

# Effect of Treatment with Cadmium on Structure-Function Relationships in Rat Liver Mitochondria: Studies on Oxidative Energy Metabolism and Lipid/Phospholipids Profiles

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**Abstract** Effects of treatment with a single intraperitoneal injection of cadmium (Cd) on oxidative energy metabolism and lipid/phospholipid profiles of rat liver mitochondria were examined at the end of 1 week and 1 month. Following Cd treatment the body weight increased only in the 1 month group, whereas the liver weight increased in both groups. State 3 and 4 respiration rates in general decreased significantly, with the maximum effect being seen with succinate. The 1 week Cd group showed decreased respiratory activity with glutamate, pyruvate + malate, and succinate as the substrates. In the 1 month Cd-treated group respiration rates recovered with glutamate and pyruvate + malate but not with succinate. All cytochrome contents decreased in the 1 week Cd-treated group but recovered in the 1 month group. ATPase activity registered an increase in both Cd-treated groups. Dehydrogenase activities increased in the 1 week group but decreased in the 1 month Cd-treated group. The mitochondrial cholesterol content increased in the 1 week Cd-treated group. In the 1 week Cd-treated group the lyso-phospholipid (Lyso), sphingomyelin (SPM), and diphosphatidylglycerol (DPG) components increased. By contrast, the phosphatidylethanolamine (PE) component decreased. In the 1 month Cd-treated group the phosphatidylinositol, phosphatidylserine, and DPG components increased,

whereas the Lyso, SPM, and phosphatidylcholine components decreased. The results demonstrate that single-dose Cd treatment can have adverse effects on liver mitochondrial oxidative energy metabolism and lipid/phospholipid profiles, which in turn can affect membrane structure-function relationships.

**Keywords** Cadmium (Cd) · Oxidative energy metabolism · Cytochromes · Dehydrogenases · Mitochondrial lipid/phospholipid profile · Total phospholipid · Cholesterol · Liver mitochondria

Cadmium (Cd) is known to be a hepatotoxic element and affects the function of several tissues including the liver and kidney (Bagchi et al. 1996; Baker et al. 2003; Bompart et al. 1991; Goering et al. 1993; Valko et al. 2005). Distribution studies have shown that the adult liver accumulates larger amounts of Cd (Klaassen and Wong 1982). Reports are available which describe in detail the damage to various subcellular organelles including mitochondria following Cd exposure (Goering and Klaassen 1983, 1984; Jamall 1987). Mitochondria have been shown to be the prime target of Cd-induced hepatotoxicity (Early et al. 1992; Martel et al. 1990; Miccadei and Floridi 1993). Thus, Early et al. (1992) reported that complete destruction of the mitochondrial membrane structure occurs within 48 h after a single Cd injection. Earlier studies by Tang and Shaikh (2001) on Cd insult to mitochondria pertain to structural damage as well as impairment of the activity of certain enzymes. Other earlier studies report the in vitro effects of Cd on the above-mentioned parameters (Liu and Liun 1990; Miccadei and Floridi 1993). However, the effects of exposure to Cd on the oxidative energy metabolism of the mitochondria have received scant attention.

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The energy transduction functions of the mitochondria as well as the activities of several mitochondrial enzymes and enzyme complexes are dependent on, and have specific requirements for, lipids/phospholipids (Daum 1985). Hence it is pertinent to assess the effects of Cd exposure on lipid/phospholipid profiles of the mitochondria. This aspect assumes importance in view of the fact that, as referred to above, treatment with Cd resulted in the destruction of mitochondrial membranes (Early et al. 1992).

In view of the foregoing we carried out experiments to examine the effects of exposure to a single injection of Cd on mitochondrial structure-function relationships in experimental animals. Since Cd has the long half-life of 15–30 years in humans (Vallee and Ulmer 1972), it was of interest to examine these parameters at early and late periods after exposure to Cd. Thus experiments were performed 1 week and 1 month after injecting the adult rats with Cd. Results of the present studies suggest that Cd exposure has differential short-term and long-term effects on mitochondrial structure-function relationships. The results of these experiments are summarized in the present communication.

A survey of the literature revealed that treatment with Cd decreased cholesterol (CHL) and triglycerides (TGs) in liver mitochondria (Larregle et al. 2007). In whole kidney, CHL, TG, and total phospholipids (TPLs) decreased (El-Sharakly et al. 2008). However, as far as we are aware no in-depth studies to examine effects of Cd treatment on mitochondrial lipid/phospholipid profiles have been reported thus far.

## Materials and Methods

### Chemicals

Cadmium acetate was purchased from Loba Chemie (Mumbai, India). Sodium salts of L-glutamic acid, succinic acid, pyruvic acid, L-malic acid, oxaloacetic acid, ADP, rotenone, bovine serum albumin fraction V (BSA), 4-morpholinopropanesulfonic acid (MOPS), dichlorophenolindophenol (DCIP),  $\text{NAD}^+$ , NADH,  $N,N,N',N'$ -tetramethyl-*P*-phenylenediamine (TMPD), and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid was procured from Sarabhai Chemicals (Vadodara, India). Silica gel G was from E. Merck, Germany. All other chemicals were of analytical-reagent grade and were purchased locally.

### Animals and Treatment with Cd

Adult male albino rats (8–10 weeks old) of the Charles-Foster strain weighing between 200 and 250 g were used. Animals were injected intraperitoneally with cadmium

acetate (freshly prepared in saline) at a dose of 2 mg/kg body weight. This dose amounts to 0.84 mg Cd/kg body weight (Early et al. 1992). The animals were divided into two groups. One group of animals was killed 1 week (on day 8) after Cd treatment. The other group was killed 1 month after treatment. The controls were given 1.23 mg sodium acetate in saline/kg body weight and these animals were also divided into two groups as above and used as 1 week and 1 month controls.

