prevent it (trace 3). It was not exclusively K^+-specific, since it was also observed in NaCl-based medium (trace 4). The first phase of swelling was strongly suppressed in sucrose/mannitol-based media (traces 5 and 6), indicating the induction of permeability for small molecules (up to 200 Da) only. The second phase of swelling was triggered by the first one (compare traces 2–4 and 5, 6). The solute size exclusion measurements of pores induced in the first and second phases of lucigenin- and NADH-dependent swelling (Fig. 7B) confirmed that PEGs larger than 200 Da were unable to enter the RLM matrix in the first phase of swelling

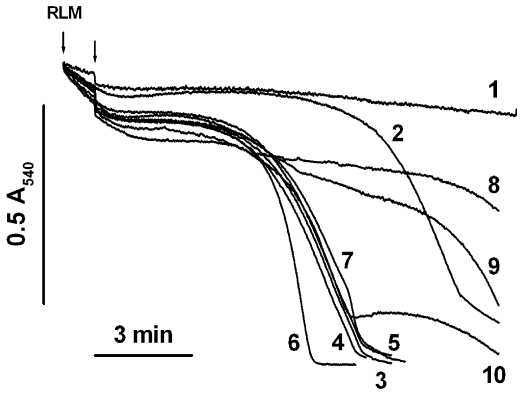

Fig. 5 – Effect of antioxidants and scavengers of ROS on the lucigenin-stimulated Ca^{2+}-dependent swelling of RLM. The conditions of incubation were as in Fig. 4A, excluding trace 1 (without addition of Ca^{2+}). The addition of 400 μM lucigenin is indicated by arrow. Traces 3–10, lucigenin-dependent swelling in the absence of other additions (trace 3) and in the presence of 110 U/ml of SOD (trace 4), 110 U/ml of SOD and 250 U/ml of catalase (trace 5), 500 μM TEMPO (trace 6), 1 mM GSH (trace 7), 40 μM BHT (trace 8), 50 μM PMC (trace 9), and 1 mM α-tocopherol (trace 10). Trace 1, lucigenin-dependent swelling in the absence of added Ca^{2+}. Trace 2, RLM without additions. The data presented are the standard original traces from one experiment of at least four identical.

