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Cu^{2+} but not Cd^{2+} ions at low concentrations increase mitochondrial inner membrane ion permeability by inducing reactive oxygen species production and lipid peroxidation

Jolita Čiapaitė[1], Zita Naučienė[1,2], Rasa Banienė[2], Odeta Buzaitė[1], Vida Mildažienė[1]*

[1] *Centre of Environmental Research, Faculty of Natural Sciences, Vytautas Magnus University, Kaunas, Lithuania*

[2] *Institute for Biomedical Research, Kaunas Medical University, Kaunas, Lithuania*

The integrity of the mitochondrial inner membrane is essential for efficient ATP production in mitochondria. In this study, we analyze the effect of heavy metal ions Cd^{2+} and Cu^{2+} at low concentrations on the mitochondrial inner membrane permeability and test the hypothesis that the mechanism of action of Cd^{2+} and Cu^{2+} ions is different due to the ability of the latter to undergo redox-cycling and consequently to stimulate the production of reactive oxygen species (ROS) and to induce lipid peroxidation. We show that 5 nmol Cu^{2+} per mg of protein but not 5 nmol Cd^{2+} per mg of protein increase membrane ion permeability in the whole range of the physiologically relevant membrane potential ($\Delta\psi$) values, especially at high $\Delta\psi$. Moreover, Cu^{2+} ions at this concentration enhance the formation of H_2O_2 in the mitochondrial matrix by 43% and induce lipid peroxidation as indicated by an increase in the amount of thiobarbituric acid reactive substances (TBARS) by 26%. Meanwhile, Cd^{2+} ions at the same concentration have no significant effect on H_2O_2 formation or lipid peroxidation. Only a higher amount of Cd^{2+} ions significantly stimulates the formation of H_2O_2. However, this is not accompanied by an increase in lipid peroxidation, which suggests that, in contrast to Cu^{2+}-induced ROS production, the mechanism of Cd^{2+}-induced ROS production most likely does not involve formation of the hydroxyl radical. In conclusion, we show that Cu^{2+} but not Cd^{2+} ions at a low concentration stimulate ROS production and induce accumulation of TBARS, suggesting that, indeed, Cu^{2+} ions increase membrane ion permeability by stimulating lipid peroxidation.

Key words: Cu^{2+} ions, Cd^{2+} ions, mitochondria, inner membrane ion permeability, reactive oxygen species, lipid peroxidation

Abbreviations
DCF-DA – 2',7'-dichlorofluorescin diacetate; DCPIP – 2,6-dichrophenolindophenol; MDA – malondialdehyde; ROS – reactive oxygen species; TBA – 2-thiobarbituric acid; TBARS – thiobarbituric acid reactive substances; $\Delta\psi$ – mitochondrial transmembrane electric potential difference.

INTRODUCTION

Copper is required for various cellular functions including energy production and the protection of cells from damage by reactive oxygen species (ROS)[a]. Under physiological conditions copper metabolism in the body is tightly regulated at the level of action of ceruloplasmin and metallothionein [1]. In turn, cadmium is not a normal constituent of the cell, although it occurs naturally in the environment. Despite the difference in the occurrence, excess intake of both copper and cadmium in the diet, with drinking water, by smoking or due to environmental contamination is toxic and carcinogenic [2] and may lead to accumulation of both metal ions in the liver and kidney, eventually impairing the function of these vital organs.