In a given set of experiments six to eight animals were used in each group. Independent sets of experiments were performed to assess the different parameters.

### Isolation of Mitochondria

Isolation of mitochondria was essentially according to the procedures described previously, with some modifications (Patel and Katyare 2006a, b). Thus, briefly, at the end of the experimental period animals were killed by decapitation and the liver was quickly removed and placed in a beaker containing chilled (0–4°C) isolation medium consisting of 250 mM sucrose containing 5 mM MOPS and 1 mM EDTA, all at pH 7.4; 0.25 mg BSA/ml isolation medium was included. Liver tissue was minced with a pair of scissors and washed repeatedly with the isolation medium to remove adhering blood. The tissue was then homogenized using a Potter-Elvehjem-type glass-Teflon homogenizer to obtain a 10% (w/v) homogenate. After removal of nuclei and cell debris at 650 *g* for 10 min, mitochondria were sedimented by centrifugation at 7500 *g* for 10 min, washed once by gentle suspension in the isolation medium and resedimentation, and, finally, suspended in the isolation medium to give a protein concentration in the range of 25–30 mg/ml. All steps in the isolation procedure were carried out at 0–4°C in a Sorvall RC 5B plus centrifuge (Patel and Katyare 2006a, b).

The postmitochondrial supernatant ( $S_1$ ) was subjected to a further centrifugation at 12,000 *g* for 10 min to sediment the light mitochondrial fraction. The resulting supernatant ( $S_2$ ) was then centrifuged at 100,000 *g* for 1 h to sediment the microsomal fraction. The supernatant ( $S_3$ ) was used as the cytosolic fraction (Patel and Katyare 2006a, b).

### Oxidative Phosphorylation

Measurements of oxidative phosphorylation were carried out at 25°C using a Clark-type oxygen electrode as described previously (Patel and Katyare 2006a, b). Briefly, the respiration medium (total volume, 1.6 ml) consisted of 225 mM sucrose, 20 mM KCl, 10 mM MOPS, pH 7.4, 5 mM potassium phosphate buffer, pH 7.4, 0.2 mM EDTA, and 160 µg of BSA (i.e., 0.1 mg BSA/ml). After introducing mitochondria (4–8 mg protein, depending on the

substrate used) into the electrode chamber, respiration was induced by the addition of substrates. Final concentrations of the substrates used were as follows: glutamate (10 mM), pyruvate + malate (10 + 1 mM), succinate (10 mM), and ascorbate + TMPD (10 + 0.1 mM). Measurements with the latter two substrates were performed in the presence of 1  $\mu$ M rotenone. State 3 respiration rates, initiated by the addition of 80–200 nmol of ADP, and state 4 respiration rates, ensuing after the depletion of added ADP, were recorded. Calculations of ADP/O ratio and ADP phosphorylation rates were as described previously (Ferreira and Gil 1984; Katyare and Satav 1989).

#### Cytochrome Content

The contents of cytochromes were calculated from the difference spectra as described previously (Patel and Katyare 2006a, b).

#### Assay of Dehydrogenases

Measurement of glutamate dehydrogenase (GDH) activity was carried out spectrophotometrically at 25°C as described by Leighton et al. (1968). Malate dehydrogenase (MDH) activity was determined following the procedure of Ochoa (1995). Measurements of succinate DCIP reductase (SDR) activity were carried out according to the method of King (1967).

#### Assay of ATPase

ATPase activity was measured in 0.1 ml of assay medium containing 50 mM MOPS, pH 7.4, 75 mM KCl, and 0.4 mM EDTA. Assays were performed in the absence and presence of MgCl<sub>2</sub> (6 mM) and 100  $\mu$ M DNP, or a combination of both. After preincubation of the mitochondrial protein (40–60  $\mu$ g) in the assay medium at 37°C for 1 min, the reaction was initiated by the addition of ATP at a final concentration of 5 mM. The reaction was terminated after 15 min by the addition of 0.1 ml of 5% (w/v) sodium dodecyl sulfate (SDS). Estimation of released inorganic phosphate was according to procedures described previously (Katewa and Katyare 2003, 2004).

#### Analytical Method

##### *Lipid/Phospholipid Analysis*

Extraction of mitochondrial lipid using a freshly prepared chloroform:methanol mixture (2:1 v/v) was essentially as described earlier (Folch et al. 1957; Pandya et al. 2004). After extraction, the solvent was completely evaporated under a stream of nitrogen and the residue was redissolved

in a known volume of a chloroform:methanol mixture. Suitable aliquots were taken for estimation of phospholipid phosphorus (Bartlett 1954) and cholesterol (Zlatkis et al. 1953) and for thin-layer chromatography (TLC).

Separation of phospholipid classes was carried out by one-dimensional TLC using silica gel G. Conditions for chamber saturation were according to Stahl (1969). Aliquots of the reconstituted samples containing 8–10  $\mu$ g of phospholipid phosphorus were spotted on a TLC plate in a way such that the diameter of the spot was minimal. The solvent system used for separation of phospholipid classes was as described by Skipski et al. (1967) and consisted of chloroform:methanol:acetic acid:water (25:15:4:2, v/v). The areas corresponding to marked spots were carefully scraped and the silica gel was transferred to clean test tubes. Samples were digested in a sand bath using 0.5 ml of 10 N H<sub>2</sub>SO<sub>4</sub>. Tubes were allowed to cool, after which a drop of 70% perchloric acid was added. The tubes were again heated for 3–4 h, until the solution in the tubes was clear and the smell of chlorine was undetectable (Pandya et al. 2004). Analysis of phosphorus content was according to the procedure of Bartlett (1954).

Contents of individual phospholipids were computed by multiplying the total phospholipid by the percentage fraction of the said phospholipid (Pandya et al. 2004).