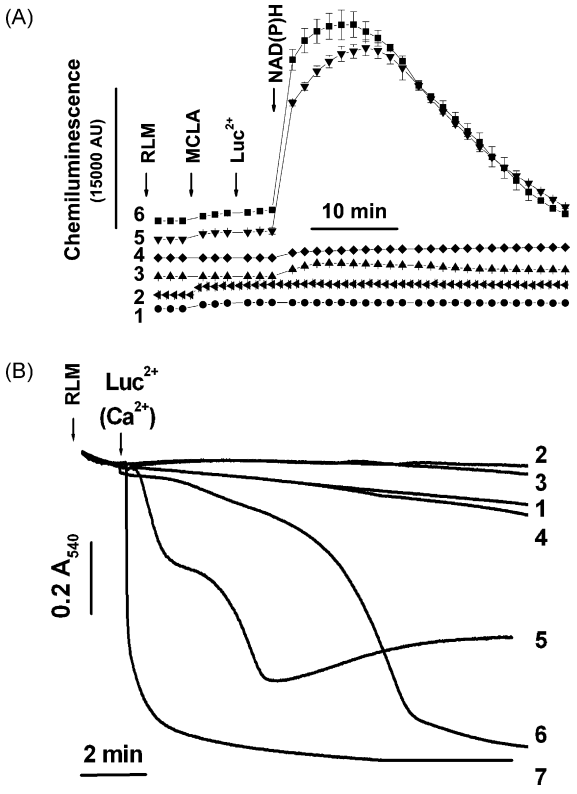

Fig. 6 – Effects of NADH and NADPH on the level of superoxide anion (A) and the swelling of RLM (B) in the presence of lucigenin. Panel A: The incubation conditions were as in Fig. 2. Where indicated, 20 μM MCLA, 100 μM lucigenin (Luc^{2+}), and 500 μM NADH and/or NADPH were added. Lucigenin-dependent chemiluminescence in RLM in the presence of NADH (trace 3), NADPH (trace 4), MCLA plus NADPH (trace 5), and MCLA plus NADH (trace 6). NADH and NADPH were added simultaneously in the absence of RLM (trace 1) or lucigenin (trace 2). The data of one of three identical experiments are presented; points on the traces are the means ± S.E.M. (n = 5). AU, arbitrary units. Panel B: RLM (1 mg protein/ml) were placed in standard medium. If noted, the incubation medium contained 250 μM NADH or NADPH; 100 μM lucigenin (Luc^{2+}) or Ca^{2+} (250 nmol/mg protein) were added where indicated by arrow. Traces 1–4, swelling in the absence of other additions, and in the presence of NADH, NADPH, and lucigenin, respectively. Traces 5 and 6, lucigenin-dependent swelling in the presence of NADH and NADPH, respectively. Trace 7, Ca^{2+}-dependent swelling. The standard traces from one of at least five identical experiments are shown.

(trace 2). The permeability of pores induced in the second phase of swelling (trace 3) was almost identical to that of the Ca^{2+}-induced mPTP (trace 1).

Fig. 8 demonstrates that mPTP blockers and antagonists: 10 μM BA (trace 3), 1 mM EGTA (trace 4), 1 μM CsA (trace 5) alone or in combination with 1 mM MgATP (trace 7), the thiol-reducing agent dithiothreitol (1 mM) (trace 6), and the monofunctional cysteine alkylator *N*-ethylmaleimide (25 nmol/mg protein) (trace 8) had little or no effect on the first (''low''-amplitude) phase of swelling. On the other hand, all agents except BA (trace 3) strongly suppressed the second (''high''-amplitude) phase of swelling. This indicates that the first phase of swelling is connected with the induction of permeability distinct from that of mPTP, whereas the second phase appears to be due to the induction of classical mPTP. The same, but delayed effects were observed in the presence of lucigenin and NAD(P)H (data not shown).

Fig. 9A shows that 1 mM EGTA (trace 3), 10 μM BA (trace 4), and 1 μM CsA (trace 5) were unable to prevent the $\Delta\Psi_m$ dissipation induced by 100 μM lucigenin and 250 μM NADH (trace 2). This was to be expected since the IMM under these conditions is permeable for low-molecular-mass solutes (see Figs. 7 and 8). Surprisingly, these agents were also unable to prevent cytochrome *c* release under these conditions (Fig. 9B). As shown in Fig. 9B, lucigenin plus NADH caused a release of cytochrome *c* from RLM in amounts equal to those released due to mPTP opening (Ca^{2+}). EGTA (+EGTA), BA (+BA), and CsA (+CsA) did not diminish the cytochrome *c* release significantly. A maximal cytochrome *c* release was observed after the treatment of RLM with the pore-forming peptide alamethicin (Alam). Lucigenin in the absence of external NADH liberated only a small portion of cytochrome *c* from RLM that were not preloaded with Ca^{2+} (Luc^{2+}). Lucigenin in the presence of CsA did not liberate any detectable amount of cytochrome *c* (CsA-Luc^{2+}). Thus, the massive lucigenin-dependent ROS production in the OMM is associated with the fast, biphasic and partly CsA-insensitive permeabilization of mitochondrial membranes. The swelling induced by lucigenin and NADH was

