Original Article

Alpha-lipoic acid protects against cadmium-induced hepatotoxicity via calcium signalling and gap junctional intercellular communication in rat hepatocytes

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(Received January 25, 2015; Accepted April 27, 2015)

ABSTRACT — This study investigated the protective effect of alpha-lipoic acid (LA) on cadmium (Cd)-induced hepatotoxicity in BRL 3A rat liver cells. We demonstrated that LA ameliorated Cd-induced cellular injury in cell viability and nuclear fragmentation in BRL 3A cells. Furthermore, LA markedly ameliorated Cd-induced gap junctional intercellular communication (GJIC) inhibition and Cx43 mRNA expression decrease, as well as disassembly of gap junctions. The gap junction blocker carbenoxolone disodium (CBX) as well as LA protected healthy cells from Cd-exposed cells in Transwell co-culture system. LA also protected BRL 3A cells against Cd-induced elevation of the intracellular concentration of free calcium ($[Ca^{2+}]_i$). Pretreatment with a chelator of intracellular Ca^{2+} BAPTA-AM or chelator of extracellular Ca^{2+} EGTA attenuated Cd-induced cytotoxicity and GJIC inhibition. CBX exacerbated the decrease in cell viability and further elevated the increase in $[Ca^{2+}]_i$ induced by Cd, whereas BAPTA-AM partly attenuated these phenomena, while EGTA had little effects. These results suggested that Cd-induced hepatotoxicity *via* GJIC inhibition and $[Ca^{2+}]_i$ elevation, which originates mainly from intracellular stores. GJIC inhibition has dual effects: (i) it restricts release of Ca^{2+} from the cell, which exacerbates the $[Ca^{2+}]_i$ elevation and cytotoxicity induced by Cd; and (ii) it protects healthy cells from their dangerous neighbors by blocking intercellular communication. Above all, our results indicated that LA partly prevented Cd-induced cytotoxicity *via* GJIC and calcium signaling in BRL 3A rat liver cells.

Key words: Alpha-lipoic acid, Calcium, Cadmium, Connexin, Hepatotoxicity, Calmodulin

INTRODUCTION

Cadmium (Cd) as a toxic metal is not an essential trace element for animals and humans (Rennolds *et al.*, 2012). The liver is the major target organ for Cd. Damage due to oxidative stress as well as signaling cascades induced by Cd can cause hepatotoxicity (Thévenod, 2009; Järup and Åkesson, 2009). However, the underlying mechanism of Cd-induced hepatotoxicity is not known.

Survival or death of cells is influenced by adjacent cells. Gap junctions provide direct communication between adjacent cells through hydrophilic and small molecules (< 1,000 Da) such as cyclic adenosine monophosphate, adenosine triphosphate (ATP), inositol triphosphate (IP3), as well as ions (Ca^{2+} , Na^+ , K^+), and cellular metabolites (Alexander and Goldberg, 2003). Gap

junctional intercellular communication (GJIC) has an important role in several biological processes, including controlling the differentiation and proliferation of cells as well as apoptosis. GJIC is regulated at several levels, including channel gating and connexin expression (Vinken *et al.*, 2011). Cd is known to disrupt the function of gap junctions of hepatocytes *in vitro* and *in vivo* (Boucherie *et al.*, 2013; Hu *et al.*, 2002).

Ca^{2+} is a messenger involved in multiple signaling cascades. Maintenance of Ca^{2+} homeostasis is essential for cell survival (Clapham, 2007). Studies have shown that Cd disrupts Ca^{2+} homeostasis to activate gene transcription and trigger the death or survival of cells (Han *et al.*, 2013; Liu *et al.*, 2014; Zou *et al.*, 2014a). Cd induces dynamin-related protein 1-dependent mitochondrial fragmentation by triggering elevation of the concentration of intracellu-

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lar calcium (i.e., $[Ca^{2+}]_i$) to promote hepatotoxicity (Xu *et al.*, 2013). Elevation of $[Ca^{2+}]_i$ can occur owing to: IP3 production; release of sequestered Ca^{2+} from intracellular stores; activation of Ca^{2+} -dependent phospholipase C; prevention of Ca^{2+} efflux *via* inhibition of Ca^{2+} -ATPase (Choong *et al.*, 2014). As the major receptor of $[Ca^{2+}]_i$, calmodulin (CaM) has an important role in apoptosis. Overexpression and antagonism of CaM can lead to the induction of apoptosis (Yu *et al.*, 2002; Wu *et al.*, 2005).

