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Glutathione mediated reductive activation and mitochondrial dysfunction play key roles in lithium induced oxidative stress and cytotoxicity in liver

Mohammad Reza Eskandari · Javad Khalili Fard · Mir-Jamal Hosseini · Jalal Pourahmad

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Abstract Lithium preparations are commonly used drug in treating mental disorders and bipolar diseases, but metal's cytotoxic mechanisms have not yet been completely understood. In this study, we investigated the cytotoxic mechanisms of lithium in freshly isolated rat hepatocytes. Lithium cytotoxicity were associated with reactive oxygen species (ROS) formation and collapse of mitochondrial membrane potential and cytochrome c release into the hepatocyte cytosol. All of the mentioned lithium-induced cytotoxicity markers were significantly ($P < 0.05$) prevented by ROS scavengers, antioxidants, mitochondrial permeability transition pore sealing agents and adenosine triphosphate generators. Hepatocyte glutathione (GSH) was also rapidly oxidized and GSH-depleted hepatocytes were more resistant to lithium-induced oxidative stress markers. This suggests that lithium is activated by GSH. Our results also showed that CYP2E1 is involved in lithium oxidative stress mechanism. Lithium cytotoxicity was also associated with mitochondrial injuries initiated by increased ROS formation resulted from metal-CYP2E1 destructive interaction or metal-induced disruption of mitochondrial electron transfer chain. Methyl donors such as betaine, methionine, or folic acid prevented lithium cytotoxicity, and this suggests that this metal is detoxified by phase II metabolic methylation. In conclusion lithium-induced cytotoxicity could be attributed to oxidative stress and mitochondrial dysfunction.

Keywords Lithium · Glutathione · Oxidative stress · Methylation · Hepatotoxicity

M. R. Eskandari · M.-J. Hosseini
Department of Pharmacology and Toxicology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran

J. K. Fard · M.-J. Hosseini · J. Pourahmad (✉)
Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, P.O.Box 14155-6153, Tehran, Iran
e-mail: j.pourahmadjaktaji@utoronto.ca

Introduction

Lithium preparations (Li^+) have been widely used as a therapeutic agent in the treatment and prophylaxis of mania, bipolar affective disorder, recurrent depression and aggressive or self-mutilating behavior (Blake et al. 2008). This mood stabilizer drug has a narrow therapeutic index and easily absorbed from the gut, distributed readily throughout the body and is excreted by kidney (British National Formulary 2006; Jefferson 2010). It induces multiple biochemical and molecular effects on neurotransmitter receptor-mediated signaling, signal transduction cascades, hormonal and circadian regulation, ion transport, and gene expression (Shaldubina et al.

2001; Al Banchaabouchi et al. 2004; Kiełczykowska et al. 2008). These effects have been widely associated with the activation of neurotrophic pathways, and neuroprotection has been the most expected and replicated biological effect associated with lithium use in both human and preclinical studies (Chiu and Chuang 2010).

Adverse reactions of lithium therapy in human associated with endocrine, gastrointestinal, dermatologic, and ophthalmologic effects. Long-duration treatment of lithium has been also found to cause nephrotoxicity and cardiovascular abnormalities (Riadh et al. 2011; Dunner 2000).There are various reports reflecting the toxic effects of lithium on liver structure and function (Aniya and Matsusaki 1983; Hunt et al. 1983; Tandon et al. 1997; Chadha et al. 2008). Lithium-treated rats indicated marked alterations in hepatic structure (Chadha et al. 2008). Lithium has been shown to influence the hepatic drug metabolizing enzyme system (Aniya and Matsusaki 1983). Cytotoxic effects of Li^+ that affect the liver tissue are manifested by the disturbances of NO, a key mediator of signaling events linked to apoptotic cell death (Tajes et al. 2008).

It was shown that lithium treatment caused alterations in the activities of Na+/K+ ATPase, hepatic phase II drug metabolizing enzymes and hepatic trace elemental profile (Tandon et al. 1997, 1999). Lithium stimulates transamination reaction in hepatic and renal tissues and inhibits the levels of serum alanine and aspartate transaminases (ALT and AST)(Ahmad et al. 2011). Lithium reduced the glutathione (GSH) levels and also increased lipid peroxidation and the activities of antioxidative enzymes, which included catalase, glutathione S-transferase, and superoxide dismutase (Malhotra and Dhawan 2008).

Oxidative stress is an overproduction of reactive oxygen species (ROS) that overwhelms the cellular antioxidant capacity (Shao et al. 2008). So it seems that increased ROS generation and oxidative stress have the central role in lithium cytotoxic mechanism. However, more detailed studies are needed to clarify the exact consequences of Li^+ induced increased ROS formation. Moreover, the whole mechanistic picture involved in lithium-induced hepatotoxicity has not yet been completely elucidated. The major objective of this study was to determine the biochemical and cellular mechanisms involved in lithium-induced hepatotoxicity in isolated rat hepatocytes.

Materials and methods

Chemicals

Lithium carbonate, rhodamine 123, collagenase (from Clostridium histolyticum), bovine serum albumin (BSA), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), *O*-phthalaldehyde, acridine orange, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), trichloroacetic acid, dimethyl sulfoxide (DMSO), 1-bromoheptane, butylated hydroxytoluene (BHT), diphenyliodonium chloride (DPI), Trypan blue and heparin were purchased from Sigma-Aldrich Co. (Taufkrichen, Germany). All other chemicals were of the highest commercial grade available.