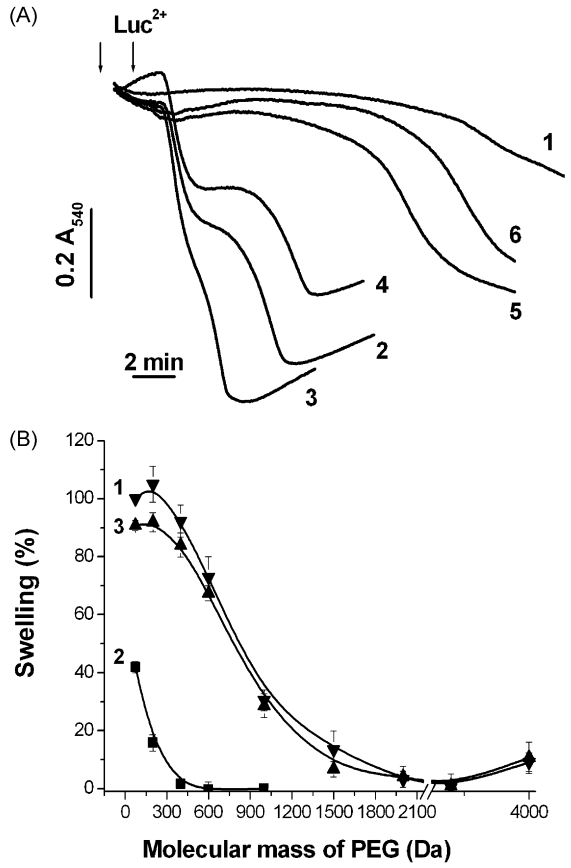

Fig. 7 – Permeability of pores in IMM, induced by lucigenin and NADH. Panel A: Lucigenin- and NADH-dependent swelling of RLM in incubation media of different composition. RLM (1 mg/ml) were added to the media with or without other additions. Where indicated, 100 μM lucigenin was added. Traces 1–3, lucigenin-dependent swelling in KCl-based medium without additions, with the addition of 250 μM NADH, and NADH plus 7.5 μM glibenclamide, respectively; traces 4–6, lucigenin- and NADH-dependent swelling in NaCl-based medium, sucrose/mannitol-based medium plus KH_2PO_4, and sucrose/mannitol-based medium plus NaH_2PO_4, respectively. The standard traces from one of at least three separate experiments are shown. Panel B: Solute size exclusion properties of lucigenin- and NADH-dependent permeability of membranes. Trace 1, CsA-sensitive permeability induced by Ca^{2+}; traces 2 and 3, permeability induced by lucigenin and NADH upon low and high amplitude swelling, respectively. The data presented are the means ± S.E.M. (n = 3).

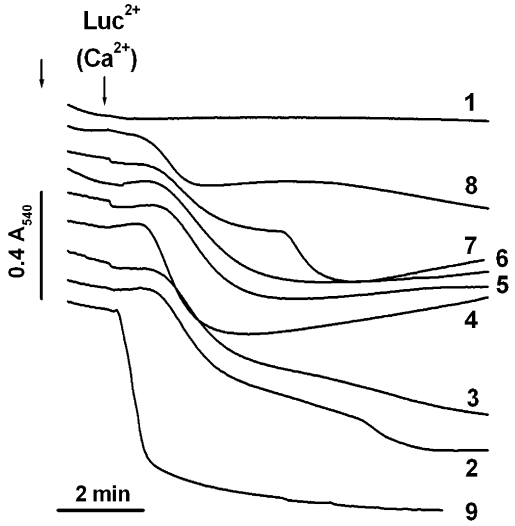

Fig. 8 – Effects of mPTP antagonists on the lucigenin- and NADH-dependent swelling of RLM. RLM (1 mg/ml) were added just before measurements. Where indicated, 100 μM lucigenin or Ca^{2+} (300 nmol/mg protein) (trace 9) were added. The incubation medium contained also 250 μM NADH (traces 2–8), 10 μM BA (trace 3), 1 mM EGTA (trace 4), 1 μM CsA (trace 5), 1 mM DTT (trace 6), 1 mM MgATP plus 1 μM CsA (trace 7), and 25 μM NEM (trace 8). The standard traces of one of at least three identical experiments are shown.

strongly $\Delta\Psi_m$-dependent, since it was prevented by the uncoupler FCCP or the complex III inhibitors myxothiazol and antimycin A. In the latter case, the swelling was reactivated by a partial restoration of $\Delta\Psi_m$ after the addition of 1 mM Mg-ATP (data not shown).