Alpha-lipoic acid (LA), found naturally in plants and animals, is a free radical scavenger and potent natural antioxidant. LA is a water- and fat-soluble, sulfur-containing compound proven as a metal chelator (Al-Majed *et al.*, 2002). Previous studies have demonstrated that LA has a protective effect on Cd-induced oxidative stress (Veljkovic *et al.*, 2012; Hussein *et al.*, 2014), however, the underlying mechanism is not clear.

In this study, we investigated the role of the Ca^{2+} signaling pathway and GJIC in Cd-induced cytotoxicity in BRL 3A cells. Furthermore, we investigated the protective effects of alpha-lipoic acid (LA) as well as the underlying mechanisms.

MATERIALS AND METHODS

Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Life Technologies, Grand Island, NY, USA). PrimeScript RT Reagent kit and SYBR Premix Ex Taq were obtained from TaKaRa Biotechnology (Dalian, China). Oligonucleotide primers were synthesized by Invitrogen (Carlsbad, CA, USA). Other reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Cell culture

The BRL 3A rat liver cell line was purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were resuspended in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin and were cultured at 37°C in 95% humidified air containing 5% CO₂. In all experiments, BRL 3A cells were treated with non-serum medium containing Cd at 90% confluence, in addition, LA was co-treated with Cd for 12 hr.

Measurement of cell viability

BRL 3A cells were cultured in flat-bottomed 96-well plates. After treatments, cells were incubated with MTT

(0.5 mg/mL) for 4 hr at 37°C. After discarding the medium, 150 µL of dimethyl sulfoxide was added to each well, and the plate shaken gently for 10 min at 37°C. The optical density (OD) of each well was read at 490 nm with a Sunrise™ Microplate Reader (Tecan, Groedig, Austria). Cell viability was expressed as the proportion of OD to that of the control. Each experiment was repeated three times.

Scrape-loading/dye transfer assay for GJIC

GJIC was measured by the scrape loading/dye-transfer method (SL/DT) as described originally by El-Fouly *et al.* (1987). In brief, after treatments for 12 hr, cells were scraped with a sharp blade dozens of times, and then incubated with Lucifer yellow (LY, 0.5 mg/mL) in the dark for 3 min at 37°C. Cells were washed three times with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich). GJIC was the mean distance traveled (µm) by LY dye from the scraped edge to neighboring cells from nine sites in each sample as measured using a Fluorescent Microscope (DMI 3000B; Leica, Solms, Germany).

Immunofluorescence and laser confocal microscopy

BRL 3A cells were grown on glass coverslips. After treatments for 12 hr, cells were fixed with 4% paraformaldehyde. Then, cells were rinsed with PBS and permeabilized with Triton X-100 (0.1% v/v), followed by incubation with 5% bovine serum albumin (BSA) in TBS with 0.1% Tween-20. Cells were incubated with antibody against Cx43 (1:200 dilution) overnight at 4°C, then incubated at room temperature for 1 hr with goat anti-rabbit IgG FITC-conjugated antibodies (1:100 dilution). Finally, cells were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:100 dilution). Coverslips were mounted onto glass slides and imaged using a Laser Scanning Confocal Microscope (LSM-710; Zeiss, Oberkochen, Germany).

RNA extraction, reverse transcription, and quantitative reverse-transcription-polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted from BRL 3A cells using TRIzol® Reagent (Invitrogen) according to manufacturer instructions. cDNA was reverse-transcribed from total RNA (900 ng) using a PrimeScript RT Reagent kit with gDNA Eraser (Takara Biotechnology). Primers were designed using Primer Premier 5 and were (forward and reverse, respectively): β -actin, CGTTGACATCCGTAAAGACCTC and TAGGAGCCAGGGCAGTAATCT; Cx43, ACTCTCGCCTATGTCTCCTCC and GTTGGTCCAC-

GATGGCTAAT; CaM, CACCATTGACTTCCCAGAG and TGTAGCCATTGCCATCCT. Gene expression was measured using a real-time PCR system (7500; Applied Biosystems, Foster City, CA, USA). Reactions were undertaken using a SYBER Premix Taq™ II kit (TaKaRa Biotechnology) according to manufacturer instructions. Annealing temperatures were 60°C for β -actin and Cx43, and 57°C for CaM. Analyses of relative mRNA expression were carried out using the $\Delta\Delta C_T$ method. Values were normalized using β -actin as an endogenous house-keeping control gene.