Animals

Male Sprague–Dawley rats weighing 280–300 g were housed in ventilated plastic cages over PWI 8–16 hardwood bedding. There were 12 air changes per hour, 12 h light photoperiod (lights on at 0800 h) and an environmental temperature of 21–23 °C with a 50–60 % relative humidity. The animals were fed with a normal standard chow diet and tap water ad libitum. All experiments were conducted according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver and viability was assessed by plasma membrane disruption determined by trypan blue (0.2 w/v) exclusion test (Pourahmad et al. 2005). Cells were suspended at a density of 10^6 cells/ml in round-bottomed flasks rotating in a water bath maintained at 37 °C in Krebs–Henseleit buffer (pH = 7.4), supplemented with 12.5 mM HEPES under an atmosphere of 10 % O_2, 85 % N_2, and 5 % CO_2. Hepatocytes were preincubated for 30 min prior to addition of chemicals. Stock solutions of all chemicals (100× concentrated for the water solutions or 1,000× concentrated for the methanolic solutions) were prepared fresh prior to use. To avoid either non-toxic or very toxic conditions in this study, we used EC50 concentrations for lithium carbonate (2 mM) which is equal to 2 mEq/l. The EC50 of a chemical in hepatocyte cytotoxicity assessment

technique (with the total 3 h incubation period) is defined as the concentration, which decreases the hepatocyte viability down to 50 % following the 2 h of incubation (Galati et al. 2000). In order to determine this value for lithium carbonate, dose–response curves were plotted and then EC50 was determined based on a regression plot of three different concentrations (Fig. 1). To incubate lithium carbonate and all other water soluble treatments with the required concentration, we added 100 µl sample of its concentrated stock solution (100× concentrated) to one rotating flask containing 10 ml hepatocyte suspension. For the chemicals, which dissolved in methanol, we prepared methanolic stock solutions (1,000× concentrated), and to achieve the required concentration in the hepatocytes, we added 10 µl samples of the stock solution to the 10 ml cell suspension. Ten microliters of methanol did not affect the hepatocyte viability after 3 h incubation (data not shown). GSH depleted hepatocytes were prepared by preincubation of hepatocytes with 200 µM 1-bromoheptane for 30 min as described by Khan and O'Brien (1991).

Cell viability

The viability of isolated hepatocytes was assessed from the intactness of the plasma membrane as determined by the trypan blue (0.2 % w/v) exclusion test (Pourahmad et al. 2010a). Aliquots of the hepatocyte incubate were taken at different time points during the 3 h incubation period.

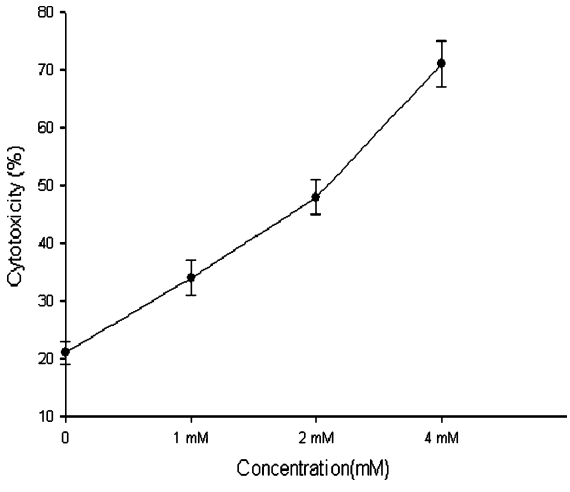

Fig. 1 The dose response curve for $EC50_{2h}$ concentration of lithium. Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 3.0 h following the addition of Li_2CO_3 (2 mM). Cytotoxicity was determined as the percentage of cells that take up trypan blue Khan and O'Brien (1991)

Determination of ROS

To determine the rate of hepatocyte ROS generation induced by lithium, DCFH-DA was added to the hepatocytes. It penetrates hepatocyte cells and is hydrolyzed to non-fluorescent dichlorofluorescin (DCFH). The latter then reacts with ROS to form the highly fluorescent dichlorofluorescin (DCF), which effluxes the cell. The fluorescence intensity of DCF was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (Pourahmad et al. 2010b).

Intracellular GSH and extra cellular GSSG assessment

GSH and GSSG were determined according to the spectrofluorometric method (Hissin and Hilf 1976). Each sample was measured in quarts cuvettes using a fluorimeter set for 350 nm excitation and 420 nm emission wavelengths.

Mitochondrial membrane potential assay

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123, has been used for estimation of mitochondrial membrane potential (Andersson et al. 1987). The amount of rhodamine 123 remaining in the incubation medium was measured fluorometrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to up take the rhodamine 123 was calculated as the difference (between control and treated cells) in rhodamine 123 fluorescence. Our data were shown as the percentage of mitochondrial membrane potential collapse (%$\Delta\Psi$m) in all treated (test) hepatocyte groups (Andersson et al. 1987).