Fig. 10 shows the mechanism of lucigenin-dependent superoxide production by reductases of xenobiotics in the OMM. Lucigenin substantially accelerated the basic oxidation of external NADH and NADPH in intact RLM with the blocked respiratory chain (Fig. 10A, traces 1 and 2). The stoichiometry of lucigenin and NAD(P)H in the reaction of oxidation was 1:>1, respectively (traces 1 and 2). This can be explained by reoxidation of the reduced form of lucigenin by the respiratory chain or oxygen. Cyanide weakly suppressed the oxidation of NADPH (trace 2) but slightly stimulated the oxidation of NADH (trace 1). The latter was accompanied by the induction of mitochondrial swelling (data not shown). Lucigenin did not oxidize NAD(P)H in the absence of RLM. Both NADPH and NADH considerably accelerated the antimycin A-, myxothiazol- and cyanide-resistant, lucigenin-supported respiration in RLM (Fig. 10B, traces 1 and 2), indicating that the NAD(P)H-dependent reductases of xenobiotics of the OMM produce autoxidizable cation radicals. In RLM inhibited with antimycin A and myxothiazol, the NADH-dependent reduction of lucigenin (decrease in absorbance at 367 nm) was drastically accelerated by cyanide (Fig. 10C, trace 2).

Thus, lucigenin is reduced by NAD(P)H-dependent reductases of xenobiotics of the OMM via the one-electron mechanism. The resulting cation radical may be reoxidized by cytochrome c and cytochrome c oxidase of the respiratory chain [32] or reduced by a second electron to form insoluble dimethylbiacridene (DBA) (Fig. 10C). A substantial portion of radicals is oxidized by oxygen to yield superoxide anions (Fig. 10A and B), causing a burst of ROS.

Table 2 summarizes the data on the effects of radical scavengers and antioxidants on MDCL in RLM in the presence of external NAD(P)H and the redox cycler. As shown in the

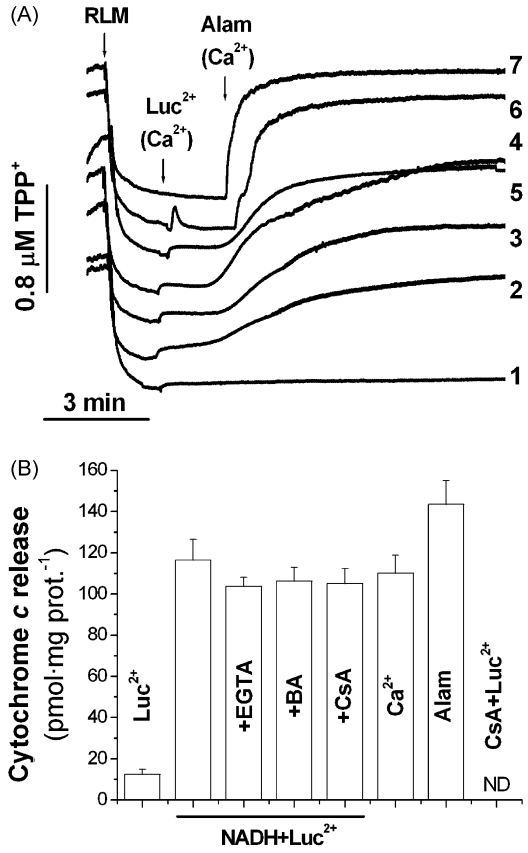

Fig. 9 – Effects of mPTP antagonists on the lucigenin-, NADH-dependent dissipation of $\Delta\Psi_m$ (A) and cytochrome c release from RLM (B). Panel A: The incubation medium contained 1.2 μM TPP^+, and, if noted, other additions. Where indicated, RLM (1 mg/ml), 100 μM lucigenin, Ca^{2+} (50 nmol/mg protein) or alamethicin (40 μg/mg protein) were added. Traces 2–5, lucigenin- and NADH-dependent dissipation of $\Delta\Psi_m$ in the medium without other additions (trace 2) and with the addition of 1 mM EGTA (trace 3), 10 μM BA (trace 4), and 1 μM CsA (trace 5). The concentration of NADH was 250 μM. Traces 1, 6, and 7, RLM treated with lucigenin, Ca^{2+} or alamethicin, respectively. The standard traces from one of at least three separate experiments are shown. Panel B: Experimental conditions as in panel A. Columns are the means ± S.E.M. of three independent experiments.