Co-culture of BRL 3A cells

To investigate the role of GJIC in Cd-induced cytotoxicity, BRL 3A cells were seeded on the inner surface of polyester membrane Transwell Cell Culture Inserts (pore size, 0.4 μ m; surface area, 4.67 cm²; Corning, Corning, NY, USA) in DMEM supplemented with 10% FBS until confluent. Then inserts were removed from the culture medium and incubated in non-serum DMEM with Cd (10 μ M) for 12 hr. After that, overturning inserts, normal BRL 3A cells were seeded on the outside surface of inserts and incubated in condition medium with LA (50 μ M) or CBX (50 μ M) for 36 hr. After treatments, cells on the inside surfaces of inserts were wiped carefully with a cotton swab. Remaining cells on the outside surfaces of inserts were washed thrice with cold PBS, and then fixed in paraformaldehyde. Fixed cells were stained with Hoechst 33258. Images were obtained using an Inverted Fluorescence Microscope (Leica) at an excitation wavelength of 330–380 nm using filters of wavelength 450–490 nm.

Measurement of $[Ca^{2+}]_i$

Fluo-4/AM was used as an intracellular fluorescent probe to detect $[Ca^{2+}]_i$ in Cd-exposed BRL 3A cells. Briefly, after treatments for 12 hr, cells were harvested and incubated with Fluo-4/AM (5 mM) for 30 min at 37°C in the dark. Then, stained cells were washed three times with PBS, and analyzed immediately on a BD-FACS Aria Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). $[Ca^{2+}]_i$ levels were represented by fluorescent intensity. Excitation and emission wavelengths were 494 nm and 516 nm, respectively. Data were analyzed using Cell Quest (Becton Dickinson).

Statistical analyses

Each experiment was repeated at least thrice. Data are the mean $\pm$ standard deviation (S.D.). Data comparisons among groups were undertaken using non-parametric, one-way ANOVA followed by the Dunnett's t-test.

$p < 0.05$ was considered significant and $p < 0.01$ as extremely significant.

RESULTS

Protective effects of LA on Cd-induced changes in the viability and morphology of cells

To choose the appropriate concentration of Cd and LA for our studies, BRL 3A cells were treated with several concentrations of Cd and LA for 12 hr to determine the effect of Cd and LA on cell viability. Cd (0–10 μ M) led to a concentration-dependent decrease in cell viability (Fig. 1A and B). Viability of cells treated with ≥ 2.5 μ M Cd was extremely significantly lower than that of control cells ($p < 0.01$). LA (25 and 50 μ M) did not alter cell viability compared with control, whereas LA (100 and 200 μ M) decreased cell viability extremely significantly ($p < 0.01$). These results made us choose 2.5 μ M Cd and 50 μ M LA for subsequent studies. To verify the effect of LA on Cd-induced cytotoxicity, cells were incubated with LA and Cd for 12 hr. LA attenuated the reduction in cell viability induced by Cd ($p < 0.05$) (Fig. 1C). LA had no effect on cell morphology compared with the control, whereas Cd altered cell morphology: round and shrunken cells were observed, co-incubation with LA could reduce the morphological damage induced by Cd (Fig. 1D).

Protective effects of LA on Cd-induced GJIC inhibition in BRL 3A cells

Exposure to Cd inhibited the function of the gap junction of BRL 3A cells extremely significantly ($p < 0.01$), and LA blocked Cd-induced GJIC inhibition (Fig. 2A and B). Cx43 distribution in BRL 3A cells was observed using confocal immunofluorescence microscopy (Fig. 2C). In control and LA groups, Cx43-positive gap-junction plaques were predominantly defined spots distributed uniformly at sites of cell-cell junctions along the cell membrane. Cd caused gap-junction disassembly; Cx43 moved out of the membrane and into the cytoplasm around the nucleus. Chromatin changes in nuclei were assessed by DAPI staining. They revealed uniform nuclear staining in control and LA groups, but distorted nuclei and chromatin condensation in the Cd-treated group. These changes could be reversed by LA. Then, we investigated the mRNA expression of Cx43 using qRT-PCR. Relative expression of Cx43 significantly ($p < 0.01$) decreased induced by Cd, and LA significantly ($p < 0.01$) attenuated it (Fig. 2D). In the Transwell culture system (Fig. 2E), while being co-cultured with Cd-exposed cells on the inside surface, the viability of BRL 3A cells on the outside surface of inserts decreased and nuclei chromatin

