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Cd^{2+}-promoted mitochondrial permeability transition: a comparison with other heavy metals★

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We compared action of Cd^{2+}, Hg^{2+}, and Cu^{2+} on isolated rat liver mitochondria in the absence of added Ca^{2+} and P_i. The heavy-metal ions produced dose-dependently: (1) enhanced membrane permeabilizaton manifested in mitochondrial swelling and activation of basal respiration, (2) inhibition of uncoupler-stimulated respiration, and (3) membrane potential dissipation. Among the metals, Cu^{2+} exhibited maximal stimulatory effect on basal respiration and minimal inhibitory action on DNP-uncoupled respiration whilst Cd^{2+} promoted the strongest depression of uncoupled respiration and the largest swelling in NH_4NO_3 medium. Dithiothreitol induced a basal respiration release if added after high [Cd^{2+}] and [Hg^{2+}], and the stimulation was CsA-insensitive.

The mitochondrion is a target organelle for such heavy metal ions as Cd^{2+}, Hg^{2+}, and Cu^{2+} (Vallee & Ulmer, 1972; Zoratti & Szabo, 1995; Strubelt *et al.,* 1996). Moreover, mitochondrial dysfunction is the earliest key event in heavy metal cytotoxicity (Muller, 1986; Nieminen *et al.,* 1990; Koizumi *et al.,* 1994; Palmeira & Madeira, 1997; Santos *et al.,* 1997; Pourahmad & O'Brien, 2000). In particular, it is found that disturbance of mi-

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Abbreviations: CsA, cyclosporin A; $\Delta\Psi_{mito}$, mitochondrial membrane potential; DNP, 2,4-dinitrophenol; DTT, dithiothreitol; MMP, mitochondrial membrane permeabilization; MPT pore, mitochondrial permeability transition pore; RLM, rat liver mitochondria.

tochondrial respiration and MMP are involved in hepatorenal toxic effects of the metals (Zaba & Harris, 1976; Weinberg *et al.,* 1982a; 1982b; Leblondel & Allain, 1984; Rasheed *et al.,* 1984; Chavez *et al.,* 1988; Chavez & Holquin, 1989; Micadei & Floridi, 1993). However, the mechanism(s) underlying the heavy metal-produced mitochondrial dysfunction is not completely understood. As recognized, MPT pore-mediated MMP may determine the hepatorenal toxicity of xenobiotics *in vitro* and *in vivo* (Zoratti & Szabo, 1995; Haouzi *et al.,* 2002), with heavy metals among them (Broekemeier *et al.,* 1989; Zazueta *et al.,* 1998; 2000; Garcia *et al.,* 2000; Belyaeva *et al.,* 2001; 2002; Pourahmad *et al.,* 2001). Besides, there are data indicating that MPT pores opened by some inducers (including Hg^{2+}) operate in two modes, a regulated one that is activated by Ca^{2+} and inhibited by cyclosporin A (CsA) and Mg^{2+}, and an unregulated mode that is Ca^{2+}-independent and insensitive to CsA and Mg^{2+} (He & Lemasters, 2002). The objective of the present study was to compare mechanism(s) of action of heavy metals on *in vitro* rat liver mitochondrial function in the absence of exogenous Ca^{2+}, P_i and Mg^{2+} in assay media. In consequence, the primary targets and the sequence of events of the heavy metal-induced mitochondrial impairment were elucidated. The involvement of regulated and unregulated pores in the mitochondrial dysfunction is discussed as well.

MATERIALS AND METHODS

Isolation of mitochondria. Rat liver mitochondria (RLM) were prepared according to a standard differential centrifugation procedure in a medium containing 250 mM sucrose, 3 mM Tris/HCl (pH 7.4) and 0.5 mM EGTA that was omitted in two last washings as described previously (Belyaeva *et al.,* 2001). Protein content was determined by the Bradford method. The endogenous Ca^{2+} content of these mitochondria was 10–15 nmol/mg of protein as determined by flame photometry.