The mechanisms of cellular functional impairment by Cu^{2+} and Cd^{2+} ions share some common features. Both metal ions tend to accumulate in mitochondria and to interact with functional groups of enzymes (in particular with sulfhydryl groups) [3]. We have shown previously that Cu^{2+} and Cd^{2+} ions at low concentrations (5 μM) impair oxidative phosphorylation by inhibiting cytochrome c oxidase in isolated rat-liver mitochondria [4]. These ions at higher concentrations have been shown also to inhibit succinate dehydrogenase [5, 6]. Nevertheless, some essential differences in the mode of action of Cu^{2+} and Cd^{2+} ions can be anticipated due to inherent differences in the properties of these metal ions. Most importantly, by definition cadmium is not a transition metal and, unlike copper, cannot change its oxidation state. This renders cadmium to be less chemically reactive

* Correspondence to: Vida Mildažienė, Prof. Dr. Habil., Centre of Environmental Research, Faculty of Natural Sciences, Vytautas Magnus University, Vileikos 8, LT-44404 Kaunas, Lithuania. E-mail: v.mildaziene@gmf.vdu.lt

than copper. Instead, cadmium has been shown to interfere with cellular function by a more passive mechanism, i. e. by replacing metal ions with common oxidation state (+2), such as Mg^{2+}, Ca^{2+} and Zn^{2+} [7]. It has been demonstrated that Cd^{2+} ions are taken up into mitochondria by the Ca^{2+}-uniporter [8], whereas accumulation of Cu^{2+} ions in mitochondria proceeds via a different, energy-independent mechanism [9].

In contrast to cadmium, copper can obtain different oxidation states. The most common states are less stable copper (I) (Cu^{+}) or more stable copper (II) (Cu^{2+}). Due to the ability to change its oxidation states, copper can participate in the Haber–Weiss cycle / Fenton reaction similarly to iron when superoxide radicals ($O_2^{\bullet-}$) or H_2O_2 are present in the environment. This leads to formation of a highly reactive hydroxyl radical ($HO^{\bullet}$) [10] which can interact with biological macromolecules including membrane phospolipids.

Under physiological conditions, the inner mitochondrial membrane is almost impermeable to small charged molecules and ions. This property is essential for the maintenance of the electrochemical proton gradient generated by the mitochondrial respiratory chain which serves as the driving force of ATP synthesis by ATP synthase. Consequently, the alterations in membrane ion permeability are expected also to affect the efficiency of ATP production. The aim of this study was to compare the effects of Cu^{2+} and Cd^{2+} ions at low concentrations (5 μM) on the inner mitochondrial membrane ion permeability and to test the hypothesis that Cu^{2+} ions may impair mitochondrial function by inducing ROS formation followed by an increased lipid peroxidation.

MATERIALS AND METHODS

Materials

Rotenone, malonate, oligomycin, creatine, creatine phosphokinase, 2′,7′-dichlorofluorescin diacetate, 2-thiobarbituric acid, succinate, ATP were from Sigma-Aldrich. $CdCl_2$ and $CuCl_2$ were from Merck. Sucrose, KCl, Tris, KH_2PO_4 and $MgCl_2$ were from Roth.

Isolation of mitochondria

Mitochondria were isolated from the liver of male Wistar rats, using a standard differential centrifugation procedure as described in [11]. Protein was estimated by the Biuret method [12] using bovine serum albumin as the standard. Protein concentration in the mitochondrial suspension was 65–75 mg/ml.

Measurement of mitochondrial inner membrane ion permeability

The kinetics of mitochondrial inner membrane ion permeability was assessed in a closed, stirred and thermostated (37 °C) glass vessel equipped with a Clark-type oxygen electrode and a tetraphenylphosphonium ion-sensitive electrode as described in [13]. Briefly, mitochondria (1 mg/ml mitochondrial protein) supplemented with 5 mM succinate (+ 2 μM rotenone) as a respiratory substrate were incubated for 3 minutes with or without 5 μM of $CdCl_2$ (5 nmol Cd^{2+} per mg of protein) or 5 μM of $CuCl_2$ (5 nmol Cu^{2+} per mg of protein) in an assay medium containing 110 mM KCl, 20 mM Tris, 5 mM KH_2PO_4, 50 mM creatine, excess of creatine kinase, 2.3 mM $MgCl_2$, 1 μg oligomycin per mg of mitochondrial protein and 1 mM ATP, pH 7.2. During the incubation, the oxygen uptake rate and mitochondrial transmembrane electric potential difference ($\Delta\psi$) were measured simultaneously. The dependence of membrane ion permeability on $\Delta\psi$ was determined by titration of the oxygen uptake rate with malonate (inhibitor of succinate dehydrogenase) and simultaneous measurement of $\Delta\psi$ as described in [13].