table, SOD alone or in combination with catalase almost completely removed the superoxide generated by the OMM. Catalase alone slightly inhibited MDCL, indicating the involvement of oxygen species other than superoxide [28]. TEMPO also strongly suppressed lucigenin-dependent MDCL in the initial period after NAD(P)H addition. However, in the course of superoxide generation by ''outer'' dehydrogenases, TEMPO rapidly lost its antioxidant effects (figures in parentheses). The scavengers of lipid radicals and peroxides that are able to penetrate the membranes: BHT, α-tocopherol, and PMC had a less pronounced effect on the level of NAD(P)H-dependent superoxide than SOD. PMC, similarly to TEMPO, retained the

Table 2 – Effect of antioxidants and scavengers of ROS on the ROS level in RLM

	MCLA-derived chemiluminescence (%)	
	Lucigenin + NADH	Lucigenin + NADPH
Control	100 (21200 AU)	100 (18550 AU)
SOD	7.2 ± 0.8	11.0 ± 0.6
Catalase	73.5 ± 3.5	75.4 ± 4.1
SOD + Catalase	4.3 ± 2.1	6.2 ± 3.1
TEMPO	40.0 ± 5.0 (126 ± 8.9)	25.6 ± 4.4 (45.8 ± 13.7)
BHT	108 ± 5.8 (80.8 ± 11.2)	76.4 ± 5.8
α-Tocopherol	89.5 ± 10.6	81.8 ± 6.6
PMC	72.8 ± 4.5 (98.5 ± 2.1)	61.9 ± 8.5 (103 ± 0.5)
GSH	43.1 ± 0.6	42.6 ± 1.5

Incubation conditions were as in Fig. 2. Concentrations of antioxidants were as in the legend to Table 1. The figures display the relative changes in MDCL after 10 or 20 (figures in parentheses) min of incubation. 100% of chemiluminescence corresponds to instrumental arbitrary units (AU). Values are the means ± S.E.M. of three separate experiments ($n = 15$). The maximal inhibitory effect of each antioxidant was significant ($P < 0.05$), for α-tocopherol $P < 0.05$, (Wilcoxon test).

antioxidant properties only over a short period of time after the initiation of massive ROS production by NAD(P)H. GSH quenched MDCL by ~60%. Thus, NADH and NADPH stimulated the massive superoxide production in the outer (accessible for SOD and catalase) compartments of mitochondria in the presence of lucigenin.

Surprisingly, none of the following antioxidants and combinations prevented or markedly delayed the induction of permeability of mitochondrial membranes: SOD (60–150 U/ml) ± catalase (110–500 U/ml), 250 μM–1 mM TEMPO, 250 μM–1 mM α-tocopherol (or α-tocopherol–succinate and α-tocopherol–acetate), and 0.5–2 mM GSH. Catalase slightly delayed lucigenin- and NADPH-induced permeability, but only in aged mitochondria with unstable $\Delta\Psi_m$ (data not shown). On the contrary, BHT at relatively low concentrations (15–40 μM) and, to a lesser extent, 50 μM PMC not only delayed swelling but also reduced its amplitude regardless of the nature of the electron donor (Fig. 11, traces 6 and 7). These results are not consistent with the data on the effects of antioxidants on the level of ROS (superoxide) produced by dehydrogenases of the OMM (see Table 2).

4. Discussion

In the present study we showed that the effect of lipophilic cationic dye lucigenin on the mitochondrial ROS production and the rates and mechanisms of membrane permeabilization depends on the site of its reduction in mitochondria. The activation of lucigenin in the inner membrane caused only slight changes in mitochondrial functions. The activation of the dye by the NAD(P)H-dependent reductases of the OMM caused a burst of ROS production, as well as a fast, partly CsA-insensitive membrane permeabilization and cytochrome c release.