Mitochondrial swelling. Heavy metal-induced membrane permeabilization of isolated mitochondria was determined from their swelling in a medium containing 125 mM NH_4NO_3 and 5 mM Tris/NO_3 (pH 7.4) after addition of Cd^{2+}, Hg^{2+}, or Cu^{2+} (as chloride salts). As known, NH_4^+ ions are capable of crossing the membrane only as protons and freely diffusing molecules of NH_3. The nitrate anion (NO_3^-) is also freely permeant through the inner mitochondrial membrane, so the monitoring of mitochondrial swelling in NH_4NO_3 medium makes it possible to estimate the proton membrane permeability of the mitochondria. Rotenone (5 μM) and oligomycin (5 μg/ml), if not otherwise mentioned, were present in all the media; 5 mM Tris/succinate (Succ) was administered where indicated in figures. Reactions were carried out at 20°C in a 2.5 ml chamber filled with incubation medium and contained 2.5 mg of mitochondrial protein. The swelling was monitored as apparent absorbance changes at 520 nm with the help of recording spectrophotometer, as before (Belyaeva *et al.,* 2001). The results shown are representative of a series of four experiments.

Mitochondrial respiration. The oxygen consumption rate of mitochondria was monitored at 26°C with a Clark oxygen electrode in a thermostatic closed chamber of 1.2 ml with magnetic stirring. Mitochondria (1 mg/ml of protein) were added to a medium containing 100 mM KCl, 20 mM Tris/HCl, 5 mM Tris/succinate (pH 7.3), rotenone (5 μM), and oligomycin (5 μg/ml). Cd^{2+}, Hg^{2+}, or Cu^{2+} (as chloride salts) at the concentrations tested was added to the medium one to two minutes after mitochondria; the basal respiration rate was estimated both in the absence and in the presence of different MPT effectors, which were added to the assay medium either from

the start of the experiment or during incubation. Besides, at the end of each experiment, 32 μM DNP was administered to the medium and the uncoupled respiration rate was determined. The oxygen consumption rate values are presented as ng atom O/min per mg of mitochondrial protein. The results shown are representative or average of a series of at least three experiments.

Radiometric assay. The influence of heavy metals on the uptake of $^{137}Cs^+$ by valinomycin-treated and succinate-energized mitochondria was determined by the Millipore filtration method, the details of which were described earlier (Belyaeva *et al.,* 2002). In particular, the kinetics of the latter process is well characterized by coefficients of distribution (r_t) of the tracer between energized mitochondria and medium. These coefficients are calculated from equation: $r = A_{MT}/A_{MD}$, where A_{MT} is radioactivity in 1 g of mitochondrial protein and A_{MD} is radioactivity in 1 ml of the medium. Efflux of $^{137}Cs^+$ from isolated mitochondria was estimated by the time ($t_{1/2}$) required for the coefficients (r_t) to decrease twice compared to those in the steady state. As known, the uptake of $^{137}Cs^+$ by energized mitochondria in the presence of valinomycin (estimated by the coefficient r_t) characterizes the energetic condition of isolated mitochondria and indirectly the mitochondrial membrane potential ($\Delta\Psi_{mito}$) value. This method of $\Delta\Psi_{mito}$ monitoring is especially convenient for determination of the dynamics of $\Delta\Psi_{mito}$ dissipation. The assay medium contained 140 mM sucrose, 50 mM Tris/acetate (pH 7.4), 2.5 mM Tris/succinate, 4 μM rotenone, 1 μg/ml oligomycin, and 0.01 μM valinomycin. Mitochondria (0.5 mg protein/ml) were incubated at 20°C. Experiments were repeated five times, with data presented being representative. For preparation of the assay media, we used, when necessary, sucrose refined with the help of a cationite KU-2-8 column, and twice recrystalized KCl. All other chemicals were purchased from Sigma. $^{137}Cs^+$ was provided by ISOTOPE (Moscow, Russia).