Fig. 1. Protective effects of LA on Cd-induced cytotoxicity in BRL 3A cells. A-C: Viability of BRL 3A cells treated with non-serum medium containing Cd (0–10 μM) and LA (0–200 μM) for 12 hr, and co-treated with LA (50 μM) and Cd (2.5 μM) for 12 hr was evaluated by the MTT assay. Results are the mean ± S.D. (n = 9). * $p < 0.05$, ** $p < 0.01$ compared with control group and # $p < 0.05$ as indicated. D: Morphologic alterations in BRL 3A cells treated with 50 μM LA and 2.5 μM Cd for 12 hr. Images were taken with a Leica inverted phase-contrast microscope (4006) equipped with a Quick Imaging system. Scale bar: 100 μm.

became condensed (and even nuclear fragmentation could be observed), these injuries could be ameliorated with LA or 50 μM CBX (prototypical blocker of gap junctions) added in the co-culture medium.

Protective effects of LA on Cd-induced elevation of $[Ca^{2+}]_i$ in BRL 3A cells

Cd induced significant increase in $[Ca^{2+}]_i$ ($p < 0.01$), and LA could rescue the $[Ca^{2+}]_i$ elevation ($p < 0.05$) (Fig. 3A). 5 μM BAPTA-AM (a chelator of intracellular Ca^{2+}) or 100 μM EGTA (a chelator of extracellular Ca^{2+}) attenuated the reduction in cell viability induced by Cd (Fig. 3B and C). CaM is a multifunctional Ca^{2+} -binding protein located in the cytoplasm and in various cellular organelles. We carried out qRT-PCR analyses to ascertain if CaM might be involved in Cd-induced cytotoxicity in BRL 3A cells. Cd markedly down-regulated the CaM expression and LA prevented it ($p < 0.01$) (Fig. 3D). These results suggested that Cd-induced cytotoxicity can

be attributed to elevation of $[Ca^{2+}]_i$. CaM may play an important part in Cd-induced cytotoxicity, and the protective effect of LA is mediated by its ability to block $[Ca^{2+}]_i$ elevation.

Interaction between GJIC and Ca^{2+} signaling plays an important part in Cd-induced cytotoxicity in BRL 3A cells

To assess the interaction between GJIC and Ca^{2+} signaling after Cd-induced cytotoxicity, CBX and EGTA were administered in combination with Cd. Co-treatment with Cd (2.5 μM) and CBX (50 μM), or co-treatment with Cd (2.5 μM), CBX (50 μM) and EGTA (100 μM), extremely significantly or significantly ($p < 0.01$, $p < 0.05$) decreased cell viability and exacerbated $[Ca^{2+}]_i$ elevation compared with treatment with 2.5 μM Cd alone, respectively, whereas co-treatment with Cd (2.5 μM), CBX (50 μM), and BAPTA-AM (5 μM) significantly attenuated the $[Ca^{2+}]_i$ elevation induced by Cd in BRL 3A cells

Fig. 2. Protective effects of LA on Cd-induced GJIC inhibition. A, B: Effect of LA on Cd (2.5 μ M)-induced down-regulation of GJIC in BRL 3A cells. Scale bar = 100 μ m. Mean distance of LY spread from the side of the scraped edge from six sites in each sample was obtained for quantification; C: Effect of LA on Cd (2.5 μ M)-induced Cx43 distribution in BRL 3A cells. Cells were stained for Cx43 (green) and nuclei were counterstained with DAPI (blue). Scale bar = 10 μ m. D: Effect of LA on Cd (2.5 μ M)-induced decreases in expression of Cx43 mRNA in BRL 3A cells. Values are the mean $\pm$ S.D. (n = 9). ** p < 0.01 compared with the control group, # p < 0.05, ## p < 0.01 compared with the group indicated. E: BRL 3A cells influenced by Cd (10 μ M)-exposed cells in a Transwell culture system. Cells were divided between the two sides of the insert according to their treatment. Scale bar = 100 μ m. Arrows indicate distorted nuclei and chromatin condensation.