The cytotoxicity of cationic dyes is often related to the autoxidation of their radical forms and ROS-dependent mitochondrial damage [4,9,17]. The dication lucigenin shares

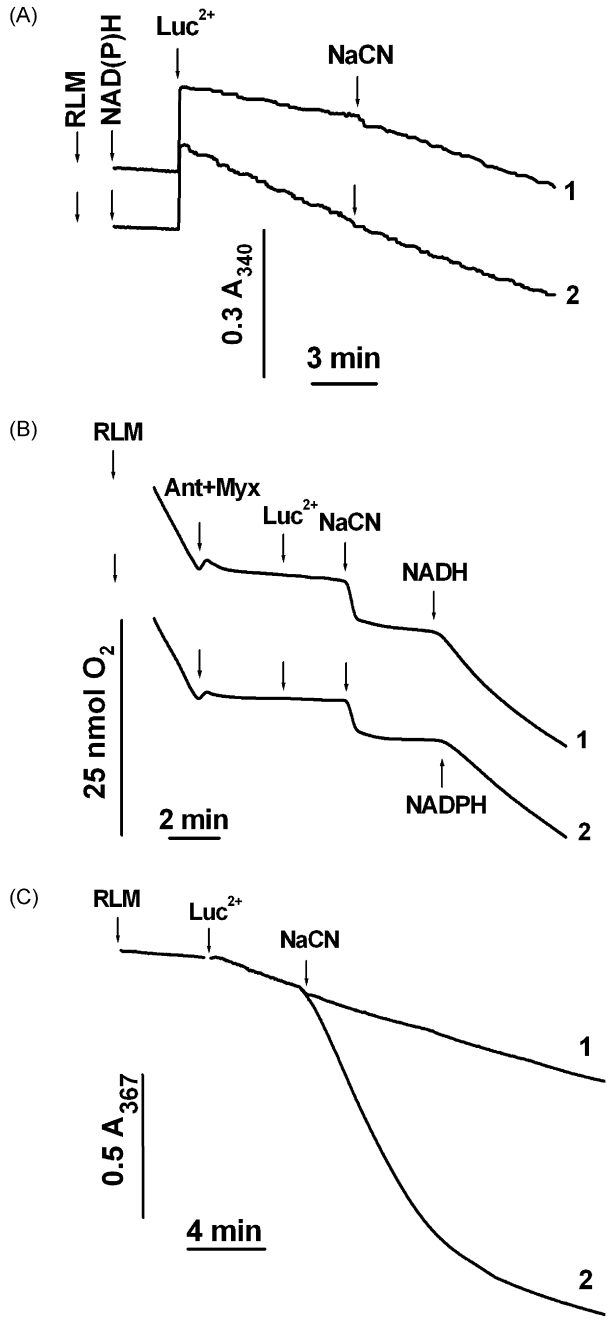

Fig. 10 – The mechanism of lucigenin reduction and superoxide anion generation by NAD(P)H-dependent reductases of xenobiotics of the outer mitochondrial membrane. Panel A: Effect of lucigenin on the oxidation of added NAD(P)H by RLM. RLM (1 mg protein/ml) were added (arrow) to standard medium supplemented with antimycin A (0.5 μg/mg protein) and 1 μM myxothiazol. Where indicated, 100 μM NADH (trace 1), 100 μM NADPH (trace 2), 25 μM lucigenin (Luc^{2+}), and 0.5 mM NaCN (traces 1 and 2) were added. Panel B: Effect of lucigenin and NAD(P)H on oxygen consumption in RLM treated with respiratory chain inhibitors. Incubation medium as in panel A, but initially without inhibitors. RLM (2 mg protein/ml) were added just before measurements. Where indicated, antimycin A (Ant) (0.5 μg/mg protein), 1 μM myxothiazol (Myx), 100 μM lucigenin (Luc^{2+}), 0.5 mM NaCN, 250 μM NADH (trace 1), and NADPH (trace 2) were injected. Panel C: Effect of cytochrome c oxidase inhibitor on the rate of lucigenin reduction in RLM. Incubation medium as in panel A, with the addition of 500 μM NADH. RLM (1 mg/ml) were added just before measurements. Where indicated, 25 μM lucigenin added to sample and reference cuvettes (traces 1 and 2) and 500 μM NaCN (trace 2) were added. The standard data from one of at least three separate experiments are shown.