RESULTS AND DISCUSSION

Using isolated organelles as a model, we compared the action of low (5–10 μM) and high (20–50 μM) concentrations of several heavy metals (Cd^{2+}, Hg^{2+}, and Cu^{2+}) on rat liver mitochondria function. As seen from Fig. 1, all the metals induced swelling of non-energized mitochondria in NH_4NO_3 medium in a dose-dependent manner; however, they did it in a different way. In particular, at equimolar concentrations of the heavy-metal ions applied (20 μM), Cd^{2+} produced maximal proton MMP (Fig. 1, trace 3). In comparison with Cd^{2+}, Hg^{2+} showed a smaller amplitude and much slower kinetics of the swelling (Fig. 1, trace 5). In turn, Cu^{2+} action was characterized by a slow initial mitochondrial swelling rate followed by its further rapid release (Fig. 1, trace 7).

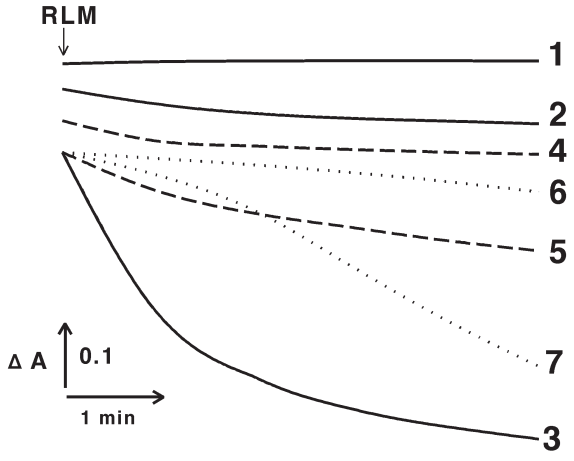

Figure 1. Heavy-metals induced rat liver mitochondrial swelling in 125 mM NH_4NO_3 medium.

The following additions were made: 1, none (control); 2, 10 μM Cd^{2+}; 3, 20 μM Cd^{2+}; 4, 10 μM Hg^{2+}; 5, 20 μM Hg^{2+}; 6, 10 μM Cu^{2+}; 7, 20 μM Cu^{2+}.

A comparison of the influence of the heavy metals on the mitochondrial respiratory function was conducted as well (Fig. 2). As found, the metals affected differently the basal and DNP-uncoupled respiration of the mitochondria, and all the effects were dose-dependent. Already at 10 μM, Cd^{2+} strongly inhibited the

uncoupler-stimulated respiration rate (Fig. 2A, trace 3), whilst the same concentrations of Hg^{2+} (Fig. 2B, trace 3) and Cu^{2+} (Fig. 2C, trace 3) decreased it less than by half (compare with trace 4). At this concentration, Cu^{2+} produced the highest stimulation of the basal respiration rate. The sustained activation of the resting state respiration obtained under these conditions was equal to 150–200% for Cu^{2+} and 30–50% for Cd^{2+} and Hg^{2+} (relative to corresponding control). 20 μM Cu^{2+} (Fig. 2C, trace 2) and Hg^{2+} (Fig. 2B, trace 2) produced sustained activation of the basal respiration rate as well and for Cu^{2+} it exceeded 300%. At the same [Cd^{2+}], only transient stimulation of the basal respiration was obtained (Fig. 2A, trace 2). At a higher (50 μM) concentration, Cu^{2+} produced a sharp transient stimulation of the resting state respiration (comparable to that observed at 20 μM Cu^{2+}), and then a moderate sustained activation took place (Fig. 2C, trace 1). In the case of Cd^{2+}, a transient potent activation of the basal respiration was followed by its depression (Fig. 2A, trace 1). Hg^{2+} at 50 μM induced a strong inhibition of the resting state respiration, without any transient stimulation observed (Fig. 2B, trace 1). In turn, the uncoupler-stimulated respiration was practically stopped by this concentration of Cd^{2+} or Hg^{2+}, and was substantially depressed by Cu^{2+}.

