Interaction of cadmium and copper ions with Complex I of the respiratory chain in rat liver mitochondria

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We have experimentally determined the effect of Cd^{2+} and Cu^{2+} ions on the activity of the respiratory subsystem in rat liver mitochondria oxidizing different substrates – succinate and glutamate + malate. The results showed that glutamate + malate oxidation is more sensitive to the toxic effect of Cd^{2+} than succinate oxidation, meanwhile Cu^{2+} ions inhibit the oxidation of both substrates to the same degree. This difference might be explained by a different interaction of Cd^{2+} and Cu^{2+} ions with Complex I in the respiratory chain. To elucidate this, we measured the activity of the Complex I by NADH oxidation in freeze-fractured mitochondria in the presence of various concentrations of Cd^{2+} and Cu^{2+} ions. The results showed that both metal ions inhibited Complex I, however, the inhibition of Complex I by Cu^{2+} ions was stronger than by Cd^{2+}. This finding does not make clear why Cd^{2+} is a more effective inhibitor of glutamate + malate than succinate oxidation, whereas the Cu^{2+}-induced inhibition is very similar in the case of both substrates. The reasons for this difference are located beyond Complex I and remain to be elucidated.

Key words: respiration, mitochondria, cadmium ions, copper ions, Complex I (NADH-ubiquinone oxidoreductase complex)

INTRODUCTION

Cadmium has been recognized as one of the most toxic environmental and industrian pollutants. It has a very long biological half-life (about 30 years in mammals), and slow excretion of Cd^{2+} contributes to Cd^{2+} accumulation in living cells, causing damages in the liver, kidneys, lungs and nervous system [5, 7]. Copper is an essential transition metal that serves as a prosthetic group in many important metalloenzymes [2]. It has a specific route of absorption and metabolism and is stored by the liver in the complex with protein metallothionein. However, the overload of Cu^{2+} provoked by various inherited defects in copper metabolism or by oral ingestion of Cu^{2+} salts causes hepatic disorders [6].

One of the cellular mechanisms for resistance to metal ion toxicity includes their sequestration within the organelles. Large amounts of various metal ions may be accumulated by mitochondria. Both Cd^{2+} and Cu^{2+} ions interact with important functional groups of a variety of enzymes in the matrix or in the inner mitochondrial membrane. It has been previously shown that Cd^{2+} directly inhibits succinate dehydrogenase and the respiratory chain in isolated liver mitochondria [4, 13]. We have demonstrated that both Cu^{2+} and Cd^{2+} cause a significant dysfunction of the respiratory subsystem of oxidative phosphorylation in rat liver mitochondria oxidizing succinate [1, 13]. In the present study, we compared the toxic effects of two metal ions on the mitochondrial respiratory chain in the case of oxidation of succinate and glutamate + malate. Glutamate + malate oxidation follows a more complex pathway, which supplies reducing equivalents to the respiratory chain mostly in the form of NADH, and therefore Complex I (NADH dehydrogenase or NADH–ubiquinone oxidoreductase complex) becomes involved in electron transport. Succinate oxidation proceeds via Complex II (succinate–ubiquinone oxidoreductase) which is regularly much less sensitive to toxic agents than Complex I. The aim of our experiments was to detect the possible effects of Cu^{2+} and Cd^{2+} on the activity of Complex I and to evaluate the contribution of Complex I to changes induced by the metal

ions in the overall flux through the respiratory subsystem.

MATERIALS AND METHODS

Mitochondria were isolated by a standard differential centrifugation procedure from the liver of male Wistar rats weighing 275–300 g. The isolation was performed in a cold room (0–4 °C). The animal was killed according to the rules defined by the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Licence N.0006). The liver was quickly removed and placed into ice-cold isotonic (0.9%) KCl solution. The tissue was cut into small pieces and homogenized with a glass-teflon homogeniser. The homogenisation medium contained 10 mM Tris-HCl, 250 mM sucrose, 3 mM EGTA and 4 mg/ml bovine serum albumine (BSA), pH 7.7 (at 2 °C). The homogenate was centrifuged at 750 × g for 5 min, and the supernatant was centrifuged at 7000 × g for 10 min. The mitochondrial pellet was suspended in a buffer containing 250 mM sucrose, 5 mM Tris-HCl, pH 7.3 (at 2 °C). The final centrifugation was done at 7000 × g for 10 min. The mitochondrial pellet was resuspended in the latter buffer to approximate the protein concentration of 50 mg/ml. During the experiment the mitochondrial preparation was stored on ice. Protein concentration was determined by the biuret method [9]. The BSA standard solution was used for quality control.

