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Original Contribution

CADMIUM INHIBITS THE ELECTRON TRANSFER CHAIN AND INDUCES REACTIVE OXYGEN SPECIES

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Abstract—Recent research indicates that cadmium (Cd) induces oxidative damage in cells; however, the mechanism of the oxidative stress induced by this metal is unclear. We investigated the effects of Cd on the individual complexes of the electron transfer chain (ETC) and on the stimulation of reactive oxygen species (ROS) production in mitochondria. The activity of complexes II (succinate:ubiquinone oxidoreductase) and III (ubiquinol:cytochrome *c* oxidoreductase) of mitochondrial ETC from liver, brain, and heart showed greater inhibition by Cd than the other complexes. Cd stimulated ROS production in the mitochondria of all three tissues mentioned above. The effect of various electron donors (NADH, succinate, and 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol) on ROS production was tested separately in the presence and in the absence of Cd. ESR showed that complex III might be the only site of ROS production induced by Cd. The results of kinetic studies and electron turnover experiments suggest that Cd may bind between semiubiquinone and cytochrome b_{566} of the Q_0 site of cytochrome *b* of complex III, resulting in accumulation of semiubiquinones at the Q_0 site. The semiubiquinones, being unstable, are prone to transfer one electron to molecular oxygen to form superoxide, providing a possible mechanism for Cd-induced generation of ROS in mitochondria. Published by Elsevier Inc.

Keywords—Cadmium, Electron transfer chain, Reactive oxygen species, Mitochondria, Complex III, Q cycle, Free radicals

INTRODUCTION

Cadmium (Cd) is a relatively abundant nonessential element that is widely used as a color pigment in paints, in electroplating and galvanizing, and in batteries. It is also a by-product of zinc and lead mining and smelting [1]. Cd is found in foods (vegetables, grains, and cereals), water, and tobacco leaves. Cd accumulates unevenly in human tissues, and is concentrated primarily in lungs, liver, kidneys, brain, heart, and testes [1,2].

A variety of mechanisms have been attributed to Cd-induced toxicity. Cd interferes with the intracellular signaling network and gene regulation at multiple levels [3]. It has been reported that Cd induces alterations in activities of antioxidant enzymes such as superoxide dismutase [4] and catalase [5]. Cd is a highly toxic metal, in particular, it was reported to produce cardiotoxicity [6], hepatotoxicity [7], as well as neuropathological and neurochemical alterations in the central nervous system resulting in irritability and hyperactivity [8,9]. Lipid peroxidation is also associated with Cd toxicity [5]. In addition, Cd has been implicated in tumorigenesis [10].

There is an increasing body of evidence that the toxicity of Cd may be associated with the production of reactive oxygen species (ROS) [11]. It has also been found that Cd induces oxidative stress in cultured human cells [1,12]. Although formally Cd belongs to the group of transition elements, it almost always adopts only one oxidation state, which is 2+. Thus, in most chemical reactions it behaves similarly to main-group metals. In particular, it does not induce production of ROS through a Fenton-like reaction [13]. The mechanism by which Cd induces ROS formation is not yet known [14]. Previous studies have indicated

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that treatment of cells with Cd results in specific mitochondrial alterations [15–18]. Cd exposure also leads to mitochondrial dysfunction in the renal cortex of rats [19].

Mitochondria are the major source of ROS production in cells. The ROS produced include the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$) [20,21]. About 1–4% of total mitochondrial oxygen consumed is incompletely reduced and leads to the production of ROS [22,23]. Experimental evidence indicates that the impairment of electron transfer through NADH:ubiquinone oxidoreductase (complex I) and ubiquinol:cytochrome *c* oxidoreductase (complex III) may induce superoxide formation [20,21].

Mitochondrial production of ROS is thought to play an adverse role in many pathologic states of organs, including heart and brain [24,25], and in apoptotic cell death [26,27]. Oxidative stress induced by Cd can negatively affect DNA, RNA, and ribosome synthesis, and inactivate enzyme systems [28–30]. Understanding of the mechanism of ROS production by Cd is therefore important for adequate risk assessment of Cd exposures from both the occupational and environmental health perspectives. It could also help in the development of new strategies for the prevention of Cd toxicity.

In the present work, we investigated the inhibitory properties of Cd with respect to individual complexes of ETC in mitochondria of guinea pig liver, brain, and heart. Exposure to Cd resulted in ROS production in mitochondria from the above three tissues. The complexes studied were complex I, complex II (succinate:ubiquinone oxidoreductase), complex III, complex IV (cytochrome *c* oxidase), and complex V (ATP synthase). Our results show that complex II and complex III are more sensitive to Cd than complexes I, IV, and V, in all three tissues tested. Using substrates specific for each complex, we demonstrated that Cd could induce ROS generation in only complex III. Kinetic studies indicated that Cd might bind to the Q_o site of complex III, between the semiubiquinone and heme b_{566}, which is different from the binding site of myxothiazol [31,32]. Possibly this binding prevents electron delivery from semiubiquinone to heme b_{566}, and promotes the accumulation of semiubiquinone at the Q_o site. The accumulated semiubiquinones are unstable and prone to donation of electrons to molecular oxygen, thus forming superoxide anion. These findings contribute to the explanation of the mechanism by which Cd induces the formation of ROS in cells.