It was found also that all the metals promoted a sharp dose-dependent decrease in steady-state $^{137}Cs^{+}$ uptake level of valinomycin-treated and succinate-energized mitochondria (reflecting $\Delta\Psi_{mito}$ dissipation) with different kinetics of $^{137}Cs^{+}$ efflux (Fig. 3). Values of $t_{1/2}$ (see Materials and Methods) averaged from five different mitochondrial preparations and describing the kinetics for r_t (i.e., the tracer distribution coefficients) were equal to 1, 2, and 4.5 min in experiments using 5 μM of Hg^{2+}, Cd^{2+}, and Cu^{2+}, respectively (Fig. 3, traces 3), with deviation of $t_{1/2}$ not exceeding 10%. It is evident that the data correlate well with the findings obtained in the swelling and respiratory experiments (see above).

Thus, the heavy metal-produced mitochondrial dysfunction is attributed to (1) an enhanced membrane permeabilizaton that manifested in mitochondrial swelling and activation of basal respiration, (2) an inhibition of uncoupler-stimulated respiration, and (3) a

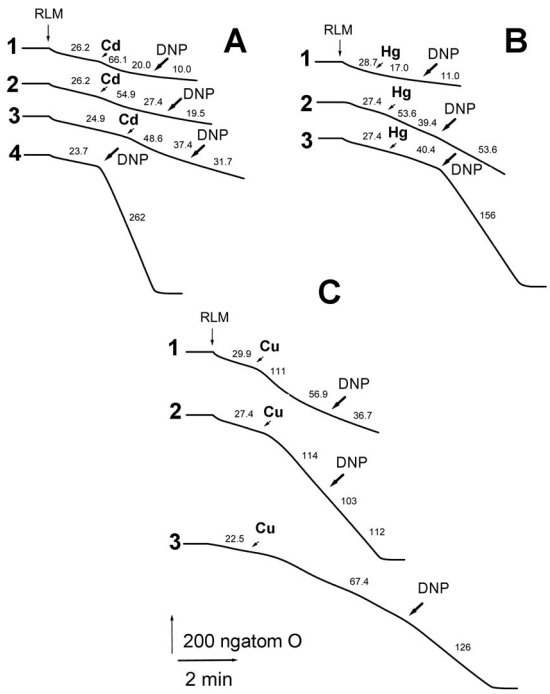

Figure 2. Action of heavy metals on basal and DNP-uncoupled respiration rate of isolated rat liver mitochondria.

The following additions were made: (A) 1, 50 μM Cd^{2+}; 2, 20 μM Cd^{2+}; 3, 10 μM Cd^{2+}; 4, none (control); (B) 1, 50 μM Hg^{2+}; 2, 20 μM Hg^{2+}; 3, 10 μM Hg^{2+}; (C) 1, 50 μM Cu^{2+}; 2, 20 μM Cu^{2+}; 3, 10 μM Cu^{2+}.

$\Delta\Psi_{mito}$ dissipation. Among the metals, Cu^{2+} exhibited the maximal stimulatory effect on the basal respiration and the minimal inhibitory action on DNP-uncoupled respiration whilst Cd^{2+} promoted the strongest depression of the uncoupled respiration and the largest swelling in NH_4NO_3 medium. Hg^{2+} induced similar effects as Cd^{2+} but all of them were less profound, except the $\Delta\Psi_{mito}$ loss. Hence, it is clear that both the mitochondrial respiratory chain and the membrane perme-

ability are the primary targets of the deleterious influence of the heavy metals. Moreover, the data obtained here underscore the sequence of events taking place during the disturbance by the metals of the mitochondrial function. In particular, it looks like $\Delta\Psi_{mito}$ collapse is the primary event in the Cd^{2+} and Hg^{2+}-induced mitochondrial dysfunction in which the direct inhibitory effect on the respiratory chain components seemingly plays a dominant role, whereas the Cu^{2+}-promoted $\Delta\Psi_{mito}$ dissipation is likely a consequence of its direct enhancing effect on the membrane permeability that prevails in the Cu^{2+}-produced mitochondrial dysfunction. Moreover, our preliminary data as well as reports by other authors (Leblondel & Allain, 1984; Wojtczak *et al.,* 1996; Garcia *et al.,* 2000) indicate that mitochondrial ATP-sensitive K^+ channel could be involved in Cu^{2+}-induced mitochondrial dysfunction. This issue is under investigation in our group.