Determination of cytochrome c release

Release of cytochrome c from mitochondria into cytosol is a key initiating step in both apoptotic and

necrotic cell death processes in intact cells (Gogvadze et al. 2006). Detection of cytochrome c release was determined by Cytochrome c ELISA Kit (Quantikine M., R&D Systems, Abingdon, UK) according to the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique and is designed to accurately quantify natural rat or mouse cytochrome c in cell lysates and subcellular fractions.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD as the post hoc test. Levene's test was used to check the homogeneity of variances. Results were presented as mean ± SD of triplicate samples. The triplicate samples were representative of three separate rats ($n = 3$). The minimal level of significance chosen was $P < 0.05$.

Results

At least 80–90 % of the control cells were viable following 3 h of incubation. The $EC50_{2h}$ found for Li^+ (i.e., 50 % membrane lysis in 2 h) was 2 mM. As shown in Table 1, Li^+ (2 mM) significantly increased hepatocyte membrane lysis comparing to control hepatocytes ($P < 0.05$). Hepatocyte membrane lysis was determined by trypan blue exclusion test. In addition, when hepatocytes were incubated with lithium at this $EC50_{2h}$ concentration of 2 mM, ROS formation determined by the oxidation of DCFH-DA to DCF was significantly ($P < 0.05$) increased (Table 2). Both lithium induced cytotoxicity and increased ROS formation were prevented by antioxidants and ROS scavengers (BHT, deferoxamine a Fe^{2+} chelator and mannitol), mitochondrial permeability transition (MPT) pore sealing agents (carnitine and cyclosporine), and natural methyl donors (methionine, betaine, and folic acid) (Tables 1, 2). On the other hand, inactivating hepatocyte methyl transferase (the enzyme is involved in biomethylation) with sodium selenite and also preincubating hepatocytes with hypomethylating agents (azacytidine, DMSO) significantly ($P < 0.05$) increased lithium toxicity as well as ROS generation. Our results also showed that lithium induced cytotoxicity and ROS formation were significantly ($P < 0.05$) increased by GSH and GSH synthesis stimulator, trifluoperazine. However, depleting hepatocyte GSH beforehand protected the hepatocyte against lithium induced cytotoxicity and ROS formation (Tables 1, 2). All of the reagents used in

Table 1 Effect of antioxidants, ROS scavenger, MPT pore sealing agents, methyldonors, hypomethylators, and CYP2E1 inhibitors on lithium induced hepatocyte lysis

Addition	Cytotoxicity (%)		
	Incubation time		
	1 h	2 h	3 h
Control	18 ± 1	21 ± 2	25 ± 2
+Li (2 mM)	36 ± 2[a]	48 ± 3[a]	68 ± 4[a]
+BHT (50 μM)	25 ± 2[b]	31 ± 3[b]	37 ± 3[b]
+Deferoxamine (200 μM)	26 ± 2[b]	34 ± 3[b]	42 ± 4[b]
+Mannitol (50 mM)	25 ± 2[b]	35 ± 2[b]	44 ± 3[b]
+Carnitine (2 mM)	28 ± 2[b]	31 ± 2[b]	38 ± 4[b]
+Cyclosporine (2 μM)	25 ± 1[b]	30 ± 3[b]	38 ± 3[b]
+Chloroquine (100 μM)	28 ± 3[b]	37 ± 3[b]	50 ± 4[b]
+Methylamine (30 mM)	30 ± 2[b]	36 ± 3[b]	42 ± 4[b]
+3-Methyladenine (5 mM)	28 ± 2[b]	36 ± 3[b]	50 ± 3[b]
+Monensin (10 μM)	27 ± 2[b]	35 ± 2[b]	48 ± 3[b]
+Methionine (1 mM)	23 ± 1[b]	33 ± 3[b]	44 ± 4[b]
+Betaine (2 mM)	28 ± 3[b]	36 ± 3[b]	51 ± 4[b]
+Folic acid (100 μM)	27 ± 2[b]	35 ± 2[b]	43 ± 3[b]
+Sodium selenite (4 μM)	50 ± 3[b]	67 ± 5[b]	90 ± 4[b]
+Azacytidine (2.5 μM)	48 ± 4[b]	66 ± 4[b]	89 ± 5[b]
+DMSO (150 μM)	49 ± 4[b]	69 ± 5[b]	88 ± 5[b]
+GSH (2 mM)	48 ± 3[b]	67 ± 5[b]	94 ± 6[b]
+Trifluoperazine (15 μM)	47 ± 2[b]	65 ± 4[b]	92 ± 5[b]
+Phenylimidazole (300 μM)	27 ± 1[b]	29 ± 2[b]	31 ± 2[b]
+Diphenyliodonium chloride(50 μM)	24 ± 2[b]	26 ± 2[b]	30 ± 3[b]
+4-Methylpyrazole (500 μM)	26 ± 2[b]	27 ± 3[b]	33 ± 3[b]
GSH depleted hepatocytes + Li (2 mM)	26 ± 2[b]	38 ± 3[b]	46 ± 4[b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 3.0 h following the addition of Li_2CO_3 (2 mM). Cytotoxicity was determined as the percentage of cells that take up trypan blue Khan and O'Brien (1991). GSH depleted hepatocytes were prepared as described by Khan and O'Brien (1991)

Values are expressed as mean ± SD of three separate experiments ($n = 3$)

[a] Significant difference in comparison with control hepatocytes ($P < 0.05$)