The respiration and membrane potential of mitochondria were measured in a closed, stirred and thermostated 1.5 ml vessel equipped with both by a Clark type oxygen electrode (Rank Brothers LTD, Cambridge, UK) and a tetraphenylphosphonium (TPP^+)-selective electrode (A. Zimkus, Vilnius University, Lithuania). The experiments were performed at 37 °C using 5 mM succinate (+2 μM rotenone) or 5 mM glutamate + 5 mM malate as substrate. Mitochondrial concentration in the probe was 1.0 mg/ml. The rate of Mitochondrial respiration corresponding to the rate in state 3 was registered after addition of 1 mM ATP to the incubation medium containing 110 mM KCl, 20 mM Tris-HCl, 5 mM KH_2PO_4, 50 mM creatine, excess of creatine kinase, 1 mM $MgCl_2$, pH 7.2. TPP^+ (133–266 nM) was added for the membrane potential measurements.

We estimated the dependence of the flux through the respiratory subsystem (J_R) on $\Delta\Psi$ by titrating mitochondrial respiration in state 3 with the inhibitor of phosphorylation system (oligomycine 5–25 ng/mg mitochondrial protein). The kinetic changes induced by Cd^{2+} and Cu^{2+} on the level of the respiratory subsystem were determined by modular kinetic analysis [10].

The activity of Complex I was measured spectrophotometrically following the kinetics of NADH reduction at 340 nm [12] in fractured mitochondria (by rapidly freezing-thawing liver mitochondria, repeated 4 times). Measurements were performed at 37 °C in the presence of 110 mM KCl, 20 mM Tris-HCl, 5 mM KH_2PO_4, 1 mM $MgCl_2$, antimycin A (1 μg/ml), 0.1 mg/ml NADH, fractured mitochondria (0.2 mg mitochondrial protein/ml), pH 7.2. Reaction was started after 3 min preincubation by adding 100 μg/ml CoQ. Enzymatic activity was calculated using the extinction coefficient of NADH 6.81 mM^{-1} cm^{-1}.

Statistical analysis. Data are obtained from 3–5 experiments carried out on differet mitochondrial preparations. The data points are expressed as mean (averaged for two or three repetitive runs) ± standard error of the mean (SEM). The effect of a toxic agent was analysed on the same mitochondrial preparations by comparing their functional parameters in the absence of Cd^{2+} and Cu^{2+} and in the presence of Cd^{2+} or Cu^{2+} (paired experiments). Statistical analysis of the data was done using Student's t test. Statistical significance was assumed at $p < 0.05$.

RESULTS

The effect of metal ions at a low concentration (up to 5 μM) which does not yet compromise the membrane permeability barrier nor mitochondrial swelling was studied in our experiments.

Our results show that respiration of liver mitochondria is less sensitive to Cd^{2+} ions when succinate is used as an oxidisable substrate than in the case of glutamate + malate oxidation which is one of the main physiological substrates for mitochondria in the liver. 3 μM Cd^{2+} inhibited the rate of succinate oxidation in state 3 by 15% but did not change the membrane potential. The obtained kinetic dependencies of J_R on the $\Delta\Psi$ (Fig. 1A) were not obviously shifted by this concentration of Cd^{2+}. A similar effect on glutamate oxidation in state 3 (inhibition by 18%) was obtained at a lower concentration of Cd^{2+} (1 μM). This concentration of Cd^{2+} significantly decreased $\Delta\Psi$ in state 3 (by 5 mV). The induced shift of kinetic dependencies demonstrates that the respiratory subsystem is clearly inhibited by 1 μM Cd^{2+} (Fig. 1B).