Measurement of H_2O_2 production

Isolated mitochondria were incubated with 5 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA) for 30 min. After incubation, the mitochondria were suspended in a medium containing 250 mM sucrose, 5 mM Tris-HCl (pH 7.35) and centrifuged at 7300 g for 10 min at 4 °C, and the supernatant was removed. Next, mitochondria (1 mg/ml mitochondrial protein) supplemented with 5 mM succinate and different amounts of Cd^{2+} and Cu^{2+} (0, 5 and 10 nmol per mg of mitochondrial protein) were incubated at 37 °C in an assay medium containing 110 mM KCl, 20 mM Tris, 5 mM KH_2PO_4 and 2.3 mM MgCl2, pH 7.2. The oxidation of DCF was measured spectrofluorimetrically (λ_{ex} = 485 nm, λ_{em} = 535 nm) for 3 min using GENios Pro reader (Tecan). The DCF oxidation rate (corrected for changes in mitochondria-derived background fluorescence) was expressed as relative fluorescence units / min per mg of protein.

Evaluation of lipid peroxidation

Malondialdehyde (MDA), a product of lipid peroxidation, was determined from formation of thiobarbituric acid reactive substances (TBARS) using a 2-thiobarbituric acid (TBA) assay [14]. Briefly, after 3 min of incubation with different amounts of Cd^{2+} and Cu^{2+} ions (5 and 10 nmol per mg of protein) mitochondria were washed three times with fresh assay medium to remove sucrose which interferes with the TBA assay [14]. Then, 1 ml of 0.5% TBA in 20% trichloracetic acid was added and the mixture was incubated in a boiling water bath for 30 min. Samples were centrifuged at 10000 g for 10 min at 4 °C. The absorbance of a sample was measured at 532 nm and 600 nm (to correct for turbidity) and the amounts of TBARS formed (expressed as nmol TBARS per mg of protein) were calculated using the molar extinction coefficient $1.56 \times 105\ M^{-1} \cdot cm^{-1}$.

Data presentation and statistical analysis

Data were obtained from three to four experiments carried out on independent mitochondrial preparations. Data are expressed as means ± SEM. The statistical significance of Cd^{2+} and Cu^{2+} ion effects was evaluated using Student's t test (paired). The differences were assumed to be statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Cu^{2+} but not Cd^{2+} ions increase mitochondrial inner membrane ion permeability

In this study, we have assessed the effect of Cu^{2+} and Cd^{2+} ions at a low concentration that does not yet induce mitochondrial swelling. To evaluate the kinetics of membrame ion permeability, we determined the relationship of oxygen uptake and $\Delta\psi$ by taking mitochondria in state 4 (i. e. the state of no active ATP synthe-

sis, which is achieved by addition of the ATP synthase inhibitor olygomycin) and progressively limiting the supply of electrons to the respiratory chain by increasing the concentration of the succinate dehydrogenase inhibitor malonate [13]. In the absence of ATP synthesis, mitochondrial oxygen uptake is solely determined by membrame ion permeability – the more permeable the membrane, the higher oxygen uptake rate. Figure1 shows the effects of Cd^{2+} and Cu^{2+} ions at concentration of 5 nmol per mg of protein on the mitochondrial inner membrane ion permeability in the whole range of physiologically relevant $\Delta\psi$ values. It is evident that Cd^{2+} ions at this concentration had no significant effect on membrane ion permeability as indicated by similar oxygen uptake rates in the presence and in the absence of Cd^{2+} ions at any given $\Delta\psi$ value (Fig. 1A). A completely different situation emerges in case of Cu^{2+} ions. A significant increase in oxygen uptake rate was observed at any given $\Delta\psi$ value in the presence of Cu^{2+} ions at the same concentration of 5 nmol per mg of protein if compared to control (Fig. 1B). The increase of membrane permeability to ions was more prominent at higher $\Delta\psi$ values corresponding to state 4. In state 4, Cu^{2+} ions stimulated the oxygen uptake rate by 42% and caused a decrease in $\Delta\psi$ by 11 mV. The finding that Cu^{2+} ions decrease $\Delta\psi$, in combination with our previous finding that Cu^{2+} ions at the same concentration inhibit cytochrome c oxidase [4], suggests that stimulation of oxygen uptake rate is indeed caused by an increase of membrane ion permeability but not by a direct stimulation of cytochrome c oxidase by Cu^{2+} ions.