[b] Significant difference in comparison with Li_2CO_3 treated hepatocytes ($P < 0.05$)

Table 2 Effect of antioxidants, ROS scavenger, MPT pore sealing agents, methyldonors, hypomethylators, and CYP2E1 inhibitors on lithium induced ROS formation

Addition	ROS formation (DCF)		
	Incubation time		
	15 min	30 min	60 min
Control	379 ± 23	408 ± 31	420 ± 32
+Li (2 mM)	801 ± 59[a]	671 ± 47[a]	635 ± 49[a]
+BHT (50 μM)	452 ± 28[b]	477 ± 31[b]	434 ± 29[b]
+Deferoxamine (200 μM)	442 ± 29[b]	470 ± 35[b]	436 ± 30[b]
+Mannitol (50 mM)	445 ± 38[b]	525 ± 43[b]	435 ± 30[b]
+Carnitine (2 mM)	427 ± 28[b]	428 ± 35[b]	421 ± 27[b]
+Cyclosporine (2 μM)	396 ± 25[b]	453 ± 33[b]	448 ± 39[b]
+Methionine (1 mM)	416 ± 35[b]	544 ± 45[b]	445 ± 34[b]
+Betaine (2 mM)	486 ± 39[b]	447 ± 29[b]	491 ± 37[b]
+Folic acid (100 μM)	452 ± 29[b]	480 ± 33[b]	437 ± 40[b]
+Sodium selenite (4 μM)	999 ± 80[b]	993 ± 79[b]	949 ± 77[b]
+Azacytidine (2.5 μM)	975 ± 70[b]	968 ± 56[b]	935 ± 61[b]
+DMSO (150 μM)	988 ± 79[b]	980 ± 76[b]	938 ± 68[b]
+GSH (2 mM)	950 ± 32[b]	877 ± 61[b]	872 ± 45[b]
+Trifluoperazine (15 μM)	932 ± 23[b]	864 ± 46[b]	860 ± 39[b]
+Phenylimidazole (300 μM)	460 ± 26[b]	433 ± 32[b]	424 ± 33[b]
+Diphenyliodonium chloride(50 μM)	381 ± 25[b]	432 ± 30[b]	423 ± 25[b]
+4-Methylpyrazole (500 μM)	392 ± 28[b]	436 ± 31[b]	428 ± 28[b]
GSH depleted hepatocytes + Li + (2 mM)	482 ± 35[b]	5,448 ± 50[b]	436 ± 41[b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 1.0 h following the addition of Li_2CO_3 (2 mM). Dichlorofluorescein (DCF) formation was expressed as fluorescent intensity units (Pourahmad and O'Brien 2000). GSH depleted hepatocytes were prepared as described by Khan and O'Brien (1991)

Values are expressed as mean ± SD of three separate experiments ($n = 3$)

[a] Significant difference in comparison with control hepatocytes ($P < 0.05$)

[b] Significant difference in comparison with Li_2CO_3 treated hepatocytes ($P < 0.05$)

cytotoxicity study including antioxidants and ROS scavengers, MPT pore sealing agents, methyl donors, methyl transferase inhibitor, hypomethylating agents, and 1-bromoheptane (used for GSH depleting) did not significantly ($P < 0.05$) increase hepatocyte membrane lysis and ROS formation at concentrations used while incubated alone in isolated hepatocytes (data not shown).

As shown in Table 3, incubation of hepatocytes with lithium (2 mM) caused rapid hepatocyte GSH depletion. Most of the lithium induced hepatocyte GSH depletion could be attributed to the expulsion of GSSG (Table 3). Also pretreatment of hepatocytes with antioxidants and ROS scavengers (BHT, deferoxamine, and mannitol), MPT pore sealing agents (carnitine and cyclosporine), and natural methyl donors (methionine, betaine, and folic acid) significantly ($P < 0.05$) prevented both lithium induced hepatocyte intracellular GSH decrease and extracellular GSSG increase (Table 3). On the other hand, methyl transferase inhibitor (sodium selenite) and hypomethylating agents (azacytidine, DMSO) potentiated hepatocyte GSH depletion. All of these protective agents did not show any significant effect ($P < 0.05$) on hepatocytes GSH/GSSG status at concentrations used (data not shown).

As shown in Table 4, lithium (2 mM) induced mitochondrial membrane potential collapse during 2 h of incubation which was prevented by antioxidants and ROS scavengers (BHT, deferoxamine and mannitol) suggesting that lithium induced mitochondrial membrane potential decrease was subsequent of ROS

Table 3 Effect of antioxidants, ROS scavenger, MPT pore sealing agents, methyldonors, hypomethylators, and CYP2E1 inhibitors on lithium induced glutathione depletion