##### *Determination of Membrane Fluidity*

Membrane fluidity determinations were carried out at 25°C in a Shimadzu RF 5000 spectrophotofluorimeter using DPH as the probe as detailed earlier (van Blitterwijk et al. 1981; Mehta et al. 1991; Bangur et al. 1995; Pandya et al. 2004). The instrument has a program for calculation of fluorescence polarization (p), from which the value of fluorescence anisotropy (r), limiting hindered anisotropy (r<sub>∞</sub>), and the static component of fluidity (S) can be calculated.

##### *Protein Estimation*

Protein estimation was according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

##### Statistical Evaluation

Results are given as mean  $\pm$  SE. Statistical evaluation of the data was by Students' *t*-test.

## Results

Following exposure to a single dose of Cd in the 1 week group there was no change in body weight, while in the 1

**Table 1** Effect of treatment with cadmium on the body and liver weight of rats

Group	Final body weight (g)	Liver weight	
		g	% body weight
1 week			
Control	259.5 ± 3.2	8.71 ± 0.15	3.36 ± 0.05
Cd treatment	258.0 ± 4.8	9.63 ± 0.21 <sup>b</sup>	3.73 ± 0.06 <sup>b</sup>
1 month			
Control	258.5 ± 3.6	8.53 ± 0.25	3.30 ± 0.10
Cd treatment	292.0 ± 6.3 <sup>b</sup>	9.57 ± 0.21 <sup>a</sup>	3.28 ± 0.06

Note: Experimental details are given in the text. Results are the mean ± SE of 12–16 independent experiments in each group

<sup>a</sup>  $P < 0.01$ ; <sup>b</sup>  $P < 0.001$  compared to the corresponding control

month group the body weight increased by 13% (Table 1). In both groups the liver weight increased, by 11–12% (Table 1). Thus, the relative liver weight increased by 11% in the 1 week group, whereas it was unaltered in the 1 month group due to the proportionate increase in body weight (Table 1).

In the next set of experiments, the effects of Cd exposure on mitochondrial oxidative phosphorylation were examined. In the 1 week group, state 3 respiration rates with glutamate and pyruvate + malate as the respiratory substrates decreased by 17% and 31%, respectively. With succinate as the substrate the state 3 respiration rate decreased maximally by 38%, whereas no effect on respiratory activity was seen with the ascorbate + TMPD couple. State 4 respiration rates with the four substrates decreased by 19–24%. However, the ADP/O ratios were

unchanged. ADP phosphorylation rates with glutamate, pyruvate + malate, and succinate registered a decrease consistent with the lowering of the corresponding state 3 respiration rates (Tables 2 and 3).

In the 1 month group the state 3 respiration rate with glutamate was unaffected, indicating recovery. However, state 3 respiration rates with pyruvate + malate and succinate decreased by 31% and 38%, and state 4 respiration rates decreased by 17% and 31%, respectively. Respiratory activity was not affected with ascorbate + TMPD as the electron donor system. ADP/O ratios were unchanged. The changes in state 3 respiration rates were also reflected in ADP phosphorylation rates (Tables 2 and 3).

Figure 1 shows typical difference spectra of mitochondrial cytochromes, and data on cytochrome contents are reported in Table 4. As can be noted, only in the 1 week Cd-treated group did the contents of all cytochromes, i.e., aa<sub>3</sub>, b, and c + c<sub>1</sub>, decrease: by 31%, 41%, and 61%, respectively. However, the values recovered in the 1 month Cd-treated animals and were in fact somewhat higher for the latter two cytochromes, although the increase was not statistically significant (Table 4).

In the next set of experiments the ATPase activity was determined. In the 1 week group, treatment with Cd resulted, in general, in an increase in the basal and Mg<sup>2+</sup>-stimulated ATPase activities, which were about 2 and 1.75 times higher. By contrast, DNP-stimulated ATPase activity decreased by 17%, without any change in Mg<sup>2+</sup> + DNP-stimulated ATPase activity. In the 1 month Cd-treated group, under all experimental conditions the ATPase activity was high, with the increase ranging from 28% to 124% (Table 5).

**Table 2** Effect of treatment with cadmium on oxidative phosphorylation in rat liver mitochondria using glutamate or pyruvate + malate as the substrate

Substrate	Group	ADP/O ratio	Respiration rate (nmol O <sub>2</sub> /min/mg protein)		RCR	ADP phosphorylation rate (n mole/min/mg protein)
			+ADP	−ADP		
Glutamate	1 week					
	Control (23)	3.14 ± 0.16	24.50 ± 1.67	9.33 ± 0.51	2.71 ± 0.14	156.74 ± 10.19
	Cd treatment (12)	3.06 ± 0.20	13.35 ± 0.88 <sup>b</sup>	6.67 ± 0.28 <sup>b</sup>	1.99 ± 0.06 <sup>b</sup>	81.70 ± 6.35 <sup>b</sup>
	1 month					
	Control (12)	3.08 ± 0.19	22.77 ± 1.27	8.81 ± 0.56	2.65 ± 0.18	140.30 ± 9.34
	Cd treatment (18)	3.04 ± 0.18	18.96 ± 1.79	7.43 ± 0.60	2.58 ± 0.10	115.90 ± 7.56 <sup>a</sup>
Pyruvate + malate	1 week					
	Control (15)	3.10 ± 0.25	23.50 ± 1.44	12.06 ± 0.96	2.06 ± 0.14	151.32 ± 20.36
	Cd treatment (9)	3.16 ± 0.19	16.14 ± 0.58 <sup>b</sup>	9.76 ± 0.32 <sup>a</sup>	1.66 ± 0.06 <sup>a</sup>	102.18 ± 5.58 <sup>b</sup>
	1 month					
	Control (14)	3.17 ± 0.09	20.46 ± 1.10	13.16 ± 0.92	1.59 ± 0.10	131.30 ± 8.44
	Cd treatment (20)	3.07 ± 0.14	17.98 ± 1.60	10.34 ± 0.78 <sup>a</sup>	1.71 ± 0.06	110.70 ± 7.52