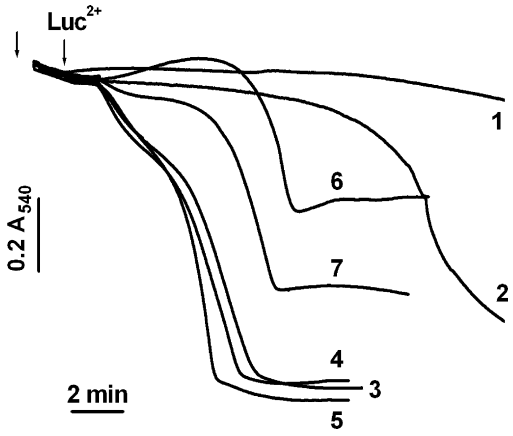

Fig. 11 – Effect of antioxidants and scavengers of ROS on the lucigenin-dependent swelling of RLM in the presence of external NADH. RLM (1 mg protein/ml) (arrow) were placed to a standard medium just before measurements. Where indicated, 100 μM lucigenin was added. Trace 1, RLM without additions; trace 2, after the addition of lucigenin; traces 3–7, lucigenin- and NADH-dependent swelling in the medium without other additions (trace 3) and after the addition of 110 U/ml SOD plus 250 U/ml catalase (trace 4), SOD, catalase and 500 μM TEMPO (trace 5), 40 μM BHT (trace 6), and 50 μM PMC (trace 7). Concentration of NADH was 250 μM. The standard traces from one of at least five identical experiments are presented.

a considerable structural similarity with a number of cationic dyes that are known as potent antitumor agents (Fig. 1A). Lucigenin induces ROS production in various biological systems, including mitochondria [21,22]. Lucigenin is accumulated by mitochondria in a $\Delta\Psi_m$-dependent fashion and can shuttle following the changes in $\Delta\Psi_m$ [23,33]. The key intermediate of lucigenin is a cation radical (lucigenin that accepted one-electron), which can react with superoxide anion to yield unstable dioxetane, and after decomposition, *N*-methylacridone and a quantum of chemiluminescence. Alternatively, the cation radical can be oxidized by molecular oxygen or reduced by a second electron, yielding superoxide anion or water-insoluble DBA, respectively ([34] and references herein). Previously, we found that lucigenin is reduced by complexes I and II via a two-electron mechanism, and subsequent one-electron oxidation in complex III is necessary for cation radical formation [35].

In the majority of studies on the effects of cationic dyes on mitochondria, the possibility of activation of dyes by dehydrogenases of the OMM was not considered. However, the OMM contains NADH- and NADPH-dependent redox systems (probably, NADH:cytochrome b5 oxidoreductase (EC 1.6.2.2) and NADPH:cytochrome P450 oxidoreductase-like protein (EC 1.6.2.4) [17,19,20]) able to reduce and activate cationic and anionic xenobiotics of various structure (Fig. 1B) and generate ROS with high rates in the presence of appropriate redox-cycling acceptors [13–18]. Since the cytosolic NADPH/$NADP^+$ ratio is high and the NADH/NAD^+ ratio can increase in some states (e.g., ischemia), oxidoreductases of the OMM may operate under physiological and pathophysiological conditions. Therefore, the contribution of NAD(P)H-dependent oxidoreductases of the OMM to the mitochondrial damage caused by cationic dyes deserves careful study.

The measurement of ROS production by NAD(P)H-dependent reductases of xenobiotics of the OMM presents considerable difficulties. The components of a biological object often interfere with the components of measuring systems: radicals of quinone-like compounds directly reduce cytochrome *c* [32], and NAD(P)H interacts with peroxidase and/or electron donors [36] in peroxidase-based assays. MCLA, a highly specific and sensitive probe, is a rare exception. In spite of its substantial intrinsic luminescence in solution (Fig. 2, Table 1), its chemiluminescence in mitochondrial suspension is almost exclusively ROS-dependent [28]. In the present study, the level of ROS was assessed using several complementary methods: the recording of the MCLA- and lucigenin-derived chemiluminescence, of cyanide-sensitive respiration, and of NAD(P)H oxidation. This approach is not suitable for measurements of lucigenin-dependent ROS in the absence of NAD(P)H since cyanide activates the cation radical formation in complex III via the mechanism other than that operating in normal conditions [35].