Addition	Intracellular GSH (μM) 3 h	Extra cellular GSSG (μM) 3 h
Control	33.1 ± 1.9[b]	1.5 ± 0.1[b]
+Li (2 mM)	13.2 ± 0.6[b]	7.6 ± 0.5[b]
+BHT (50 μM)	30.3 ± 1.7[b]	2.4 ± 0.2[b]
+Deferoxamine (200 μM)	31.0 ± 2.6[b]	2.5 ± 0.2[b]
+Mannitol (50 mM)	29.4 ± 1.5[b]	1.7 ± 0.1[b]
+Carnitine (2 mM)	27.4 ± 1.9[b]	1.9 ± 0.2[b]
+Cyclosporine (2 μM)	31.4 ± 2.0[b]	2.4 ± 0.2[b]
+Methionine (1 mM)	29.5 ± 2.2[b]	2.9 ± 0.1[b]
+Betaine (2 mM)	30.9 ± 1.9[b]	2.8 ± 0.2[b]
+Folic acid (100 μM)	29.6 ± 2.2[b]	2.1 ± 0.1[b]
+Sodium selenite (4 μM)	10.5 ± 0.9[c]	9.2 ± 0.6[c]
+Azacytidine (2.5 μM)	10.7 ± 0.8[c]	9.1 ± 0.5[c]
+DMSO (150 μM)	10.8 ± 0.5[c]	9.2 ± 0.5[c]
+Phenylimidazole (300 μM)	28.1 ± 1.8[c]	2.3 ± 0.1[c]
+Diphenyliodonium chloride (50 μM)	29.4 ± 2.6[c]	2.2 ± 0.2[c]
+4-Methylpyrazole (500 μM)	28.9 ± 1.9[c]	2.4 ± 0.1[c]

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 2.0 h following the addition of Li_2CO_3 (2 mM). Intracellular GSH and extra cellular GSSG were fluorometrically determined as described by Hissin and Hilf (1976)

Values are expressed as mean ± SD of three separate experiments ($n = 3$)

[a] Significant difference in comparison with control hepatocytes ($P < 0.05$)

[b] Significant difference in comparison with Li_2CO_3 treated hepatocytes ($P < 0.05$)

formation. Lithium induced hepatocyte mitochondrial membrane potential collapse was also prevented by methyl donors (methionine, betaine, and folic acid) and also depleting GSH beforehand protected hepatocytes against lithium induced GSH depletion. However, lithium induced mitochondrial membrane potential decline was increased by methyl transferase inhibitor (sodium selenite), hypomethylating agents (azacytidine, DMSO), GSH, and trifluoperazine (GSH synthesis stimulator) (Table 4). All of these mentioned inhibitors did not show any significant effect ($P < 0.05$) on hepatocytes mitochondrial membrane

Table 4 Mitochondrial membrane potential decline during lithium induced hepatocyte injury

Addition	%ΔΨm		
	Incubation time		
	15 min	30 min	60 min
+Li (2 mM)	41 ± 2	55 ± 3	65 ± 5
+BHT (50 μM)	7 ± 1[a]	8 ± 1[a]	11 ± 1[a]
+Deferoxamine (200 μM)	6 ± 1[a]	6 ± 1[a]	10 ± 2[a]
+Mannitol (50 mM)	6 ± 1[a]	8 ± 1[a]	15 ± 1[a]
+Carnitine (2 mM)	7 ± 1[a]	8 ± 1[a]	9 ± 1[a]
+Cyclosporine (2 μM)	7 ± 1[a]	7 ± 1[a]	9 ± 1[a]
+Methionine (1 mM)	10 ± 1[a]	11 ± 2[a]	15 ± 2[a]
+Betaine (2 mM)	8 ± 1[a]	8 ± 1[a]	10 ± 1[a]
+Folic acid (100 μM)	6 ± 1[a]	6 ± 1[a]	6 ± 1[a]
+Sodium selenite (4 μM)	55 ± 4[a]	72 ± 3[a]	87 ± 2[a]
+Azacytidine (2.5 μM)	52 ± 2[a]	68 ± 2[a]	82 ± 2[a]
+DMSO (150 μM)	54 ± 3[a]	70 ± 5[a]	84 ± 4[a]
+GSH (2 mM)	77 ± 6[a]	85 ± 4[a]	96 ± 2[a]
+Trifluoperazine (15 μM)	74 ± 5[a]	82 ± 6[a]	99 ± 1[a]
+Phenylimidazole (300 μM)	7 ± 1[a]	7 ± 1[a]	8 ± 1[a]
+Diphenyliodonium chloride (50 μM)	6 ± 1[a]	7 ± 1[a]	8 ± 1[a]
+4-Methylpyrazole (500 μM)	9 ± 1[a]	8 ± 1[a]	8 ± 1[a]
GSH depleted hepatocytes + Li (2 mM)	13 ± 3[a]	15 ± 3[a]	17 ± 3[a]

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 1.0 h following the addition of Li_2CO_3 (2 mM). Mitochondrial membrane potential was determined as the difference in mitochondrial uptake of the rhodamine 123 between control and treated cells. Our data were shown as the percentage of mitochondrial membrane potential collapse (%ΔΨm) in all treated (test) hepatocyte groups (Pourahmad and O'Brien 2001). GSH depleted hepatocytes were prepared as described by Khan and O'Brien (1991)

Values are expressed as mean ± SD of three separate experiments ($n = 3$)

[a] Significant difference in comparison with Li_2CO_3 treated hepatocytes ($P < 0.05$)

potential at concentrations used while incubated alone in hepatocytes (data not shown).