(Fig. 4A and B). Subsequently, GJIC was measured: BAPTA-AM and EGTA blocked the GJIC inhibition induced by Cd, whereas CBX intensified Cd-induced GJIC inhibition (Fig. 4C and D). These results suggested that Cd elevates $[Ca^{2+}]_i$ mainly from intracellular stores, and that blocking $[Ca^{2+}]_i$ elevation can ameliorate functional disorders of gap junctions.

DISCUSSION

The liver has been identified as the target of Cd-mediated toxicity (Dudley *et al.*, 1984). At the cellular level, Cd has been associated with oxidative stress and apoptosis (Zhou *et al.*, 2009). In the present study, we demonstrated that Cd induces cytotoxicity in BRL 3A cells by inhibiting GJIC and triggering elevation of $[Ca^{2+}]_i$ by stimulating Ca^{2+} release from intracellular stores. In addition, we explored the possible protective effect of LA. Our results showed that LA may be a functional chemopreventative agent which could ameliorate the cytotoxic

effects of Cd *via* GJIC and Ca^{2+} signaling.

GJIC is one of the most important mechanisms of cellular communications. The liver was one of the first organs in which GJIC was studied (Vinken *et al.*, 2008). Cd is known to inhibit GJIC in the mouse liver in a time- and concentration-dependent manner, and this inhibition is correlated with a decrease in the expression of connexins (Jeong *et al.*, 2000). In the present study, Cd induced inhibition of GJIC and down-regulation of expression of Cx43 mRNA in BRL 3A cells (Fig. 2A, B and D). Correspondingly, the cellular Cx43 decreased and scattered by Cd (Fig. 2C), LA partly alleviate these injury. Our results imply that LA is involved in the protective mechanisms against GJIC inhibition-induced liver injury. Studies have suggested that apoptotic cells can spatially extend apoptosis to healthy cells through communication of cell-death signals *via* GJIC (Decrock *et al.*, 2009). Transwell inserts (pore size, 0.4 μ m) were used in a co-culture system in the present study. Both sides of cells could not cross through the insert membrane but shared small-size

Fig. 3. Protective effects of LA on Cd-induced $[Ca^{2+}]_i$ elevation in BRL 3A cells. A: Cd (2.5 μ M)-induced elevation of $[Ca^{2+}]_i$ in BRL 3A cells was diminished by LA treatment (12 hr). Results are the mean fluorescence. Each bar represents mean $\pm$ S.D. ($n = 4$). B, C: BRL 3A cells were pretreated with BAPTA-AM (5 μ M) or EGTA (100 μ M) for 30 min, followed by treatment with 2.5 μ M Cd for 12 hr. The cell viability was evaluated by the MTT assay. Results are the mean $\pm$ S.D. ($n = 9$). D: Effect of LA on Cd (2.5 μ M)-induced decreases in expression of CaM mRNA in BRL 3A cells. Values are the mean $\pm$ S.D. ($n = 9$). ** $p < 0.01$ compared with the control group, # $p < 0.05$, ## $p < 0.01$ compared with the group indicated.

substances could do so *via* hemi-channels and/or establishment of gap junctions (cells cannot cross through the insert membrane if the pore size is $< 3 \mu$ m). CBX is a GJIC blocker, and had a protective effect on the injury induced by Cd-exposed cells (Fig. 2E). Similar findings have been reported in PC12 cells (Guo *et al.*, 2014). Our data allowed us to infer that GJIC inhibition has a protective effect that blocks communication of cell-death signals from injured cells to healthy cells.

Ca^{2+} is a universal messenger that regulates many aspects of the physiologic and pathologic functions of cells (Berridge *et al.*, 2000). If cells are activated by extracellular stimuli, $[Ca^{2+}]_i$ can rise by more than five-fold *via* Ca^{2+} influx from outside of the cells and/or release from intracellular stores (Ishikawa *et al.*, 2011).

Studies have demonstrated that $[Ca^{2+}]_i$ elevation is a common signal during Cd-induced apoptosis and death in several cell types (Yuan *et al.*, 2013; Son *et al.*, 2010; Xu *et al.*, 2013). We demonstrated that Cd induces $[Ca^{2+}]_i$ elevation in BRL 3A cells (Fig. 3A). We used BAPTA-AM to chelate intracellular Ca^{2+} to demonstrate that $[Ca^{2+}]_i$ elevation has a crucial role in Cd-induced cytotoxicity in BRL 3A cells (Fig. 3B). We found that chelating extracellular Ca^{2+} with EGTA and co-treatment with LA also elicited protective effects (Fig. 3A and C). We hypothesized that EGTA disrupts Ca^{2+} homeostasis and impels Ca^{2+} removal from the cytoplasm, and LA is involved in the protective mechanisms against $[Ca^{2+}]_i$ elevation-induced liver injury. $[Ca^{2+}]_i$ elevation induces cellular injury by activating a signaling cascade that is mediated primarily *via*