By measuring the MDCL, we showed that the rate of lucigenin-dependent ROS production in the respiratory chain is approximately 10–20 times lower than in the OMM. The estimation of ROS production in the OMM by measurements of lucigenin-dependent NAD(P)H oxidation and cyanide-resistant respiration gave figures up to 15–20 nmol H_2O_2 min^{-1} mg $protein^{-1}$. The values of the same order were obtained for other redox cyclers [13,14]. However, it is widely accepted that the rates of ROS production by the respiratory chain of intact mitochondria is in the range of 0.022–1.5 nmol H_2O_2 min^{-1} mg $protein^{-1}$ [37]. Thus, the data on the level of ROS in RLM obtained by MDCL and other methods agreed well. Since superoxide anion can be extruded from the intermembrane space of mitochondria through the voltage-dependent anionic channel (VDAC) [38], the analysis with the use of antioxidants does not exclude any of the OMM surfaces as being responsible for the massive superoxide production.

The higher rates of lucigenin-dependent ROS production in the OMM than in the IMM corresponded to higher rates of permeabilization of mitochondrial membranes. Moreover, upon the activation of lucigenin in the OMM, the permeabilization of mitochondrial membranes became biphasic and partly insensitive to the antagonists of mPTP (CsA, EGTA, BA, dithiothreitol, *N*-ethylmaleimide, MgATP). It is important that the cationic dye was able to liberate cytochrome *c* even in the presence of mPTP antagonists and absence of Ca^{2+}, i.e., under conditions close to physiological. The dynamics and sensitivity of the lucigenin- and NAD(P)H-dependent mitochondrial permeabilization to inhibitors of mPTP were very similar to those induced by peroxynitrite anion [39], tetraalkyl thiuram disulfides [40], and polycationic peptides mastoparan [29], p13II protein [41], and BTM-P1 [42]. Interestingly, the activity of p13II or BTM-P1 critically depends on the presence in the sequence of four or five positively charged residues of lysine and arginine [41,42] whose functions could not be ascribed only to the targeting of peptide to mitochondria [41].

Nevertheless, the absence of the effect of most antioxidants and scavengers (both able and unable to penetrate the membranes) on the permeabilization of membranes regardless of the site of lucigenin activation (IMM or OMM) is inconsistent with the strong suppression of ROS production by SOD and catalase or TEMPO, observed under these conditions. The extensive protective effect observed in the presence of the phenolic antioxidants BHT and PMC seems to be due to the direct action on the regulatory site of the permeability transition pore, but not to their antioxidant properties. Earlier this possibility was verified in experiments with the BHT analogue 3,5-di-*tert*-butyltoluene, which lacks the antioxidant properties [31]. Thus, it is unlikely that the permeabilization of membranes in the presence of lucigenin is mediated by ROS independent of the site of lucigenin reduction. The most probable regulator of the permeability of mitochondrial membranes is the cation radical of lucigenin, which is the main product of lucigenin reduction by NAD(P)H-dependent reductases of xenobiotics in the OMM (see Fig. 10). The permeabilization of mitochondrial membranes by lucigenin in the presence of NAD(P)H is a $\Delta\Psi_m$-dependent process. The cation radical of lucigenin, unlike DBA [43], is charged and soluble in water, and its movements must be affected by $\Delta\Psi_m$. Moreover, in contrast to the products of lucigenin oxidation dioxetane and *N*-methylacridone, the effect of the cation radical must not be diminished by ROS scavengers and antioxidants. Lucigenin per se should also be excluded since its activation in the OMM dramatically accelerated the permeabilization of membranes. It is important to mention that the cytotoxicity of other redox-cycling dyes may also be mediated by their radical forms and attenuated when radicals are oxidized by oxygen to yield ROS [19].

Taken together, these data support the idea that the possibility of NAD(P)H-dependent generation of toxic forms of cationic dyes in the OMM should be considered in the evaluation of these agents as potential antitumor drugs. One must also take into account that the permeabilization of membranes and the release of cytochrome *c* under these conditions could be CsA-insensitive, as well as Ca^{2+}- and ROS-independent.

Acknowledgements

This study was supported by grants to Kruglov from the Russian Foundation for Basic Research (RFFI 05-04-49650) and to Kruglov and Saris from the Magnus Ehrnrooth Foundation in Helsinki and the Medical Society in Finland.

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