Note: Experimental details are given in the text. Results are the mean ± SE of the number of observations indicated in parentheses

<sup>a</sup>  $P < 0.05$ ; <sup>b</sup>  $P < 0.001$  compared with the corresponding untreated group

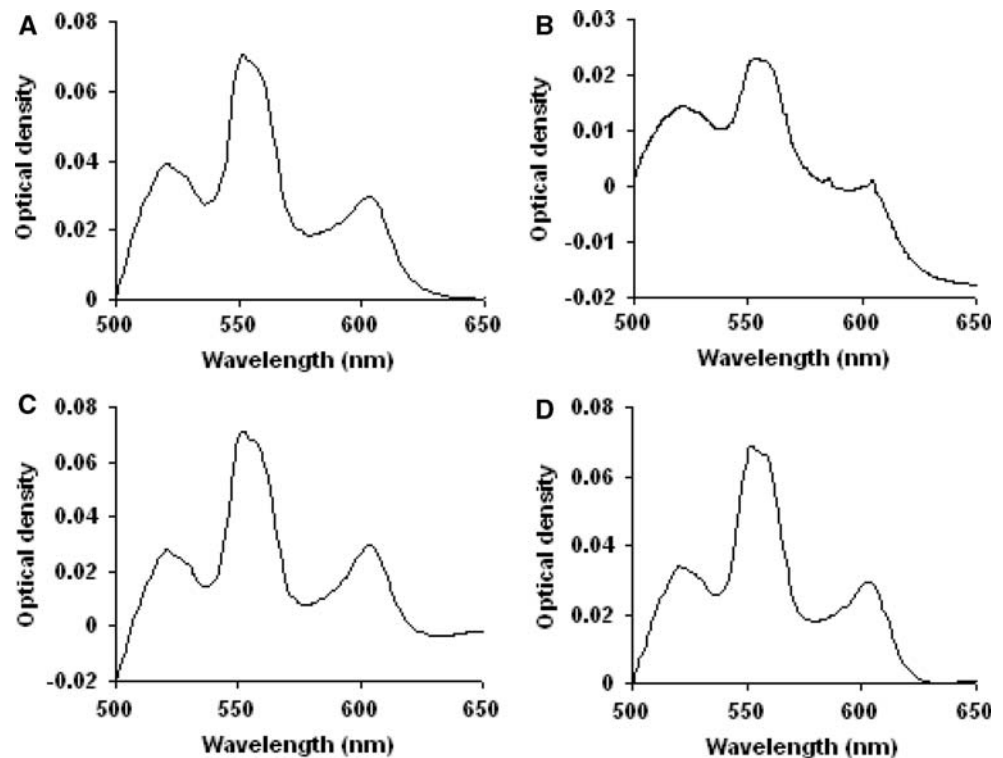
**Table 3** Effect of treatment with cadmium on oxidative phosphorylation in rat liver mitochondria using succinate or ascorbate + TMPD as the substrate

Substrate	Group	ADP/O ratio	Respiration rate (nmol O <sub>2</sub> /min/mg protein)		RCR	ADP phosphorylation rate (nmol/min/mg protein)
			+ADP	-ADP		
Succinate	1 week					
	Control (19)	2.01 ± 0.11	71.77 ± 4.83	40.28 ± 1.82	1.81 ± 0.08	310.14 ± 21.52
	Cd treatment (12)	1.70 ± 0.16	44.55 ± 4.37 <sup>c</sup>	30.51 ± 3.28 <sup>a</sup>	1.47 ± 0.04 <sup>a</sup>	142.48 ± 11.42 <sup>c</sup>
	1 month					
	Control (14)	2.02 ± 0.16	72.85 ± 4.64	37.97 ± 2.62	2.02 ± 0.18	293.70 ± 22.82
	Cd treatment (23)	2.09 ± 0.07	43.31 ± 2.51 <sup>c</sup>	24.74 ± 1.62 <sup>c</sup>	1.76 ± 0.05	177.20 ± 8.68 <sup>c</sup>
Ascorbate + TMPD	1 week					
	Control (29)	0.34 ± 0.03	31.08 ± 1.04	23.92 ± 1.08	1.33 ± 0.03	20.88 ± 1.42
	Cd treatment (16)	0.32 ± 0.03	29.51 ± 1.66	23.64 ± 1.63	1.28 ± 0.04	18.68 ± 1.22
	1 month					
	Control (16)	0.44 ± 0.03	30.86 ± 1.64	23.31 ± 1.73	1.37 ± 0.04	26.10 ± 1.50
	Cd treatment (12)	0.44 ± 0.04	27.06 ± 1.69	21.16 ± 1.35	1.29 ± 0.03	23.64 ± 2.18

Note: Experimental details are given in the text. Results are the mean ± SE of the number of observations indicated in parentheses.

<sup>a</sup>  $P < 0.02$ ; <sup>b</sup>  $P < 0.002$ ; <sup>c</sup>  $P < 0.001$  compared with the corresponding untreated group

**Fig. 1** Typical cytochrome spectra of rat liver mitochondria: 1 week (a) and 1 month (c) control groups; 1 week (b) and 1 month (d) cadmium-treated groups. The ordinate represents optical density units, and the abscissa represents wavelength (nm). The plots are typical of four independent experiments in each group



GDH activity was unchanged in the 1 week Cd group but decreased by 31% in the 1 month Cd-treated group (Fig. 2a). While mitochondrial MDH activity showed a 27% increase in the 1 week Cd-treated group, paradoxically in the 1 month Cd group this activity decreased by 45% (Fig. 2b). SDR activity became 2.74 times higher in the 1 week Cd-treated group but returned to normal after

1 month of Cd treatment (Fig. 2c). Cytosolic MDH activity increased significantly (1.74-fold) in the 1 week Cd-treated group, whereas it registered a 43% decrease in the 1 month Cd-treated group (Fig. 2d).