MATERIALS AND METHODS

Materials

Horse heart cytochrome *c* (type IV), antimycin A, DB (2, 3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone), DCIP (dicholorophenolindophenol), DMPO (5, 5-dimethyl-L-pyrroline *N*-oxide), DPPH (1,1-diphenyl-2-picrylhydrazyl), $CdCl_2$, and other chemicals used in this work are all reagent grade and were purchased from Sigma Company (St. Louis, MO, USA). Myxothiazol and dodecyl maltoside (DM) were from Fluka Chemical Corporation (Milwaucee, WI, USA) and Anatrace Inc. (Maumee, OH, USA), respectively. DBH_2 is reduced DB. The spin trap DMPO was purified by charcoal adsorption and vacuum distillation.

Animals used

These studies were conducted in facilities accredited fully by the Association for the Assessment and Accreditation of Laboratory Animal Care International and were approved by the institutional Animal Care and Use Committee. Male Dunkin–Hartley guinea pigs (600–700 g) were used. The guinea pigs were sacrificed according to a standard protocol. The animals were first anesthetized intraperitoneally (i.p.) with sodium pentobarbital (65 mg/kg). Once it was determined that animals were unresponsive to any stimuli, they were exsanguinated by severing the abdominal aorta. The liver, brain, and heart were recovered.

Preparation of mitochondria

The liver, brain, and heart mitochondria of guinea pig were isolated as described previously [19,33]. The liver, brain, and heart were minced over ice and rinsed twice with ice-cold isolation buffer (30 mM Tris–HCl, 250 mM mannitol, 100 mM KCl, and protease inhibitors, pH 7.0). The washed tissues were homogenized in ice-cold buffer at a ratio of 10% (w/v), and centrifuged at 600*g* for 10 min. The supernatant was collected and centrifuged at 15,000*g* for 30 min. The resulting pellet containing the mitochondria was washed twice with the isolation buffer. Finally, the washed pellet was suspended in isolation buffer and stored at −80°C.

Enzyme assays

Activities of complex I and complex III were determined as described previously [33,34]. Complex II activity was assayed in 30 mM phosphate, 100 mM KCl, 2 mM KCN, 50 μM DCIP, 200 μM succinate, and 0.1% DM, pH 7.0. The reduction of DCIP was monitored at 605 nm. Complex IV activity was determined in 30 mM phosphate, 100 mM KCl, 6 μM ferrocytochrome c, and 0.1% DM, pH 7.0. The reaction was monitored at 550 nm [35]. All reactions were monitored either in a Cary 50 Bio UV-spectrophotometer or in a UV-2401 PC UV-VIS recording spectrophotometer at room temperature.

Reversible binding of Cd to mitochondria

The enzyme activity of complex III of liver mitochondria was tested as described above. In a 2 ml reaction system, 0.8 mg mitochondrial protein was mixed with PBS reaction buffer containing 6 μM cytochrome *c*. The reaction was started by adding 20 μM DBH_2; then 10 μM $CdCl_2$, 2 mM EDTA, and 5 μM antimycin A were added one after another under stirring and the cytochrome *c* reduction was recorded.

Identification of the inhibitory site of Cd in complex III

Cytochrome *c* binding domain determination was performed in 2 ml of an assay mixture containing 30 mM phosphate, 100 mM KCl, 2 mM KCN, and 0.1% DM, pH 7.0. The final concentration of the electron donor DBH_2 ranged from 20 to 400 μM. The final concentration of the mitochondrial protein was 13.7 mg/ml. The reaction was started with addition of cytochrome *c*.

DBH_2 binding determination was done in the same reaction system as described above. The final concentration of DBH_2 was 20 μM. The reaction was started with addition of DBH_2. The concentration range of cytochrome *c* selected was 5 to 40 μM.

Michaelis–Menten enzyme kinetics of complex III for DBH_2 and cytochrome *c* was measured separately, at Cd concentrations of 1 and 4 μM. The data were plotted as Eddie–Hofstee plots for DBH_2 and cytochrome *c*.

Cytochrome reduction assay

Reduction of cytochromes b_{566}, b_{562}, and c_1 was assayed in 50 mM Tris–HCl (pH 7.4) containing 100 mM KCl, 0.1 % DM, 1.5 mg/ml mitochondrial protein, and specific inhibitors of complex III (5 μM antimycin A or 5 μM myxothiazol). The reaction was initiated by addition of 20 mM succinate and was monitored at 566 nm for cytochrome b_{566}, 562 nm for cytochrome b_{562}, and 520 nm for cytochrome c_1 respectively [31,36].

Free radical measurements with electron spin resonance

ESR spin trapping was used to detect short-lived free radical intermediates as described before [37]. This technique involves the addition-type reaction of a short-lived radical with a paramagnetic compound (spin trap) to form a relatively long-lived free radical product (spin adduct), which can then be studied using conventional ESR. The intensity of the signal is used to measure the amount of short-lived radicals trapped, and the hyperfine couplings of the spin adduct are generally characteristic of the original trapped radicals. Spin trapping is the method of choice for detection and identification of free radical generation due to its specificity and sensitivity. All ESR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments Inc., Billerica, MA, USA) and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and DPPH as reference standards. The relative radical concentration was estimated by multiplying half of the peak height by $(\Delta H_{pp})^2$, where ΔH_{pp} represents peak-to peak width. An Acquisitive program (Bruker Instruments Inc.) was used for data acquisitions and analyses. Mitochondrial protein (the amount in the reaction is indicated in the figure legend) was suspended in PBS containing 0.1% DM, 5 mM glutamate/malate, 100 μM NADH, and 100 mM succinate. In the presence or absence of Cd (10 μM), DMPO (200 mM) was added in test tubes to a final volume of 1.0 ml. The reaction mixture was then transferred to a flat cell for ESR measurement. The scavenger experiments were performed in the presence of SOD and Cd. The final concentration of SOD was 0.5 mg/ml. The concentrations in the reaction mixture given in the figure legends are final concentrations. Experiments were performed at room temperature under ambient air. Protein concentration was determined by the method of Lowry et al. [38].

Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by Student's *t* test at a significance level of $p < .05$.

RESULTS

Effects of Cd on complexes of ETC

The effects of Cd on the enzyme activities in four complexes of ETC are shown in Fig. 1. Generally, complexes II and III from mitochondria of liver, brain, and heart are more sensitive to Cd than complexes I and IV. Maximum inhibition was 50–60% for complex II and 30–77% for complex III, respectively. Maximum inhibition of complexes I and IV was only 22–30% and 16–20%, respectively, much lower than those of complexes II and III.

In liver mitochondria, the maximum inhibition for complexes II and III was achieved in the presence of 20 μM $CdCl_2$ and the enzyme activities of complex II and III were inhibited by 58 and 75%, respectively (Fig. 1A). Maximum inhibition of complexes I and IV was only 24 and 16%, respectively. The inhibition of enzyme activity of complexes of ETC of brain mitochondria by Cd showed a profile similar to that of liver mitochondria. The activities of complexes II and III were more inhibited on exposure to Cd; however, the enzymatic activity of neither complex I nor complex IV was significantly inhibited by Cd (Fig. 1B). ETC complexes of heart

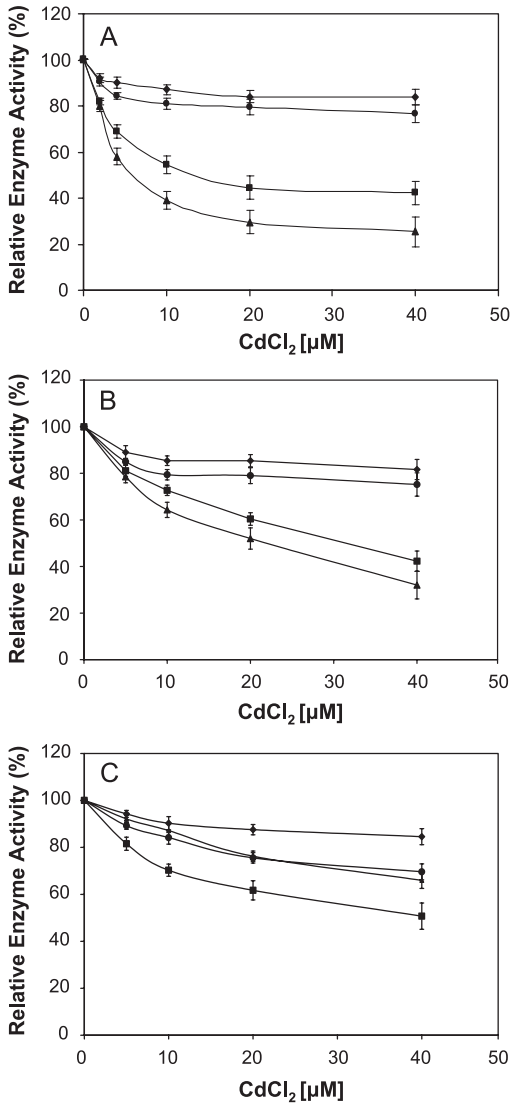

Fig. 1. Inhibition of enzyme activities of complexes I–IV of mitochondria separated from liver (A), brain (B), and heart (C) by Cd. Enzyme assays for complexes I to IV are described under Materials and Methods. The reaction volume was 1 ml containing 0.8 mg mitochondrial protein. The mitochondria were preincubated with Cd for 10 min at room temperature before enzyme activity determinations. (●) Complex I. (■) Complex II. (▲) Complex III. (♦) Complex IV. Data represent means ± SD ($n \geq 4$).

mitochondria were less sensitive to Cd compared with ETC complexes of liver and brain mitochondria (Fig. 1C). In heart mitochondria, complex II was the most inhibited enzyme, being inhibited by 50%. The enzyme activities of complexes I, III, and IV were inhibited by 30, 30, and 20%, respectively.

Inhibitory effect of Cd on complex III is reversible

To examine whether the inhibition of complex III by Cd is reversible, EDTA was added to liver mitochondria in the presence of Cd. As indicated in Fig. 2, addition of EDTA reversed the inhibitory effect of Cd on complex III. More than 90% of the enzyme activity was recovered by adding 2 mM EDTA to the mitochondria that were pretreated with 10 μM $CdCl_2$. The reaction can be completely inhibited by adding antimycin A as shown in Fig. 2, indicating that the cytochrome *c* reduction is due to the electron transfer through complex III.