To examine the involvement of cytochrome P450s in lithium cytotoxicity mechanism, we evaluated the effects of NADPH P450 reductase (DPI) and CYP2E1 inhibitors (phenylimidazole, 4-methylpyrazole) on the all the measured cytotoxicity parameters. These agents significantly ($P < 0.05$) prevented lithium-induced cytotoxicity, ROS generation, GSH depletion, decline

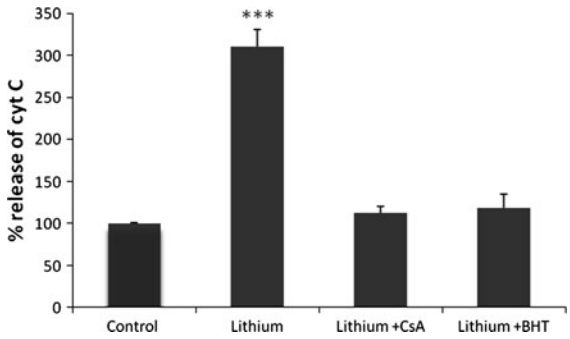

Fig. 2 Effect of lithium-induced cytochrome c release in isolated rat hepatocyte. Cytochrome c release was measured using an ELISA kit. *Each histogram* represents the mean ± SEM of three different determinations. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

of mitochondrial membrane potential and acridine orange release. All these reagents did not show any significant effect on the mentioned cytotoxicity markers at concentrations used (data not shown).

Induction of MPT is supposed to be resulted from opening of the related pores in mitochondrial membrane leading to the release of cytochrome c (Gogvadze et al. 2006). In order to confirm the occurrence of this intra-mitochondrial protein release into the cytosol, cytochrome c release in lithium treated rat hepatocyte was determined spectrophotometrically at wavelength of 450 nm with an ELIZA reader. As shown in Fig. 2, lithium (2 mM) induced release of cytochrome c from mitochondria into the cytosol following 1 h of incubation in isolated rat hepatocyte before cytotoxicity ensued. Lithium induced cytochrome c release was prevented by both MPT pore sealing agent (cyclosporine A) and antioxidant (BHT) suggesting that lithium induced cytochrome c expulsion was subsequent of MPT pore opening due to ROS formation. Cyclosporine A and BHT did not caused any significant effect on the hepatocyte mitochondrial cytochrome c release at concentrations used (data not shown).

Discussion

Lithium toxicity represents a state of increased oxidative stress, which is mainly based on the evidence of increased lipid peroxidation, or by indirect evidence of reduced antioxidant reserve, such as SOD and catalase enzymes, in animal models (Tandon et al. 1998). Previous studies also revealed that liver is one of the main targets for lithium toxicity (Aniya and Matsusaki 1983; Hunt et al. 1983; Tandon et al. 1997; Chadha et al. 2008). Although, the cellular and molecular mechanisms involved in the lithium induced liver toxicity have not yet been completely understood. One of the strongest mechanistic hypotheses in lithium induced hepatotoxicity is oxidative stress (Chadha et al. 2008). Oxidative stress is a result of imbalance between the production and removal of ROS, signal molecules initiating cell death pathways, and indicates overproduction of these substances or significant depletion of antioxidant defense molecules (Halliwell 2006; Chirino and Pedraza-Chaverri 2009).

Our results showed that lithium induced sharp rise in hepatocytes ROS formation and cell membrane lysis (cytotoxicity) after incubation in isolated rat hepatocytes. Our data also supported the hypothesis that lithium induced cytotoxicity is directly related to ROS formation which could be a key player in the mechanism underlying lithium induced liver injury. Overproduction of ROS or significant depletion of antioxidant defense molecules (e.g., GSH) causes oxidative stress in different cells (Valko et al. 2007). Our results showed that when isolated hepatocytes were incubated with lithium, GSH depletion was occurred as a consequence of ROS formation. A previous study also reported that lithium treated rats showed a significant decrease in the levels of reduced GSH, catalase and glutathione-*S*-transferase, following 2–4 month of daily lithium treatment (Chadha et al. 2008).

GSH is an intracellular antioxidant that prevents intracellular ROS formation and oxidative stress. As an antioxidant, it is involved in cell protection from the noxious effect of oxidative stress, both directly and as a cofactor of GSH peroxidases (GPxs), and these reactions generate oxidized GSH (GSSG) (Pompella et al. 2003). On the other hand, some recent studies have also highlighted the ability of GSH to promote oxidative processes by participating in metal ion mediated reactions eventually leading to the formation of ROS and free radicals. A crucial role in these phenomena is played by membrane bound γ-glutamyltransferase (GGT) activity (Pompella et al. 2003). It was already reported that lithium treatment could significantly increase GPx and GGT activities, and decrease GSSG reductase activity in protein-deficient rats (Chadha et al. 2008). Our results showed that

lithium caused rapid hepatocyte GSH depletion following incubation in hepatocyte which is quite in agreement of the previous work regarding the metals impact on the activities of GPx and GSSG reductase. On the other hand, lithium also promoted oxidative stress when incubated with GSH and GSH synthesis stimulator, trifluoperazine. Cytotoxicity, ROS formation and mitochondrial membrane damage induced by lithium was interestingly potentiated by GSH and trifluoperazine. Also, depleting hepatocyte GSH beforehand prevented the hepatocytes against lithium induced cytotoxicity, ROS generation, mitochondrial membrane damage which reminds toxic reactions of GSH with some other metals including copper, chromium, uranium, cisplatin, and thallium (Pourahmad and O'Brien 2000, 2001; Pourahmad et al. 2006, 2010a, b, c, d; Riadh et al. 2011).