What is the main cause of the MMP promoted by the metals tested under the applied experimental conditions (i.e., in the absence of added Ca^{2+}, P_i and Mg^{2+} in assay medium)? Previously, we showed that Cd^{2+}-produced MMP checked under identical experimental conditions was partially sensitive to CsA, a potent pharmacological inhibitor of regulated MPT pores (Belyaeva *et al.,* 2001; 2002). Recently, our evidence has been confirmed and extended by Curti and colleagues (Dorta *et al.,* 2003). As to the Cu^{2+}- and Hg^{2+}-induced MMP, we found here that it was completely insensitive to CsA as estimated polarographically (Fig. 4) and by measuring the swelling of both non-respiring and succinate-energized mitochondria in NH_4NO_3 medium in the presence and in the absence of

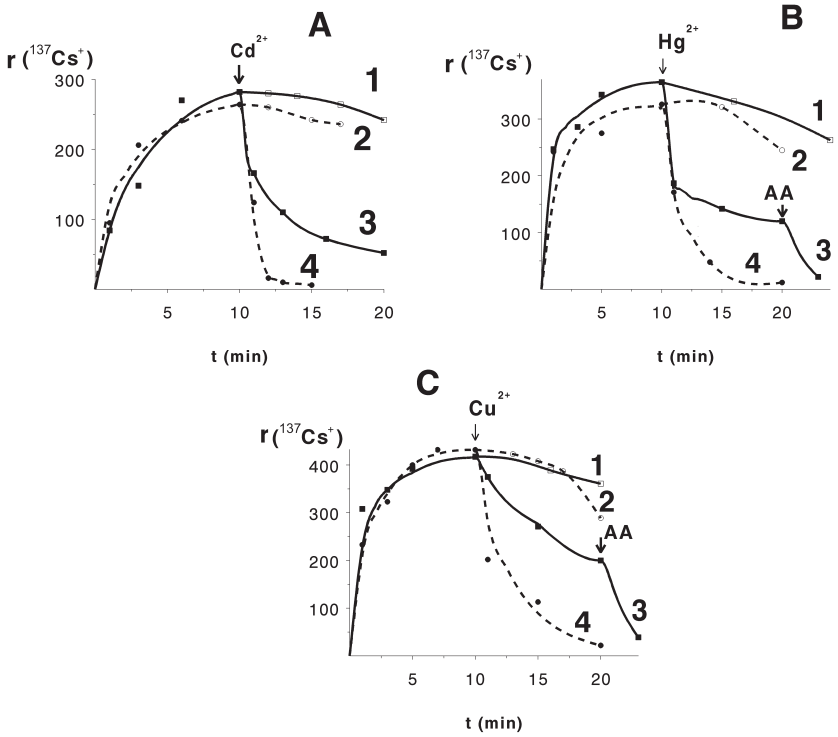

Figure 3. Action of heavy metals on $^{137}Cs^+$ distribution between respiring rat liver mitochondria and incubation medium.

The following additions were made: 1, none (control 1); 2, none (control 2); 3, 5 μM Cd^{2+} (A), 5 μM Hg^{2+} (B), 5 μM Cu^{2+} (C); 4, 10 μM Cd^{2+} (A), 10 μM Hg^{2+} (B), 10 μM Cu^{2+} (C). Antimycin A, AA, (1 μM) was administered where indicated by arrows.

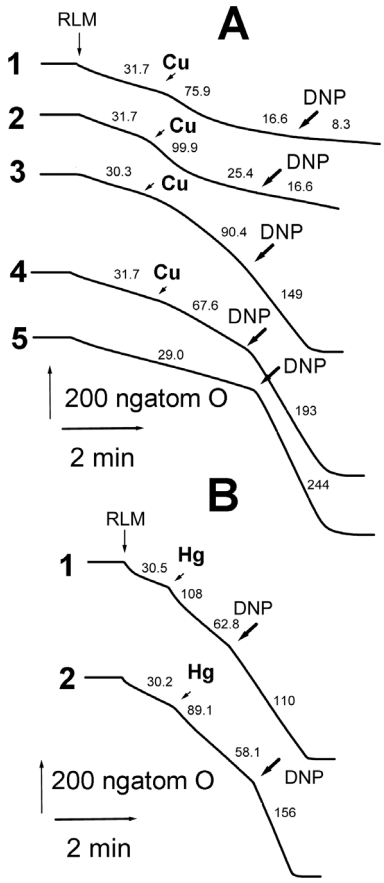

Figure 4. Influence of cyclosporin A (CsA) on the disturbance of rat liver mitochondrial respiratory function by heavy metals.