Identification of the inhibitory site of Cd on complex III

Complex III of ETC is present in the mitochondrial inner membrane and catalyzes electron transport from ubiquinol (QH_2) to cytochrome *c*, coupling the translocation of protons across the membrane [39]. The complex contains a ubiquinol (electron donor) binding site and a cytochrome *c* (electron acceptor) binding site. To understand the mechanisms of Cd inhibition of complex III, we tested whether Cd interferes with the binding of the electron donor hydroubiquinone (DBH_2) and the electron acceptor cytochrome *c*. The enzyme kinetics of complex III for DBH_2 and cytochrome *c* were measured at two concentrations of Cd, 1 and 4 μM, and the results were represented by Eddie–Hofstee plots. The parallel lines for DBH_2 and cytochrome *c* indicate that K_m values of the enzyme were unchanged in the presence of Cd (Figs. 3A and 3B). This suggests that Cd does not interfere with binding of the substrate, DBH_2 and cytochrome *c*, to the enzyme, i.e., that binding of Cd to complex III is noncompetitive.

Inhibition of single turnover of cytochrome reduction

To learn whether Cd is involved in the Q_i site (ubiquinone-reducing site) or the Q_o site (ubiquinol-oxidizing site) of complex III, the binding position of Cd was explored. The accepted electron transfer pathway of Q cycle in complex III is described in Fig. 4. The

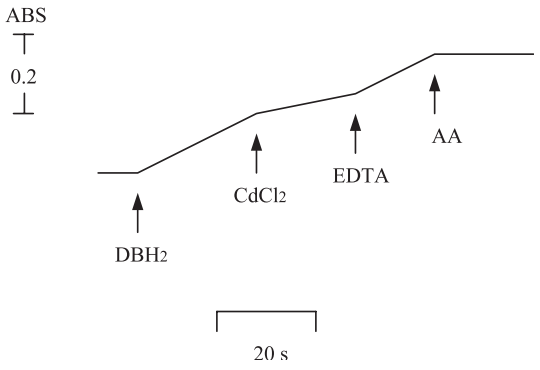

Fig. 2. Reversible inhibition of complex III by Cd. Enzyme activity of complex III was determined by monitoring the cytochrome *c* reduction at 550 nm using DBH_2 as substrate as described previously [31,32]. The reaction was started by adding 20 μM DBH_2. Inhibition of cytochrome *c* was obtained by adding $CdCl_2$ (10 μM). Addition of EDTA (2 mM) restored the activity, and addition of 5 μM of antimycin A (AA) completely blocked the reduction of cytochrome *c*.

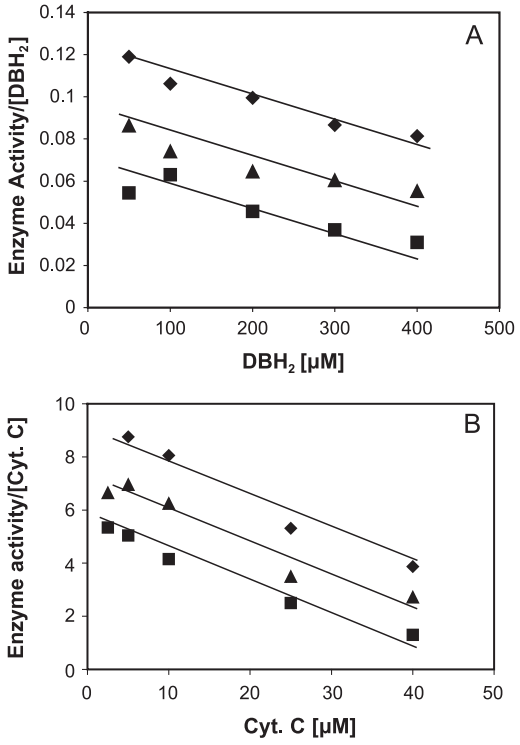

Fig. 3. Eddie–Hofstee plots of complex III of liver mitochondria. (A) The enzyme activity of complex III was determined in the presence of 0.8 mg mitochondrial protein and 12 μM cytochrome *c* at various concentrations of DBH_2. (♦) No $CdCl_2$ was added. (▲) 1 μM $CdCl_2$. (■) 4 μM $CdCl_2$. (B) Activity of complex III was determined at a fixed concentration of DBH_2 (20 μM) and various concentrations of cytochrome *c*. (♦) No $CdCl_2$. (▲) 1 μM $CdCl_2$. (■) 4 μM $CdCl_2$.

experimental approach involved measuring the effects of Cd on the reduction rates of cytochromes b_{566}, b_{562}, and c_1 in the presence of the specific inhibitors antimycin A (Q_i site inhibitor) and myxothiazol (Q_o site inhibitor) [40]. The results indicate that preincubation of 10 μM Cd with mitochondria markedly decreased the reduction rates of cytochromes b_{566}, b_{562}, and c_1 in the presence of antimycin A (Fig. 5). When myxothiazol was used instead of antimycin A, Cd had no effect on the reduction rates of cytochromes b_{566}, b_{562}, and c_1 (data not shown). These data suggest that Cd binds in the vicinity of the Q_o site of complex III.

To gain more evidence that Cd binds to the Q_o site of complex III, the Cd-dependent inhibition of complex III was determined in the presence of excess zinc (Zn). Zn has been shown to bind to the Q_o site of complex III and inhibit electron transfer activity [41]. If Cd binds to the same site as Zn does, competition should occur at the binding site of complex III. Double reciprocal plots for Zn inhibition of cytochrome *c* reductase activity in the absence and presence of Cd show that the V_{max} values were not changed, indicating competition between Cd and Zn for the same binding site in complex III (Fig. 6). Taken together, our results suggest that Cd binds to the Q_o site of complex III in the same mode as Zn.