The integrity of the mitochondrial inner membrane is essential for an efficient production of ATP in the process of oxidative phosphorylation, and any increase in its permeability is expected to affect negatively the efficiency of ATP synthesis and consequently to impair the cellular function. Here it can be concluded that Cu^{2+} ions had a stronger ability to uncouple substrate oxidation from ATP synthesis than Cd^{2+} ions as indicated by an increased mitochondrial inner membrane ion permeability, especially at high $\Delta\psi$, i.e. in and near state 4 (Fig. 1). Our data are in agreement with earlier findings that Cu^{2+} ions effectively increase membrane ion permeability, whereas Cd^{2+} ions are much less effective. A comparable swelling of liver mitochondria was obtained at 5 μM Cd^{2+} and 40 μM Cu^{2+} ion concentrations [15]. It has been suggested that the ability of Cd^{2+} ions to increase membrane ion permeability depends on phosphate transport and increases with a higher phosphate concentration in the medium [16]. Therefore, uncoupling can be observed at low Cd^{2+} ion concentrations (< 5 μM) under certain experimental conditions [16, 17]. However, in the present study and as noted by other authors [18], uncoupling occurs only at Cd^{2+} ion concentrations exceeding 5 μM under conditions characterized by a low phosphate concentration.

Effects of Cd^{2+} and Cu^{2+} ions on H_2O_2 production and lipid peroxidation

We hypothesized that the much stronger stimulation of inner membrane ion permeability at high $\Delta\psi$ values by Cu^{2+} ions (Fig. 1B) as compared to Cd^{2+} ions (Fig. 1A) might be explained by the ability of the former to participate in the Haber–Weiss / Fenton reactions leading to formation of hydroxyl radical and a subsequent increase in lipid peroxidation causing a loss of membrane integrity. To test this hypothesis, firstly we have assessed how Cd^{2+} and Cu^{2+} ions affect the overall production of ROS in isolated mitochondria oxidizing succinate in state 2 (the state that is virtually identical to state 4, without active ATP synthesis, although it is achieved by different means). Figure 2A shows that at a concentration of 5 nmol per mg of protein (the concentration used for assessment of metal ion effects on membrane ion permeability) Cd^{2+} ions had no significant effect on the overall pro-