Results on the lipid/phospholipid profiles of rat liver mitochondria as affected by exposure to Cd are reported in Tables 6–8. In the 1 week Cd-treated group the TPL

**Table 4** Effect of treatment with cadmium on the content of cytochromes in rat liver mitochondria

Group	Cytochrome content (pmol/mg protein)		
	aa <sub>3</sub>	b	c + c <sub>1</sub>
1 week			
Control (4)	137.0 ± 13.29	262.3 ± 19.33	294.2 ± 16.01
Cd treatment (4)	94.3 ± 6.77 <sup>a</sup>	154.7 ± 10.27 <sup>b</sup>	113.7 ± 4.09 <sup>b</sup>
1 month			
Control (4)	140.3 ± 5.06	256.2 ± 13.67	301.7 ± 33.83
Cd treatment (4)	164.8 ± 13.12	279.8 ± 20.28	330.8 ± 16.66

Note Experimental details are given in the text. Results are the mean ± SE of the number of observations indicated in parentheses

<sup>a</sup>  $P < 0.05$ ; <sup>b</sup>  $P < 0.001$  compared with the corresponding untreated group

**Table 5** Effect of treatment with cadmium on the ATPase activity in rat liver mitochondria

Group	Activity (μmol P <sub>i</sub> liberated/h/mg protein)			
	Basal	+Mg <sup>2+</sup>	+DNP	+Mg <sup>2+</sup> + DNP
1 week				
Control (12)	1.90 ± 0.17	5.26 ± 0.24	20.94 ± 0.86	17.58 ± 1.34
Cd treatment (12)	3.94 ± 0.25 <sup>c</sup>	9.21 ± 0.57 <sup>c</sup>	17.40 ± 0.77 <sup>a</sup>	16.15 ± 0.77
1 month				
Control (12)	1.98 ± 0.20	5.05 ± 0.49	19.80 ± 0.77	16.46 ± 0.62
Cd treatment (12)	4.45 ± 0.26 <sup>c</sup>	8.47 ± 0.74 <sup>b</sup>	25.30 ± 1.56 <sup>a</sup>	23.83 ± 1.49 <sup>c</sup>

Note: Experimental details are given in the text. Results are the mean ± SE of the number of observations indicated in parentheses

<sup>a</sup>  $P < 0.01$ ; <sup>b</sup>  $P < 0.002$ ; <sup>c</sup>  $P < 0.001$  compared with the corresponding untreated group

content did not change, whereas the CHL content increased significantly (by 166%). In the 1 month Cd-treated group the TPL content decreased by 11%, whereas the CHL content increased by 18%. Consequently, the TPL/CHL (mole:mole) ratio decreased in both Cd-treated groups, signifying increased membrane rigidity (Table 6).

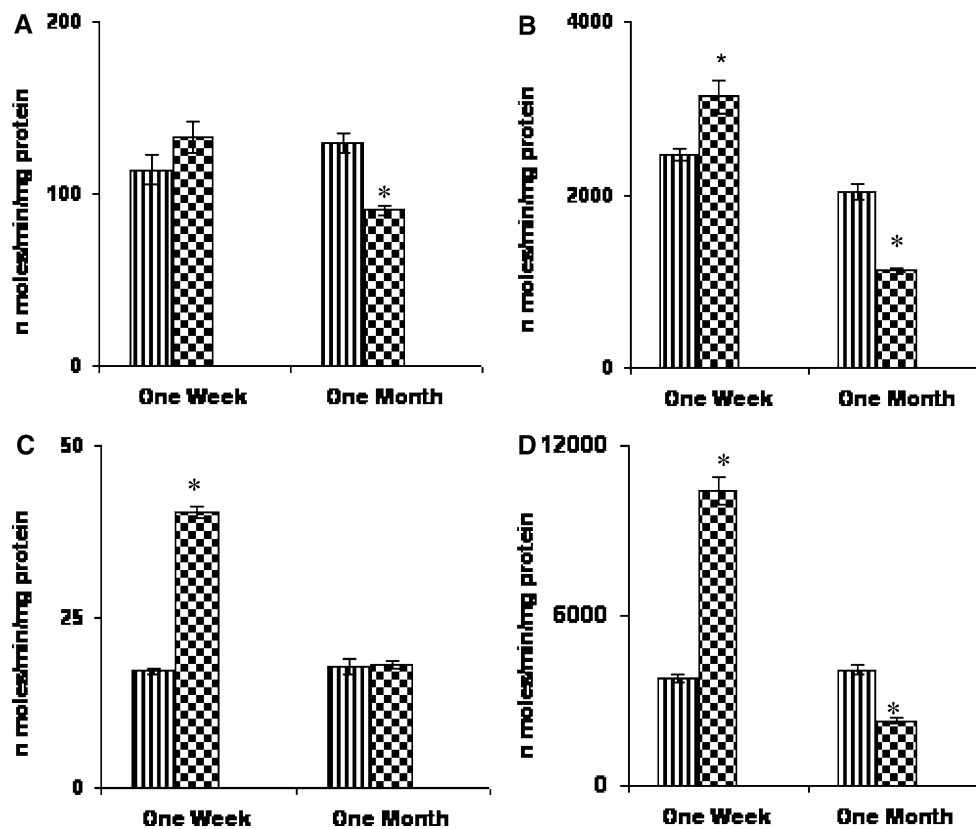
Analysis of the phospholipid profile revealed that Cd exposure in the 1 week group had the generalized effect of increasing the lysophospholipid (Lyso) and sphingomyelin (SPM) components, which almost doubled; the diphosphatidylglycerol (DPG) component increased by 34%. By contrast, the phosphatidylethanolamine (PE) component decreased by 36%. For the 1 month group the phosphatidylinositol (PI), phosphatidylserine (PS), and DPG components registered increases (of 24%–42%), whereas Lyso, SPM, and phosphatidylcholine (PC) registered decreases (of 12%–44%). Interestingly, the PE component was restored to normality (Table 7). The computed contents of the individual phospholipid classes were generally consistent with the above changes (Table 8).