ESR results

It has been reported that impairment of the enzyme activities of complexes I and III is the main source of ROS generation in mitochondria. Because enzyme activ-

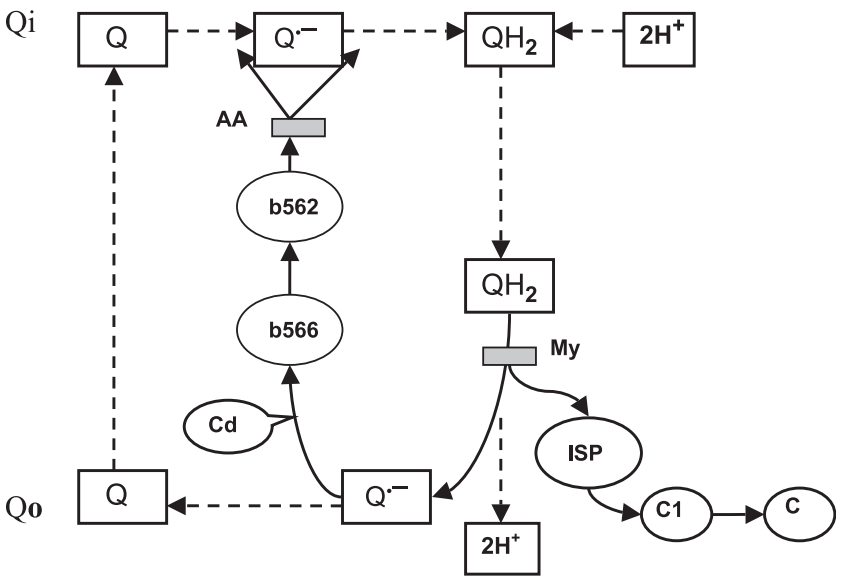

Fig. 4. Hypothesized protonmotive Q-cycle path of electrons from ubiquinol to cytochrome *c* through the redox prosthetic groups of complex III. Dashed lines show molecular movement. Electron transfers are shown by solid arrows. Q_i represents the negative side of the membrane (center N), where the ubiquinone is reduced. Q_o represents the positive side of the membrane (center P), where the ubiquinol is oxidized. The possible binding position of Cd is located between b_{566} and $Q^{\bullet-}$ of the Q_o site. AA, antimycin A; My, myxothiazol. Solid rectangles show the inhibitory locations of antimycin A and myxothiazol.

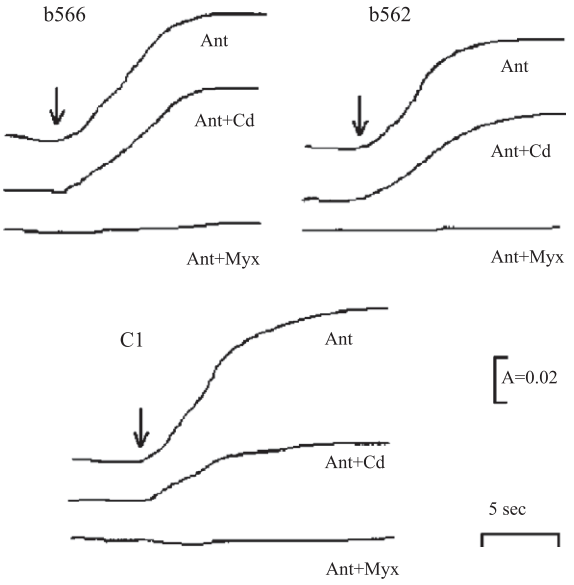

Fig. 5. Effect of Cd on antimycin-insensitive pre-steady-state reduction kinetics of cytochromes b_{566}, b_{562}, and c_1. The reaction mixture consisted of 50 mM Tris–HCl (pH 7.4), 100 mM KCl, 0.1 % DM, 1.5 mg/ml mitochondrial protein, and specific inhibitors of complex III (5 μM antimycin A or 5 μM myxothiazol). Reduction of cytochrome *b* and c_1 was started by adding 20 μM DBH_2 (final concentration) with stirring. Reduction of cytochromes b_{566}, b_{562} and c_1 was measured at 566, 562, and 520 nm, respectively. Ant, presence of 5 μM antimycin A only; Ant +Cd; 5 μM antimycin A and 10 μM Cd; Ant+Myx, 5 μM antimycin A and 5 μM myxothiazol. The arrows indicate the addition of DBH_2 to start the reaction.