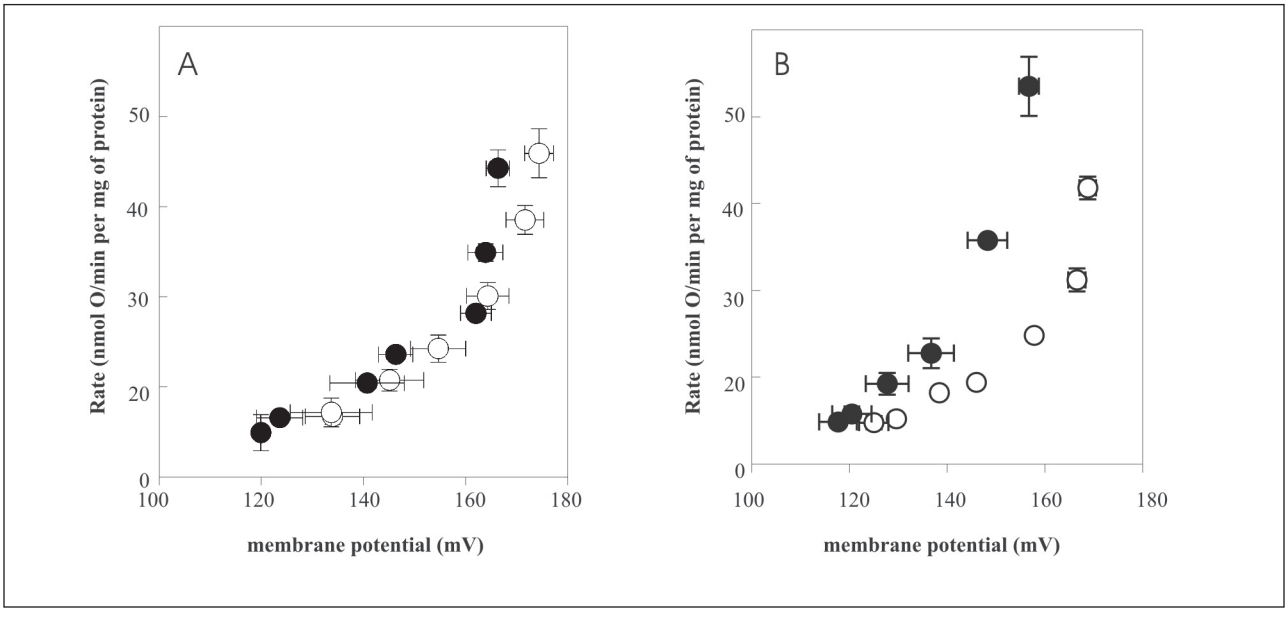

Fig. 1. The effect of Cd^{2+} (A) and Cu^{2+} (B) ions on mitochondrial inner membrane ion permeability in isolated rat-liver mitochondria respiring on succinate. Membrane ion permeability was measured as oxygen uptake rate when ATP synthesis is blocked by addition of excess oligomycin (1 μg per mg of mitochondrial protein). Different values of $\Delta\psi$ were achieved by adding increasing amounts of succinate dehydrogenase inhibitor malonate. Averages from n = 3 independent experiments ± SEM are presented. Empty symbols – control, filled symbols – 5 nmol of Cd^{2+} ions per mg of protein (A) and 5 nmol of Cu2+ ions per mg of protein (B)