GSH is able to bind metal cations, a feature likely exploited by the cell during transport and delivery of metals (Ciriolo et al. 1990). Interactions of GSH with metal ions can be more complex, with important bearings on the redox environment of the cell. Thiol compounds especially when dissociated to their thiolate anion ($R–S^-$) forms can in fact affect the reduction of metal cations, for example, iron and copper. Electrons can be then transferred in turn from metal ions to molecular oxygen, thus generating superoxide anions, which will easily dismutate to the strong prooxidant hydrogen peroxide. The sequence—a true ''redox cycling'' of metals—can proceed with even minimal concentrations of metal ions as long as electron donors (thiols) and acceptor (molecular oxygen) are available in the system (Kappus and Sies 1981). In this way, paradoxically, the ''reducing'' ability of thiols is finally turned into overall ''oxidizing'' effects (e.g., lipid peroxidation) (Tien et al. 1982; Paolicchi et al. 1999). The efficiency of individual thiol compounds in metal ion reduction is determined by the relative p*K*a's of their thiols, that is, by the ease of dissociation of their SH groups to the corresponding thiolate anions. GSH, a tripeptide consisting of glycine (Gly), cysteine (Cys), and glutamic acid (Glu), itself is rather less efficient than other thiols, an aspect which can be explained by the vicinity of the SH group of cysteine to the a-carboxyl group of glutamic acid (Paolicchi et al. 1999). The removal of glutamic acid in fact produces a marked increase in the metal-reducing ability, resulting in a promotion of oxidative processes (Tien et al. 1982; Paolicchi et al. 1999). The cleavage of the γ-glutamyl bond in GSH is physiologically affected (extracellularly) by membrane-bound GGT, the sole enzyme capable of doing it, expressed at various levels in cells and tissues. The possibility thus exists that sites of GGT activity may act as sites of promotion of metal ion reduction and redox cycling, with consequent stimulation of oxidative processes.

The following series of chemical reactions could explain the interactions of GSH as a thiol compound and Li^+. γ-Glutamyltransferase (GGT), superoxide dismutase (SOD) and glutathione peroxidase (GPxs) catalyze these reactions:

$$\mathrm{Glu-Cys-Gly} \xrightarrow{\mathrm{GGT}} \mathrm{Gly-Cys-Sh+GLU}$$

$$\mathrm{Gly-Cys-SH+Li^+ \rightarrow Gly-Cys-S^\circ + Li}$$

$$\mathrm{Li+O_2 \rightarrow Li^+ + O_2^{\circ -}}$$

$$2\mathrm{O_2^{\circ -}} \xrightarrow[2\mathrm{H^+}]{\mathrm{SOD}} \mathrm{H_2O_2 + O_2}$$

$$\mathrm{H_2O_2 + GSH} \xrightarrow{\mathrm{GPxs}} \mathrm{GSSG + H_2O}$$

It is therefore suggested that Li^+ might exert cytotoxic effects via oxygen reduction and when it is reduced by a one-electron transfer reaction, which in turn reduce O_2 to $O_2^{\circ -}$, the superoxide anion radical. Thus, these reactions result in the generation of further ROS and further toxicity. In the following another suggested mechanism could be explained by interactions of generated thiolate anion in the first reaction with the thiyl radical in the second reaction:

$$\mathrm{Gly-Cys-S^- + Gly-Cys-S^\circ \rightarrow Gly-Cys-Cys-Gly^{\circ -}}$$

$$\mathrm{Gly-Cys-Cys-Gly^{\circ -} + O_2 \rightarrow Gly-Cys-Cys-Gly + O_2^{\circ -}}$$

$$2\mathrm{O_2^{\circ -}} \xrightarrow[2\mathrm{H^+}]{\mathrm{SOD}} \mathrm{H_2O_2 + O_2}$$

$$\mathrm{H_2O_2 + GSH} \xrightarrow{\mathrm{GPxs}} \mathrm{GSSG + H_2O}$$

In these reactions the superoxide anion radical is generated and promotes oxidative stress and toxicity. These findings may provide mechanistic clues to a possible role of GSH and thiol compounds in Li^+ induced toxicity. Since previous studies supported the role of lithium at increasing cellular GSH synthesis through activation of GGT in hepatocyte (Chadha et al. 2008), we can therefore consider the trifluoperazine pretreatment effect as a potentiation of toxic