ity of complex III is significantly inhibited by Cd, we hypothesized that inhibition of electron transfer of ETC by Cd might induce ROS formation. To test this, mitochondria from liver, brain, and heart were incubated with Cd. ESR measurements were taken within 3 min of incubation of mitochondria with or without Cd. Fig. 7 shows the ESR spectra of mitochondria from the liver, brain, and heart. These spectra consist of a 1:2:2:1 quartet with hyperfine coupling of $a_H = a_N = 14.9$ G. Based on these splitting constants and the lineshape, the 1:2:2:1 quartets were assigned to a DMPO/•OH adduct. As DMPO/•OH could, in principle, arise from many sources other than •OH trapping, we performed a competition experiment using sodium formate as an •OH radical scavenger and as a source of a secondary radical to verify the presence of •OH radicals. In this competition experiment, •OH radical abstracts a hydrogen atom from formate to form a new radical, which can be trapped by DMPO to generate a new spin adduct signal. As expected, addition of formate decreased the intensity of the DMPO/•OH^- adduct signal and resulted in the appearance of a new spin adduct signal with hyperfine splittings of a_H = 15.8 G and a_N = 18.8 G. These splittings are typical of those of a DMPO/•COO^- adduct, demonstrating that the •OH radicals are indeed generated. It can be seen that the Cd-treated mitochondria from liver, brain, and heart all generated stronger ESR signals than the untreated mitochondria, indicating that the addition of Cd induced ROS generation. The intensities of ESR signals increased by 68.6, 64.6, and 37.3% for mitochondria of liver, brain, and heart, respectively, in the presence of Cd. This is inversely related to the enzyme activity inhibition rate that was shown in Fig. 1. The ESR signals induced by Cd in the three tissues tested were

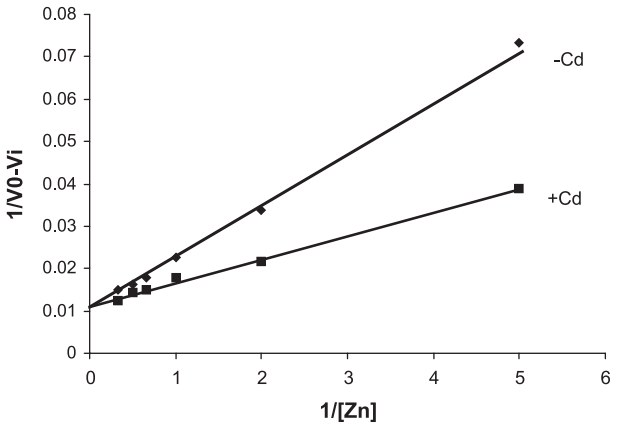

Fig. 6. Double-reciprocal plots of inhibition of complex III by Zn and Cd. Cytochrome *c* reduction activity was evaluated in the absence (◆) and presence (■) of 4 μM $CdCl_2$. The reaction conditions were as described under Materials and Methods. The final concentration of the electron donor DBH_2 was 20 μM, and that of cytochrome *c*, the electron acceptor, was 20 μM. The final concentration of the mitochondrial protein was 13.7 mg/ml. V_0 represents the rate of cytochrome *c* reduction (μmol/min·mg protein) without Cd and Zn; V_i represents the rate of cytochrome *c* reduction at the given concentration of Zn.

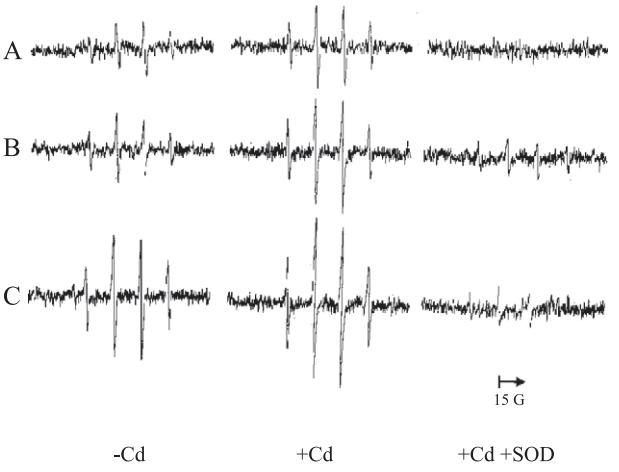

Fig. 7. ESR test to monitor Cd-induced ROS production in mitochondria. Production of ROS was measured by ESR as described under Materials and Methods. Mitochondrial protein was suspended in PBS containing 5 mM glutamate/malate, 100 μM NADH, and 100 mM succinate, ± Cd (10 μM), and DMPO (200 mM). The final volume was 1.0 ml. (A) Liver mitochondria (400 μg/ml). (B) Brain mitochondria (200 μg/ml). (C) Heart mitochondria (100 μg/ml). The ESR signal intensities in the presence of SOD are shown on the right side.

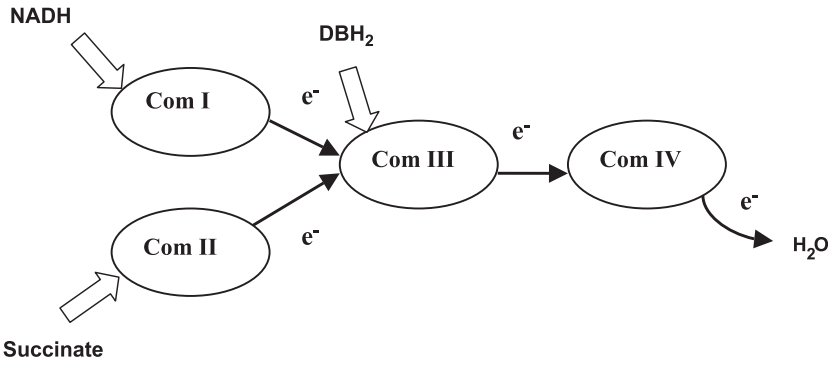

Fig. 8. Scheme of electron transfer path from complexes I and II to III and IV of ETC. The open arrows show where the electron donors NADH, succinate, and DBH_2 act. Solid arrows show the pathway of electron transfer. Com I to IV represent complexes I through IV, respectively.

significantly inhibited by SOD, a scavenger of ROS (Fig. 7, right column).