The data in Table 9 show that, despite the significant changes in the phospholipid makeup, apparently Cd exposure did not change membrane fluidity in either experimental group.

## Discussion

It is evident from the data presented that exposure to Cd affects mitochondrial structure-function relationships within 1 week of the treatment, which is reflected in terms of alterations in oxidative metabolism and lipid/phospholipid profiles, and differential effects persist even at the end of 1 month.

Thus within 1 week of Cd treatment respiration rates in general decreased significantly, with the maximum effect being seen with succinate as the respiratory substrate. The 1 month Cd group showed recovery with glutamate and pyruvate + malate as the substrates but not with succinate. Interestingly, in neither group was the respiratory activity affected with ascorbate + TMPD as the substrate couple (Tables 2 and 3). The decreased respiration rates also lowered the energy potential of the mitochondria, as reflected in terms of the ADP phosphorylation rates, with the effects being prominently noticeable in the 1 week group. Thus maximum impairment was noted in the 1 week group. Structural damage to the mitochondria was also evident in terms of increased basal and Mg<sup>2+</sup>-stimulated ATPase activities in both Cd-treated groups. However, the overall potential of ATPase for energy transduction as



**Fig. 2** Effect of treatment with cadmium on mitochondrial and cytosolic dehydrogenase activities in rat liver. Measurement of glutamate dehydrogenase (GDH) activity was carried out spectrophotometrically at 25°C in an assay system (1 ml) consisting of 125 mM potassium phosphate buffer, pH 7.4, 10 mM sodium glutamate, 0.1% Triton X-100, and 100–200 µg of mitochondrial protein as the source of the enzyme (Leighton et al. 1968). Malate dehydrogenase (MDH) activity was measured in an assay system (1 ml) containing 125 mM potassium phosphate buffer, pH 7.4, 2.5 mM oxaloacetate, 0.1% Triton X-100, and 5–15 µg of mitochondrial or cytosolic protein (Ochoa 1995). Measurements of succinate

DCIP reductase (SDR) activity were carried out using an assay system (1 ml) containing 125 mM potassium phosphate buffer, pH 7.4, 0.1 mM sodium azide, 15 mM sodium succinate, and 100–300 µg of mitochondrial protein as the source of the enzyme. After incubation at 25°C for 1 min the reaction was initiated by the addition of 10 µM DCIP and the decrease in absorbance at 600 nm was recorded at 5-s intervals (King 1967). **a** Glutamate dehydrogenase. **b** Malate dehydrogenase (mitochondrial). **c** SDR. **d** Malate dehydrogenase (cytosolic). *Striped bars*, control rats; *checked bars*, Cd-treated rats. \* $P < 0.001$  compared with the corresponding control group. Results are the mean  $\pm$  SE of 12 independent observations

**Table 6** Effect of treatment with cadmium on total phospholipid (TPL) and cholesterol (CHL) content of rat liver mitochondria

Group	TPL (µg/mg protein)	CHL (µg/mg protein)	TPL/CHL (mole:mole)
1 week			
Control (6)	158.22 $\pm$ 7.50	60.39 $\pm$ 3.81	1.31 $\pm$ 0.12
Cd treatment (7)	174.51 $\pm$ 8.87	160.49 $\pm$ 6.38 <sup>b</sup>	0.54 $\pm$ 0.02 <sup>b</sup>
1 month			
Control (8)	157.72 $\pm$ 8.92	61.71 $\pm$ 3.85	1.27 $\pm$ 0.10
Cd treatment (8)	139.76 $\pm$ 3.02	72.90 $\pm$ 3.59	0.97 $\pm$ 0.04 <sup>a</sup>

*Note:* Experimental details are given in the text. Results are the mean  $\pm$  SE of the number of observations indicated in parentheses

<sup>a</sup>  $P < 0.05$ ; <sup>b</sup>  $P < 0.001$  compared to the corresponding control

judged from the DNP- and  $Mg^{2+}$  + DNP-stimulated ATPase activities was more or less undiminished (Table 5). The pattern of dehydrogenases activities showed that in the 1 week Cd-treated group there was a general tendency to increase, which may be a compensatory mechanism. A

similar argument would apply even for ATPase activity, especially in the 1 month group. However, in the 1 month group, although the respiratory activities recovered, the GDH and MDH activities decreased. It may hence be suggested that in both the 1 week and the 1 month Cd-treated

**Table 7** Effect of treatment with cadmium on phospholipid composition in rat liver mitochondria

Phospholipid class	Composition (% of total)			
	1 week		1 month	
	Control (6)	Cd treatment (7)	Control (8)	Cd treatment (8)
Lyso	2.76 ± 0.26	5.57 ± 0.76 <sup>c</sup>	2.60 ± 0.16	1.71 ± 0.18 <sup>c</sup>
SPM	4.66 ± 0.46	10.09 ± 0.94 <sup>e</sup>	4.73 ± 0.32	2.66 ± 0.37 <sup>d</sup>
PC	44.63 ± 0.55	42.80 ± 2.04	43.82 ± 0.71	38.48 ± 1.07 <sup>d</sup>
PI	4.50 ± 0.41	3.40 ± 0.59	3.79 ± 0.27	5.03 ± 0.35 <sup>b</sup>
PS	3.06 ± 0.28	2.72 ± 0.71	4.26 ± 0.31	5.28 ± 0.39
PE	30.18 ± 1.00	19.36 ± 1.51 <sup>e</sup>	31.08 ± 1.17	32.04 ± 2.72
DPG	11.97 ± 0.61	16.05 ± 1.68 <sup>a</sup>	10.42 ± 0.90	14.80 ± 2.11