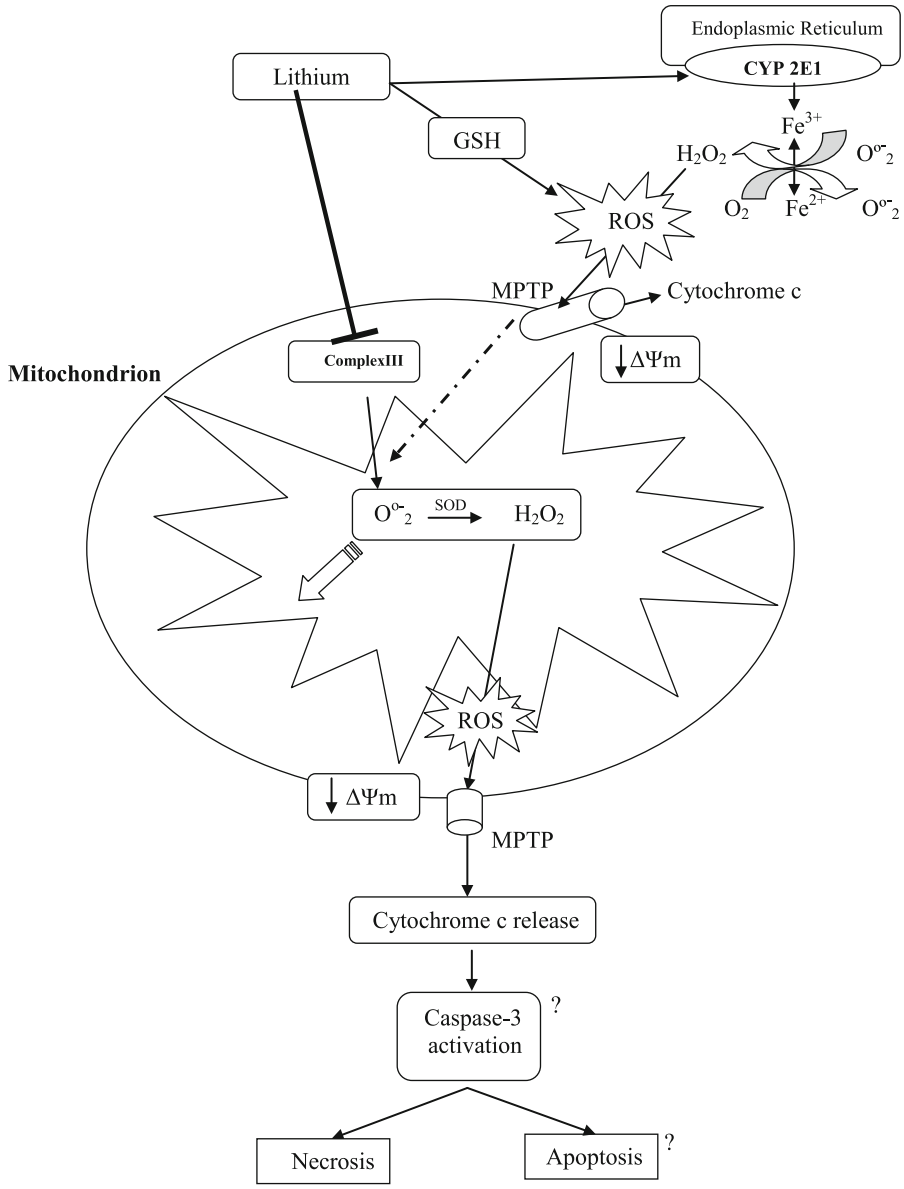

Fig. 3 Proposed mechanisms of lithium induced hepatocyte cytotoxicity. ΔΨm mitochondrial membrane potential; *MPTP* mitochondrial permeability transition pore

GSH and lithium reactions leading to more ROS production culminated in higher liver toxicity.

Our results showed that hepatocyte mitochondrial membrane potential (ΔΨm) was rapidly decreased by lithium. Lithium induced mitochondrial membrane potential decrease was prevented by antioxidants and radical scavengers indicating that mitochondrial membrane damage was a consequence of ROS formation. The ΔΨm is maintained by continuous pumping of protons from the matrix across the inner mitochondrial membrane into the intermembrane space. Because these protons in turn are used to drive the ATP synthase, a collapse of the ΔΨm invariably results in compromised ATP synthesis (Spear and Aust 1994). Any damage to mitochondrial ATP generation results in intracellular acidosis and osmotic injury. The later is the cause of plasma membrane lysis (Grimm and Brdiczka 2007).

Considering our finding that lithium induced cytotoxicity markers were significantly increased by

inactivating hepatocyte methyl transferase or preincabating with hypomethylating agents but all were prevented by methyl donors such as methionine, betaine and folic acid, we suggest that lithium detoxification pathway in rat hepatocytes is phase II metabolic methylation.

Our results showed that CYP2E1 inhibitors prevented lithium induced cytotoxicity, ROS generation, mitochondrial membrane damage and GSH depletion. Because metabolic bioactivation is not likely a process for lithium toxicity, it can therefore be suggested that lithium may undergo destructive interaction with CYP2E1 leading to release of heme and significant ROS formation. The same mechanism was previously observed with cisplatin and thallium (Pourahmad et al. 2010a, d; Eskandari et al. 2011). It was also suggested that lithium might increase uncoupling activity in CYP2E1 and thus increasing ROS generation (Boelsterli 2007). Then, release of catalytic iron from the heme of CYP2E1 and subsequent production of ROS could be responsible for the cytotoxicity mechanism initiation.

In general, Our results confirmed that lithium induced liver toxicity is resulted from different cytotoxic events including: increased ROS formation via either through destructive reaction with CYP 2E1 and release of heme molecule or direct inhibition of mitochondrial respiratory chain, and subsequent change of mitochondrial membrane conformation to opened MPT pore position and cytochrome c release into the cytosol which causes mitochondrial collapse of Ψm. Cytochrome c release is the regulating step for mitochondria-mediated cell death signaling which could lead to both apoptosis and necrosis. And finally our findings contribute to a better understanding of cytotoxic mechanisms of lithium in isolated rat hepatocytes (Fig. 3).

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