To determine the possible site(s) where ROS is produced in the presence of Cd, the following experiments were performed. The complex I electron donor NADH, the complex II electron donor succinate, and the complex III electron donor DBH_2 were separately incubated with mitochondria of brain in the presence and absence of Cd, and ROS generation was measured by ESR. Reactions without Cd were used as controls. The electron transfer pathways with different electron donors are shown in Fig. 8. It is expected that if complex I is capable of generating ROS, then the ESR signal response in the presence of NADH will be more than that in the presence of DBH_2 alone, as the total ROS generation then would be from both complexes I and III. If complex I is incapable of generating ROS, then the ESR signal response in the presence of NADH should be the same as that with DBH_2 alone. A similar explanation for complex II would be applicable to succinate. Compared with control, addition of Cd increased the intensity of the ESR signals by 55±5% with DBH_2 (Fig. 9D), by 57±4% with NADH (Fig. 9B), and by 43±3% with succinate (Fig. 9C). The results show that in the presence of Cd, NADH or succinate do not produce more ROS when compared with DBH_2. These observations suggest that complex III is the major site of ROS production induced by Cd.

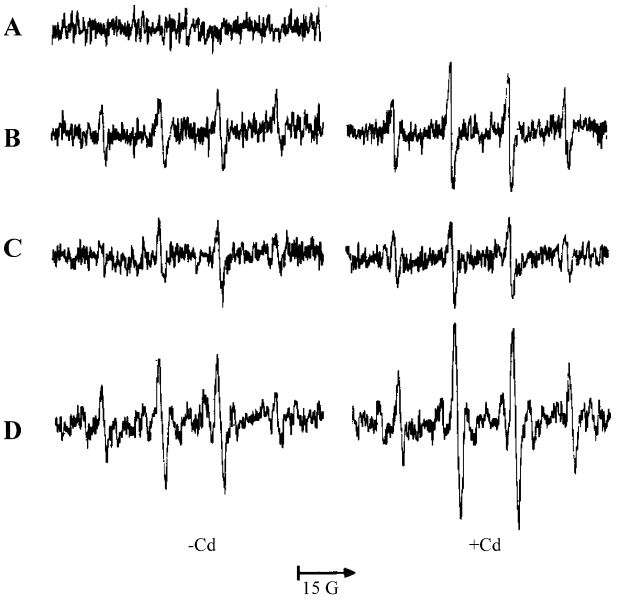

Fig. 9. Cd-induced ROS generation in brain mitochondria with NADH, succinate, and DBH_2 as the electron donors. Production of ROS was measured by ESR as described under Materials and Methods. The reaction was carried out in 0.5 ml PBS buffer containing 150 μg of mitochondrial protein and 100 mM DMPO. The reaction was initiated by adding the electron donor. (A) Baseline without Cd. The baseline with 10 μM Cd is very similar to the baseline without Cd (data not show). (B) 100 μM NADH. (C) 400 μM succinate. (D) 20 μM DBH_2. −Cd, reactions without Cd. +Cd, 10 μM Cd was added to the reaction system and the system was then allowed to incubate for 3 min at room temperature before electron donors were added. The results are representative of three separate experiments.

DISCUSSION

Cd is a relatively abundant element that is widely distributed in food, water, and the environment. It accumulates in human tissues [42]. Recent studies have indicated that Cd induces oxidative stress in cells. Cd, as the least representative member of the transition element group, does not induce production of ROS though a Fenton-like redox cycling mechanism, as is the case with chromium [13]. However, Cd does induce oxidative stress, which causes DNA strand damage, lipid peroxidation, alterations in gene expression, and apoptosis [1,29]. Free radical scavengers and antioxidants have been reported to attenuate Cd-induced toxicity [1,43].

Experimental evidence indicates that mitochondria appear to be the target organelle of Cd, [18,19]. In addition, mitochondria are able to accumulate Cd resulting in the inhibition of electron transfer and oxidative phosphorylation [19]. Alterations in mitochondrial functions have been extensively reported to be related to

many diseases such as mitochondrial encephalomyopathy lactic acidosis (MELAS) [44], Leber hereditary optic neuropathy (LHON) [45], ragged red fibers (RRF) [46], Kearms–Sayre syndrome (KSS) [47], neurodegenerative diseases such as Parkinson's disease (PD) [48–50], Alzheimer's disease (AD) [51,52], Huntington's disease (HD) [53,54], as well as aging [55,56]. Mitochondrial dysfunction is also related to cancer [57]. Therefore, it is important to determine if Cd can induce ROS generation in mitochondria and to find out the possible mechanism of generation of ROS.