The effect of Cu^{2+} on the respiratory subsystem in state 3 was very similar for both substrates 5 μM Cu^{2+} inhibited the respiration rate by 16% (decreased $\Delta\Psi$ by 11 mV) with succinate and by 17% (dropped $\Delta\Psi$ by 8 mV) with glutamate + malate. The kinetic dependence of the respiratory subsystem on $\Delta\Psi$ (Fig. 2 A, B) was shifted by Cu^{2+} towards the lower rates of respiration in the entire

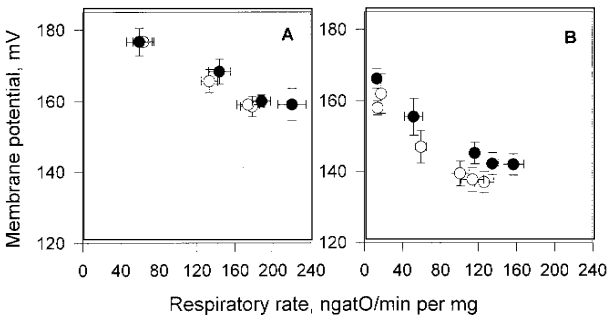

Fig. 1. Kinetic dependencies of the respiratory subsystem on the membrane potential ΔΨ in rat liver mitochondria. *A* – substrate – succinate (+ rotenone) (n = 4); ● – control, ❍ – +3 μM Cd^{2+}, *B* – substrate – glutamate + malate (n = 4); ● – control, ❍ – +1 μM Cd^{2+}

range of ΔΨ, indicating that Cu^{2+} inhibited the respiratory subsystem in both cases.

The different sensitivity of succinate and glutamate + malate oxidation in mitochondria to metal ions might be explained by the different interaction of these ions with Complex I in the respiratory chain. If the inhibition of Complex I by Cd^{2+} is much stronger than by Cu^{2+}, one might expect a more effective

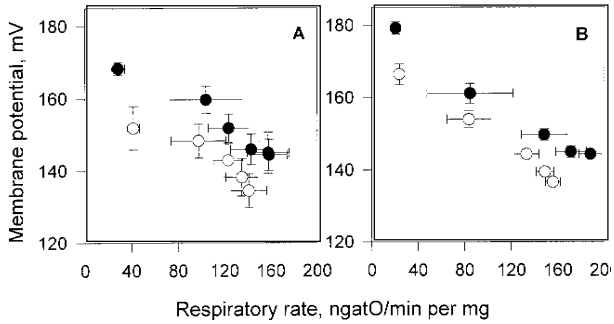

Fig. 2. Kinetic dependencies of the respiratory subsystem on the membrane potential ΔΨ in rat liver mitochondria. *A* – substrate – succinate (+ rotenone) (n = 4); ● – control, ❍ – +5 μM Cu^{2+}, *B* – substrate – glutamate + malate (n = 5); ● – control, ❍ – +5 μM Cu^{2+}

inhibition of NAD-dependent substrate oxidation by the former ion. To elucidate this, we performed the measurement of the activity of Complex I by NADH oxidation in freeze-fractured liver mitochondria in the absence of metal ions and in the presence of various concentrations of Cd^{2+} or Cu^{2+}. Our results showed that Complex I was inhibited by both metal ions (Fig. 3). The inhibition of NADH oxidation was dependent on Cd^{2+} concentration up to 10 μM (when maximal inhibition by 16% was reached) and did not increase further with increase in Cd^{2+} concentration (Fig. 3). The pattern of inhibition of Complex I by Cu^{2+} was different – the inhibition at the same concentrations of metal ion was stronger, it was dependent on Cu^{2+} concentration at least up to 15 μM and was progressively increasing with time during measurement, possibly indicating a slow irreversible binding of Cu^{2+} to Complex I.

Thus, the obtained findings do not make clear why Cd^{2+} is more a effective inhibitor of glutamate + malate than succinate oxidation, whereas Cu^{2+} inhibits oxidation of both substrates to the same degree. The reasons for this difference are located beyond Complex I, since, in contrast to the expected, Complex I appeared to be more sensitive to the toxic action of Cu^{2+} than to Cd^{2+}. The sites conferring a high sensitivity to the glutamate-oxidizing system to Cd^{2+} ions remain to be determined, but the most possible candidates for this role are glutamate dehydrogenase and the glutamate transport system operating in liver mitochondria.