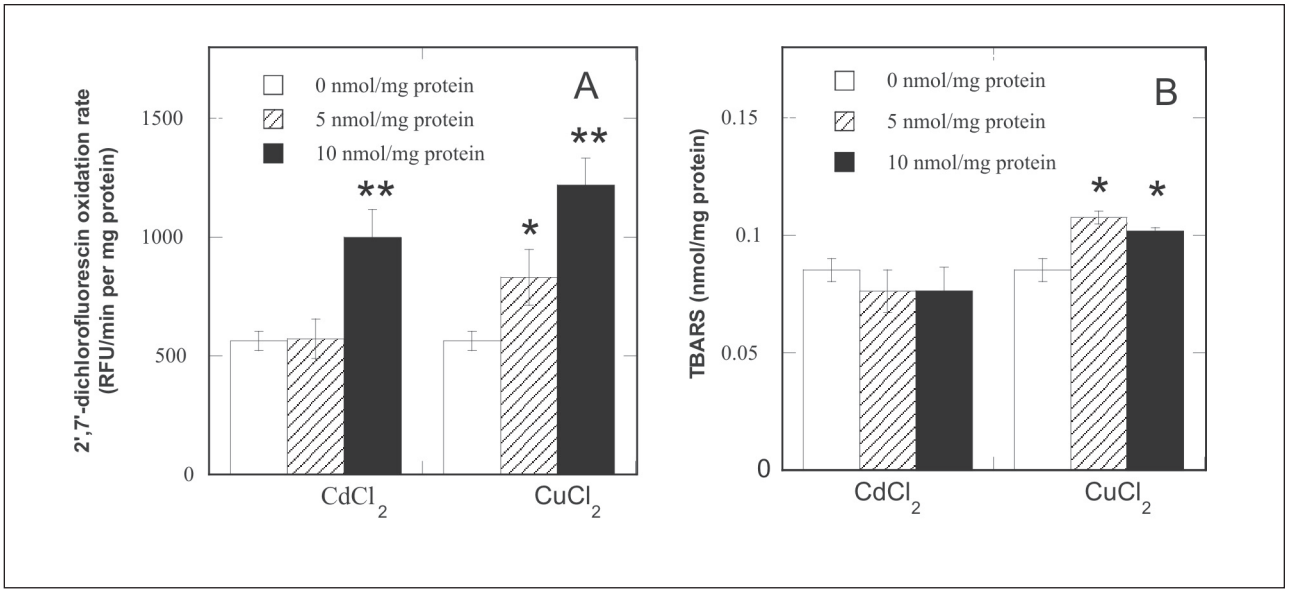

Fig. 2. The effect of Cu^{2+} and Cd^{2+} ions on H_2O_2 production (A) and lipid peroxidation (B) in isolated rat-liver mitochondria respiring on succinate Averages from n = 3 (A) and n = 4 (B) independent experiments ± SEM are presented. RFU, relative fluorescence units; TBARS, thiobarbituric acid reactive substances; * and **, statistically significant differences $p < 0.05$ and $p < 0.01$ vs. control (i.e. 0 nmol of Cu^{2+} or Cd^{2+} ions per mg of protein), respectively

duction of H_2O_2 as indicated by the unchanged oxidation rate of DCF. In turn, Cu^{2+} ions at the same concentration enhanced the oxidation rate of DCF by 43% (Fig. 2A). Increasing the content of Cd^{2+} and Cu^{2+} ions to 10 nmol per mg of protein resulted in a 1.7-fold ($p < 0.01$) and 2.1-fold ($p < 0.01$) higher DCF oxidation rate for Cd^{2+} and Cu^{2+} ions, respectively.

As the next step, we assessed the ability of both metal ions to induce lipid peroxidation in isolated liver mitochondria respiring on succinate in state 2. Figure 2B shows the effects of Cd^{2+} and Cu^{2+} ions on the formation of TBARS indicating the level of the lipid peroxidation product MDA. At the amounts tested (5 and 10 nmol per mg of protein), Cd^{2+} ions had no significant effect on the formation of TBARS. Meanwhile, Cu^{2+} ions at a concentration of 5 nmol per mg of protein significantly increased (by 26%) the amount of TBARS per mg of mitochondrial protein. Interestingly, increasing the amount of added Cu^{2+} ions to 10 nmol per mg of protein did not further increase the amount of TBARS formed (Fig. 2B).

To sum it up, we showed that Cu^{2+} ions but not Cd^{2+} ions at a concentration of 5 nmol per mg of protein stimulated the production of ROS and caused accumulation of TBARS, suggesting that, indeed, Cu^{2+} ions might increase membrane ion permeability by stimulating lipid peroxidation. Our results are in agreement with the observation that Cu^{2+} ions are much more potent inductors of lipid peroxidation than Cd^{2+} ions in intact hepatocytes [19]. Interestingly, although at higher levels (10 nmol per mg of protein) Cd^{2+} ions also stimulated ROS production, the process did not lead to an increased lipid peroxidation as indicated by a lack of accumulation of TBARS (Fig. 2B). This in turn indicated that the mechanism of Cd^{2+}-induced ROS production was different from that of Cu^{2+}-induced and most likely did not involve the formation of hydroxyl radical.