We demonstrated here that Cd inhibits mainly complexes II and III of the mitochondria obtained from liver, brain, and heart. Maximum inhibition by Cd was 60% for complex II and 77% for complex III (Fig. 1). In contrast, maximum inhibition was only 20% for complex I and 30% for complex IV (Fig. 1). As we mentioned in the Introduction, impairment of electron transfer through complexes I and III may induce superoxide formation [20,21]. The impairment of electron transfer through complex III by Cd may possibly induce ROS generation. ESR experimental results show that inhibition of ETC activity by Cd induces ROS generation in the mitochondria of all three tissues tested in the presence of electron donors of complex I, complex II, and citric acid cycle (Fig. 7). To determine the inhibitory site of Cd that is responsible for the increased production of ROS, we incubated Cd-exposed mitochondria of brain with different ETC substrates, and measured the production of ROS (Fig. 9). When NADH is used as the substrate, complex I gets electrons from NADH and then transfers them downstream to complex III. So, with NADH as substrate, ROS might be produced by complex I, or complex III, or even both. When succinate is used, complex II is first reduced and then the electrons are delivered to complex III. Therefore, the sites of ROS production with succinate as substrate might be complex II, or complex III, or both. DBH_2 is a substrate for complex III. So, production of ROS with DBH_2 as the substrate can be attributed only to complex III. NADH, succinate, and DBH_2 stimulated the intensities of the ESR signal by 57, 43, and 55%, respectively (Fig. 9). Thus, neither NADH nor succinate stimulated more ROS production compared with DBH_2. These results suggest that the Cd-induced formation of ROS in mitochondria does not depend on inhibition of complex I or II. Therefore, complex III is the only site in the ETC complex where ROS are produced in the presence of Cd. A similar conclusion was reached by Chen et al. [58] through their experiments studying ROS generation during the oxidation of complex I substrates.

The possible mechanism of ROS generation by complex III in the presence of Cd may be explained as follows. According to the generally accepted Q-cycle model, ubiquinol is oxidized at the Q_o site of complex III (Fig. 4). The two electrons carried by ubiquinol are directed into two different pathways [59]. One electron from ubiquinol is transferred through iron–sulfur proteins, cytochrome c_1, cytochrome *c*, and finally to cytochrome *c* oxidase (complex IV) where the electron is used to reduce the oxygen forming H_2O. The release of one electron converts ubiquinol to a semiubiquinone, which then donates the second electron to cytochrome b_{566}, b_{562} and finally the Q_i site of complex III. The second electron contributes to the reduction of ubiquinone at the Q_i site. The Q_i site inhibitor, antimycin A, blocks the electron transfer from b_{562} to ubiquinone, which may consequently result in the accumulation of semiubiquinone anions at the Q_o site [60]. The Q_o site inhibitor myxothiazol completely blocks oxidation of ubiquinol and no electron can go to the b hemes and cytochrome *c* of complex III [61]. However, whether myxothiazol stimulates ROS by inhibiting complex III remains controversial [62,63]. We found that the inhibition of complex III by Cd is noncompetitive, suggesting that Cd does not interfere with the binding of substrates to the complex (Fig. 3). The inhibition of complex III by Cd is not related to binding of Cd to the substrate binding sites. We propose that Cd may bind to the Q_o site of complex III based on the following observations: (a) the reduction rates of the hemes (b_{566}, b_{562}, and c_1) of complex III, in the presence of antimycin A, cannot be completely inhibited by Cd (Fig. 5); (b) Cd shows competitive binding to complex III with Zn, which has been shown to bind to the Q_o site of complex III [41] (Fig. 6).

Myxothiazol is an inhibitor of the Q_o site of complex III [61,64]. However, our data indicate that the effect of Cd is different from that of myxothiazol. First, the reversible binding of Cd to complex III indicates a loose binding of Cd, in contrast to the binding of myxothiazol to the Q_o site of complex III, which is strong and irreversible [61,64]. Second, Cd is not able to completely inhibit the electron transfer of complex III. The reduction of b hemes (b_{566}, b_{562}) and cytochrome *c* was only about 70% inhibited by Cd (Fig. 5). By contrast, myxothiazol completely blocks oxidation of ubiquinol at the Q_o site. No electron can go to the b hemes and cytochrome *c* of complex III in the presence of myxothiazol [60,61]. The results indicate that the inhibitory behaviors of Cd and myxothiazol are different, although both inhibitors bind to the Q_o site of complex III. Presumably, Cd binds between semiubiquinone and cytochrome b_{566} as reported by Miccadei and Floridi [65]. One possibility is that Cd weakly binds to the Q_o site, causing a conformational change at the Q_o site, which results in partial inhibition of the second electron transfer from semiubiquinone to b_{566} and b_{562}. Inhibition of the second electron transfer from semiubiquinone to b_{566} and b_{562}

will result in accumulation of semiubiquinone at the Q_o site of complex III. The semiubiquinones are not stable and are prone to transfer one electron to molecular oxygen to form superoxide [59,60]. This may explain why Cd can induce ROS formation in mitochondria.

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ABBREVIATIONS

complex I — NADH:ubiquinone oxidoreductase
complex II — succinate:ubiquinone oxidoreductase
complex III — ubiquinone:cytochrome *c* oxidoreductase
complex IV — cytochrome *c* oxidase
complex V — ATP synthase
DBH_2 — 2,3-dimethoxy-5-methyl-6-decyl–1,4-benzoquinol
DCIP — dichlorophenolindophenol
DM — dodecyl maltoside
DMPO — 5,5-dimethyl-L-pyrroline *N*-oxide
DPPH — 1,1-diphenyl-2-picrylhydrazyl
ESR — electron spin resonance
ETC — electron transfer chain
PBS — phosphate-buffered saline
ROS — reactive oxygen species