The following additions were made: (A) 1, 2 μM CsA plus 50 μM Cu^{2+}; 2, 50 μM Cu^{2+}; 3, 2 μM CsA plus 10 μM Cu^{2+}; 4, 10 μM Cu^{2+}; 5, none (control); (B) 1, 2 μM CsA plus 20 μM Hg^{2+}; 2, 20 μM Hg^{2+}.

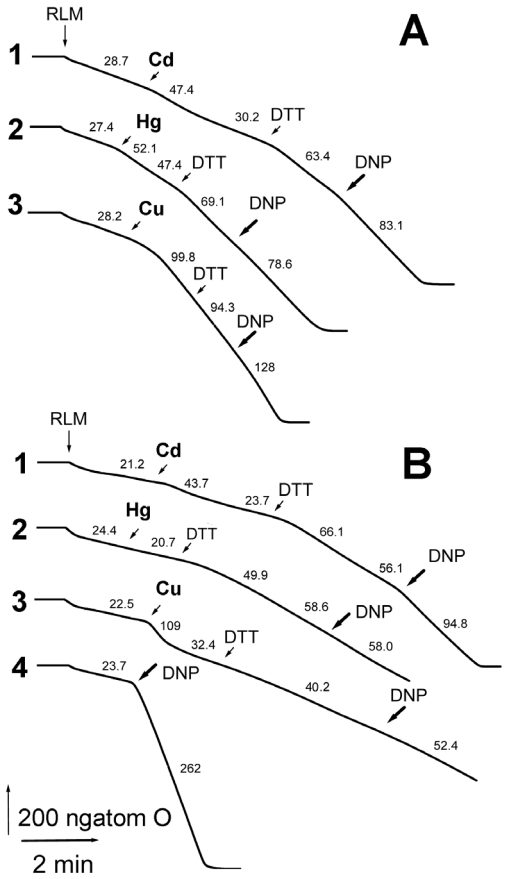

Figure 5. Effects of dithiothreitol on the heavy-metal disturbed rat liver mitochondrial respiratory function in the absence (A) or presence of CsA (B) in the medium.

The following additions were made: (A) 1, 20 μM Cd^{2+}; 2, 20 μM Hg^{2+}; 3, 20 μM Cu^{2+}; (B) 1, 50 μM Cd^{2+}; 2, 50 μM Hg^{2+}; 3, 50 μM Cu^{2+}; 4, none (control). DTT (150 μM) was administered where indicated by arrows. CsA (5 μM) was present, if not otherwise mentioned, from the start of the experiment.

CsA (not shown). Markedly, DTT, a dithiol reductant and MPT effector, produced an additional stimulatory effect on the basal respiration disturbed by the heavy metals only if it was supplemented after high [Cd^{2+}] and [Hg^{2+}], and the activation was insensitive to CsA (Fig. 5). Besides, the DNP-uncoupled respiration rate depressed by high [Hg^{2+}] and [Cu^{2+}] was not restored significantly by DTT administration either in the absence (Fig. 5A) or in the presence (Fig. 5B) of CsA in the assay medium. This is in opposite to the situation observed by us before in the case of Cd^{2+} (Belyaeva & Korotkov, 2003). Hence, both regulated and unregulated (i.e., Ca^{2+}-independent and insensitive to CsA and Mg^{2+}, see He & Lemasters, 2002) MPT pores contribute in the heavy-metal produced MMP but the involvement differs substantially depending on the metal used. This aspect of the toxicity of the heavy metals will be discussed elsewhere (Belyaeva *et al.,* in preparation).

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