In other reports, it has been suggested that Cu^{2+}- and Cd^{2+}-induced stimulation of ROS production could be evoked by a number of different mechanisms including an increased production of ROS by the mitochondrial respiratory chain and a decreased activity of antioxidant enzymes [6, 19–22]. This suggests that the effect of Cd^{2+} ions observed in our experiment may stem from the interference of Cd^{2+} ions with antioxidant enzymes in the mitochondrial matrix rather than from the direct stimulation of ROS production by the electron transfer chain in the mitochondrial inner membrane. Thus our data support the hypothesis that the cytotoxicity of copper, similarly to other transition state metals, primarily stems from its ability to undergo redox-cycling reactions and to cause generation of the superoxide anion radical and the hydroxyl radical in mitochondria, microsomes and peroxisomes, while the primary drive for toxicity of cadmium has been proposed to be the depletion of glutathione and binding of Cd^{2+} ions to sulfhydryl groups of proteins [23].

However, it should be noted that a weaker effect of Cd^{2+} ions on the mitochondrial inner membrane ion permeability, which we observed in our experiment could have been determined to some extent by a lower concentration of free Cd^{2+} ions in the assay medium compared to that of Cu^{2+} ions due to a stronger binding of the former to mitochondrial proteins.

CONCLUSIONS

Our data indicate that at low concentrations Cu^{2+} ions but not Cd^{2+} ions increase mitochondrial inner membrane ion permeability by inducing ROS production and lipid peroxidation. Cd^{2+} ions stimulate ROS production only at a much higher concentration and the mechanism appears to be different from that of Cu^{2+} ions, possibly not involving the formation of hydroxyl radical, since increased ROS formation does not result in increased lipid peroxidation.

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CU^{2+}, BET NE CD^{2+} JONAI, ESANT MAŽAI JŲ KONCENTRACIJAI, DIDINA MITOCHONDRIJŲ VIDINĖS MEMBRANOS LAIDUMĄ JONAMS SKATINDAMI REAKTYVIŲ DEGUONIES FORMŲ SUSIDARYMĄ IR LIPIDŲ PEROKSIDACIJĄ

Santrauka

Vidinės mitochondrijų membranos vientisumas yra svarbus ATP sintezės našumui mitochondrijose. Šiame darbe ištirtas mažos koncentracijos sunkiųjų metalų Cd^{2+} ir Cu^{2+} jonų poveikis vidinės mitochondrijų membranos pralaidumui ir nustatyta, kad šis poveikis yra skirtingas. Viena iš priežasčių yra ta, kad Cu^{2+} jonai gali būti įvairaus oksidacijos laipsnio, todėl jie gali sukelti reaktyvių deguonies formų susidarymą ir lipidų peroksidaciją. Nustatyta, kad esant 5 nmol/mg baltymo Cu^{2+} jonų koncentracijai, padidėja vidinės mitochondrijų membranos laidumas jonams, kai yra fiziologinės membranos potencialo reikšmės. Tokia pati Cd^{2+} jonų koncentracija membranos laidumo neveikė. Cu^{2+} jonai 43% stimuliavo H_2O_2 susidarymą mitochondrijų užpilde ir sukėlė lipidų peroksidaciją – su tiobarbitūrine rūgštimi reaguojančių produktų (TBARS) kiekis padidėjo 26%. Cd^{2+} jonai statistiškai reikšmingai didino H_2O_2 susidarymą tik esant didesnei jų koncentracijai (10 nmol/mg baltymo), tačiau tai neskatino lipidų peroksidacijos. Taigi Cd^{2+} jonų sukeltas reaktyvių deguonies formų susidarymo mechanizmas ir poveikis vidinei mitochondrijų membranai skiriasi nuo Cu^{2+} jonų. Tai gali būti aiškinama tuo, jog veikiant Cd^{2+} jonams nesusidaro hidroksilo radikalas. Apibendrinant nustatyta, kad Cu^{2+}, bet ne Cd^{2+} jonai, esant mažoms jų koncentracijosms, skatina reaktyvių deguonies formų ir TBARS susidarymą, o tai savo ruožtu nulemia didesnį membranos pralaidumą jonams.