However, the established fact that the activity of Complex I is inhibited by both toxic metal ions is an important new addition to the list of numerous mitochondrial processes affected by Cd^{2+} or Cu^{2+}. It has been previously shown that Cd^{2+} directly inhibits succinate dehydrogenase. H^+-ATPase [13], stimulates cytochrome bc_1 [6] and that Cu^{2+} inhibits phosphate transport, K^+ accumulation [2], increases membrane permeability to protons [2] and inhibits cytochrome bc_1 [6]. Thus, our study demonstrates the multi-site or pleiotropic nature of the effects of toxic pollutants on metabolic fluxes through the complex system of oxidative phosphorylation. The majority of physiological substrates in liver mitochondria are NAD-dependent and therefore their oxidation proceeds via Complex I. Therefore the revealed ability of Cd^{2+} and Cu^{2+} ions to inhibit Complex I is relevant for analysis of their toxic effects in the living cell.

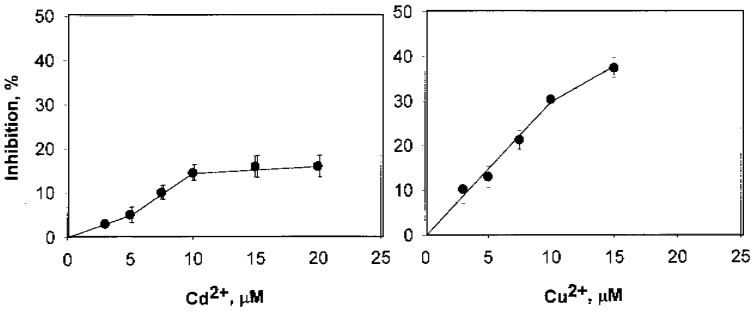

Fig. 3. Inhibition of NADH oxidation in freeze-fractioned liver mitochondria by Cd^{2+} (n = 3) and by Cu^{2+} (n = 4)

ACKNOWLEDGEMENT

This study was supported by the EC program "Copernicus" (contract No. ERBIC15 CT960307) and the Lithuanian Science and Studies Foundation.

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KADMIO IR VARIO JONŲ SĄVEIKA SU KVĖPAVIMO GRANDINĖS I KOMPLEKSU ŽIURKĖS KEPENŲ MITOCHONDRIJOSE

S a n t r a u k a

Eksperimentiškai nustatėme Cd^{2+} ir Cu^{2+} jonų poveikį kvėpavimo sistemos aktyvumui izoliuotose žiurkės kepenų mitochondrijose, oksiduojančiose skirtingus substratus – sukcinatą ir glutamatą + malatą. Rezultatai parodė, kad glutamato + malato oksidacija yra jautresnė toksiniam Cd^{2+} poveikiui nei sukcinato oksidacija, o Cu^{2+} abiejų substratų oksidaciją inhibavo vienodai. Norėdami nustatyti, ar šį poveikio skirtumą galima paaiškinti nevienoda Cd^{2+} ir Cu^{2+} sąveika su kvėpavimo grandinės I kompleksu, pagal NADH oksidacijos greitį šalčiu suardytose kepenų mitochondrijose įvertinome I komplekso aktyvumą, terpėje esant įvairioms metalų jonų koncentracijoms. Parodėme, kad abiejų metalų jonai inhibuoja kvėpavimo grandinės I komplekso aktyvumą, tačiau inhibicija buvo didesnė Cu^{2+} negu Cd^{2+} jonų atveju. Šie rezultatai neleidžia suprasti, kodėl Cd^{2+} inhibicijos laipsnis yra didesnis glutamato + malato oksidacijos atveju nei sukcinato, o Cu^{2+} sukelta inhibicija panaši abiejų substratų oksidacijos metu. Tokio skirtumo priežastys nesusijusios su I kompleksu, ir jiems nustatyti reikia papildomų tyrimų.