Fig. 4. Interaction between GJIC and Ca^{2+} signaling in Cd-induced cytotoxicity of BRL 3A cells. A: The cell viability was evaluated by the MTT assay. Results are the mean $\pm$ S.D. (n = 9). B: $[Ca^{2+}]_i$ was measured after treatment as shown in A. Results are the mean fluorescence. Each bar represents mean $\pm$ S.D. (n = 4). C, D: Effect of 50 μ M CBX, 5 μ M BAPTA-AM, and 100 μ M EGTA on Cd (2.5 μ M)-induced down-regulation of GJIC in BRL 3A cells. Scale bar = 100 μ m. * p < 0.05, ** p < 0.01 compared with the control group, # p < 0.05, ### p < 0.01 compared with the 2.5 μ M Cd group.

Ca^{2+} -binding proteins such as CaM (Wang, 1985). CaM signaling might be involved in Cd-induced cytotoxicity (Fig. 3D).

Ca^{2+} is removed from cells by the Na^{+} - Ca^{2+} exchanger and plasma-membrane Ca^{2+} -ATPase. Ca^{2+} enters cells *via* voltage-gated calcium channels, receptor-operated channels, and store-operated channels. Endoplasmic reticula and mitochondria also take part in maintaining calcium homeostasis (Gurkoff *et al.*, 2013; Berridge *et al.*, 2003). It is accepted that Ca^{2+} can pass through gap-junction channels. The GJIC blocker CBX exacerbated the decrease in Cd-induced cell viability in BRL 3A cells (Fig. 4A), which was the opposite effect compared with that of CBX in the Transwell co-culture system, one of the reasons was that CBX further elevated the $[Ca^{2+}]_i$ induced by Cd (Fig. 4B). It seems that GJIC inhibition exacerbates injury in damaged cells. Combined activity of CBX and BAPTA-AM demonstrated that chelation of intracellular Ca^{2+} ameliorated Cd-induced cell injury, whereas the combined activity of CBX and EGTA had an aggravating effect. We concluded that GJIC inhibition limits Ca^{2+}

passage, in particular extruding $[Ca^{2+}]_i$ to the outside of cells, and that Cd elevates $[Ca^{2+}]_i$ mainly from intracellular stores. Similar findings have been reported in PC12 cells (Xu *et al.*, 2011). Intercellular communication mediated by gap junctions can be regulated by multiple factors, including $[Ca^{2+}]_i$, H^{+} , CaM, voltage, and phosphorylation or expression of connexin subunits (Del Corso *et al.*, 2012; Vinken *et al.*, 2006). $[Ca^{2+}]_i$ regulates gap-junction communication by uncoupling cells from each other by electric and metabolic means (Peracchia, 2004). Similar results were noted in our study: blockade of Cd-induced $[Ca^{2+}]_i$ elevation by BAPTA-AM, and co-treatment with Cd and EGTA recovers the functions of gap junctions (Fig. 4C and D), furthermore LA has a similar effect (Fig. 2A and B). In addition, the CaM binding sites of several intracellular domains of connexins blockade gap-junction channels (Zou *et al.*, 2014b). Overall, the interaction between GJIC and calcium pathways has an important role in Cd-induced cytotoxicity.

In summary, the results obtained in the present study clearly demonstrate that exposure to Cd causes GJIC

inhibition and $[Ca^{2+}]_i$ elevation, followed by cell injury in BRL 3A cells. Increases in $[Ca^{2+}]_i$ induced by Cd originate mainly from intracellular stores. Inhibition of GJIC restricts Ca^{2+} passage out of cells, which exacerbates the $[Ca^{2+}]_i$ elevation and cytotoxicity induced by Cd. Previous studies provided evidence that LA attenuated Cd-induced cytotoxicity because of its antioxidation, in this study, LA elicits a partial protective effect on Cd-induced toxicity *via* GJIC and Ca^{2+} signaling. These results help to improve our understanding of Cd-induced hepatotoxicity.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 31372495, No. 31302058) and a project Funded by Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD)

Conflict of interest---- The authors declare that there is no conflict of interest.

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