Note: Experimental details are given in the text. Results are the mean ± SE of the number of observations indicated in parentheses

Lyso lysophospholipid, SPM sphingomyelin, PC phosphatidylcholine, PI phosphatidylinositol, PS phosphatidylserine, PE phosphatidylethanolamine, DPG diphosphatidylglycero

<sup>a</sup>  $P < 0.05$ ; <sup>b</sup>  $P < 0.02$ ; <sup>c</sup>  $P < 0.01$ ; <sup>d</sup>  $P < 0.002$ ; <sup>e</sup>  $P < 0.001$  compared to the corresponding control

**Table 8** Effects of treatment with cadmium on the content of individual phospholipid classes in rat liver mitochondria

Phospholipid class	Content (µg/mg protein)			
	1 week		1 month	
	Control (6)	Cd treatment (7)	Control (8)	Cd treatment (8)
Lyso	4.24 ± 0.31	9.74 ± 1.44 <sup>b</sup>	4.14 ± 0.49	2.38 ± 0.25 <sup>b</sup>
SPM	7.97 ± 0.61	17.56 ± 1.71 <sup>c</sup>	9.50 ± 0.81	3.69 ± 0.50 <sup>c</sup>
PC	72.75 ± 3.01	75.31 ± 6.90	78.09 ± 3.07	53.63 ± 0.54 <sup>c</sup>
PI	6.08 ± 0.45	6.18 ± 1.32	5.91 ± 0.61	7.07 ± 0.61
PS	7.10 ± 0.61	4.53 ± 1.01 <sup>a</sup>	7.10 ± 0.81	7.37 ± 0.55
PE	44.25 ± 2.29	33.27 ± 1.67 <sup>b</sup>	47.70 ± 3.41	45.00 ± 4.53
DPG	23.10 ± 1.49	27.91 ± 3.03	26.24 ± 1.71	20.62 ± 2.88

Note: Experimental details are given in the text. Results are the mean ± SE of the number of observations indicated in parentheses

<sup>a</sup>  $P < 0.05$ ; <sup>b</sup>  $P < 0.01$ ; <sup>c</sup>  $P < 0.001$  compared to the corresponding control

**Table 9** Effects of cadmium exposure on fluidity parameters of liver mitochondria

Group	Fluidity parameter			
	Fluorescence polarization, p	Fluorescence anisotropy, r	Limited hindered anisotropy, $r_x$	Order parameter, S
1 week				
Control (6)	0.185 ± 0.011	0.132 ± 0.008	0.076 ± 0.011	0.407 ± 0.036
Cd treatment (7)	0.196 ± 0.002	0.140 ± 0.002	0.086 ± 0.002	0.464 ± 0.006
1 month				
Control (8)	0.188 ± 0.002	0.134 ± 0.002	0.078 ± 0.002	0.442 ± 0.006
Cd treatment (8)	0.193 ± 0.003	0.137 ± 0.002	0.083 ± 0.003	0.454 ± 0.010

Note: Experimental details are given in the text. Results are given as the mean ± SE of the number of observations indicated in parentheses

groups the dehydrogenases were not the rate-limiting steps. It is of interest to point out here that the contents of all cytochromes decreased significantly in the 1 week Cd-treated group but recovered in the 1 month group. Thus, at least in the 1 week Cd group the cytochromes seem to be the

rate-limiting step (Table 4). Another possibility to be considered is interference by Cd with ion-sulfur (Fe-S) centers of complexes I and II. Apparently complex I recover by the end of 1 month but the effect persisted in complex II. Meiss et al. 1982 and Early et al. (1992) have shown that in adult



rats the liver mitochondria are maximally damaged within 48 h after exposure to Cd but the structural attributes recover later. The results of our present studies not only corroborate these observations but also provide further biochemical evidence of the damage to structural/functional attributes of the mitochondria following exposure to Cd. Liu and Liun (1990) reported that under *in vitro* conditions Cd caused uncoupling of mitochondria, and this effect was thought to be due to altered membrane fluidity. However, in our studies we found that the ADP/O ratios were unaltered. It is unlikely that under *in vivo* conditions the uncoupling concentrations which Liu and Liun (1990) used were not reached. Alternately, the mitochondria might have recovered their potential for energy conservation, i.e., ADP/O ratios, within a week of Cd exposure.

It is now well recognized that the crucial peptides of cytochromes  $aa_3$  and b and of ATPase are mitochondrial gene products, whereas the peptides of cytochromes c and  $c_1$  and the dehydrogenases are coded by nuclear genes (Poyton and McEwen 1996). In view of this it may be suggested that Cd action is mediated by activating/inactivating specific mitochondrial and nuclear genes. It would thus appear that in the liver both mitochondrial and nuclear genes are affected by Cd within a short duration, whereas in due course these changes revert to normality (Table 5; Figs. 1, 2).

Our results on lipid/phospholipid profiles also suggest that the short-term and long-term effects were distinctly different, and although the respiratory activity more or less normalized in the 1 month group, the lipid/phospholipid profiles did not return to normal (Tables 6–8). This would suggest that Cd exposure has long-lasting effects on membrane structure/function relationships. It is unlikely that the observed effects could be the direct consequence of diminished energy potential since the content of TPL was not influenced to a significant extent, whereas that of CHL increased in the 1 week Cd group. It is hence possible that Cd directly affected the activities of the enzymes in biosynthetic pathways as well as the transfer of CHL and phospholipids from microsomes to mitochondria. This assumption is also substantiated by the fact that the contents of Lyso and SPM increased but those of PE decreased in the 1 week Cd group; in the 1 month group Lyso, SPM, and PC decreased. This interesting possibility, however, needs to be verified by more direct experiments. Larregle et al. (2007) reported that treatment with Cd decreased CHL and TGs in liver mitochondria. In whole kidney, CHL, TGs, and total TPLs decreased (El-Sharaky et al. 2008). Our results are at variance with these observations. This is probably due to the fact that these authors subjected the animals to chronic treatment with Cd given in drinking water.

It has been reported that the activity of membrane-bound  $Na^+$ ,  $K^+$ -ATPase, which is involved in maintaining ionic

balance, was stimulated in Cd-treated animals (Dhavale et al. 1988). The increased  $Na^+$ ,  $K^+$ -ATPase activity together with the decreased ADP phosphorylation rates which we noted here could lead to a decrease in ATP content in the cell. Liu and Liun (1990) have reported a decreased ATP content and concomitant decrease in cell viability. Thus the liver cell necrosis and cell death observed in Cd-exposed rats could be correlated with this decreased content of energy currency in the cell (Meiss et al. 1982; Dudley et al. 1982). Since ATP is required for all biosynthesis processes, e.g., DNA, RNA, and protein synthesis, all these processes will be affected following Cd exposure. In fact, inhibition of RNA polymerase, protein synthesis, DNA polymerase, and DNA synthesis following Cd exposure has already been reported (Degraeve 1981; Hidalgo et al. 1976; Norton and Kench 1977; Soll et al. 1976). This could be due either to a direct effect of Cd on these enzymes, or to the decreased ATP content, or to a combination of both. A mechanism by which Cd inhibits DNA synthesis was proposed by Rana et al. (1981). Cd blocks the formation of thymidine triphosphate, a prerequisite for DNA synthesis. Theocharis et al. (1992) have shown that the enzyme thymidylate kinase, which is required to convert thymidine monophosphate to thymidine diphosphate, is also inhibited in the presence of Cd. It is known that this enzyme requires high levels of ATP, which could limit the reaction leading to inhibition of DNA synthesis (Stoll et al. 1976). In contrast to this, the mechanism(s) underlying altered lipid/ phospholipid profiles would seem not to be influenced by ATP constraint.

It has been reported that in humans Cd has a long half-life and stays in the body for about 15–30 years (Vallee and Ulmer 1972). A comparable long half-life in rats could lead to continuous release for a considerably longer period of time. This prolonged exposure due to internal release could lead to damage and subsequent dysfunction of mitochondria. However, another important fact is that the toxicity of Cd in a tissue does not always correlate with its concentration in that tissue. An example is the testes, where, although the concentration of Cd in testes was higher in immature than in adult rats—probably due, as in the case of brain, to the incompletely developed blood-testis barrier/blood-brain barrier—the testes of immature rats were resistant to the damaging effect of Cd, whereas those of adult rats were not. This suggests that the Cd level is not the only factor in determining Cd toxicity (Klaassen and Wong 1982).

Exposure to Cd at relatively high and low levels causes necrosis and apoptosis, respectively, which suggests that the mode of cell death by cadmium is dependent on the exposure level (Sato et al. 2003). However, the molecular signaling underlying cadmium-induced apoptosis remains unclear. The reports available are equivocal and suggest

that apoptosis might occur by caspase-dependent or caspase-independent apoptotic pathways through mitochondria-mediated AIF translocation into the nucleus (Shih et al. 2003; Waisberg et al. 2003). At the cellular level Cd affects proliferation and differentiation, apoptosis, and other cellular activities (Bertin and Averbeck 2006; Waisberg et al. 2003).

Results of the present studies show that the short-term effects on respiratory activity more or less normalized at the end of 1 month of Cd exposure. Paradoxically, however, the lipid/phospholipid profiles did not return to normal. The increased CHL content in the 1 week group may relate to the similar increase in microsomal CHL content which we have reported previously (Modi et al. 2008). However, the CHL content in the 1 month group was normalized despite the high CHL content of the microsomes (Modi et al. 2008), which is suggestive of impaired transport. The increased SPM content at the expense of PE in the 1 week Cd group was an interesting feature. Also, the DPG content increased significantly, which may be a compensatory mechanism toward decreased respiratory activity. On the other hand, increased Lyso could be responsible for membrane destabilization (Early et al. 1992). Likewise, the increases in Lyso and SPM also parallel the changes in microsomes. At the end of 1 month the SPM content was overcorrected and the value became lower than that in the control, which was also the case for PC. This may, once again, relate to impaired transport, especially since microsomal SPM remained elevated. SPM is synthesized in the plasma membrane (Modi et al. 2008). However, the PI component overshot the control value. All in all, even at the end of 1 month the lipid profiles did not return to normal. Paradoxically, however, the membrane fluidity did not change. Based on these findings we can say that state 3 and state 4 respiration rates and dehydrogenases activities were significantly influenced by the lipid changes.

Results of the present studies have brought out yet another aspect of Cd toxicity at the mitochondrial level. From the data presented it may be inferred that energy transduction in the rat liver is susceptible to and is damaged by Cd exposure. Thus, while the 1 week group showed maximum impairment of respiratory activity, once the damage occurs the mitochondria lose their resistance and become susceptible to Cd insult. In addition, a single exposure to Cd is sufficient to cause sustained damage to mitochondrial oxidative phosphorylation, and after a longer period of time has elapsed following the entry of Cd the organelles may not be able to restore normal structure-function relationships. The results reported here thus provide new insight into the early and sustained damaging effects of Cd exposure.

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