Biochemical and Biophysical Research Communications 459 (2015) 713–719

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Autophagy and gap junctional intercellular communication inhibition are involved in cadmium-induced apoptosis in rat liver cells

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ARTICLE INFO

Article history:
Received 28 January 2015
Available online 14 March 2015

Keywords:
Cadmium
Gap junctional intercellular communication
Autophagy
Hepatotoxicity

ABSTRACT

Cadmium (Cd) is known to induce hepatotoxicity, yet the underlying mechanism of how this occurs is not fully understood. In this study, Cd-induced apoptosis was demonstrated in rat liver cells (BRL 3A) with apoptotic nuclear morphological changes and a decrease in cell index (CI) in a time- and concentration-dependent manner. The role of gap junctional intercellular communication (GJIC) and autophagy in Cd-induced apoptosis was investigated. Cd significantly induced GJIC inhibition as well as downregulation of connexin 43 (Cx43). The prototypical gap junction blocker carbenoxolone disodium (CBX) exacerbated the Cd-induced decrease in CI. Cd treatment was also found to cause autophagy, with an increase in mRNA expression of autophagy-related genes Atg-5, Atg-7, Beclin-1, and microtubule-associated protein light chain 3 (LC3) conversion from cytosolic LC3-I to membrane-bound LC3-II. The autophagic inducer rapamycin (RAP) prevented the Cd-induced CI decrease, while the autophagic inhibitor chloroquine (CQ) caused a further reduction in CI. In addition, CBX promoted Cd-induced autophagy, as well as changes in expression of Atg-5, Atg-7, Beclin-1 and LC3. CQ was found to block the Cd-induced decrease in Cx43 and GJIC inhibition, whereas RAP had opposite effect. These results demonstrate that autophagy plays a protective role during Cd-induced apoptosis in BRL 3A cells during 6 h of experiment, while autophagy exacerbates Cd-induced GJIC inhibition which has a negative effect on cellular fate.

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1. Introduction

Cadmium (Cd) is a toxic heavy metal that is harmful to humans and animals, and is commonly found in air, soil, sediments and water. It is known to target multiple organ systems, particularly the kidneys and liver [1]. A number of studies have demonstrated that Cd induces apoptosis in many cells and tissues [2,3], and several signaling pathways (the Ca^{2+} pathway, Mitogen-activated protein kinase pathway, phosphatidylinositol-3-kinase (PI3K)-Akt pathway and nuclear factor-κB (NF-κB) pathway) are involved in Cd-induced apoptosis [4]. However, the underlying molecular mechanism of Cd-induced cytotoxicity is still poorly understood.

Gap junctional intercellular communication (GJIC) is one of the most important cellular communications and plays an important role in many biological processes [5,6]. Gap junctions are formed from two connexons on adjacent cells, with each connexon comprised of six connexins (Cx). There are more than 20 Cx species known to be present in animals and humans. GJIC involves the passive diffusion of small (<1000 Da) hydrophilic substances (e.g., cyclic adenosine monophosphate, adenosine triphosphate, inositol triphosphate, ions and cellular metabolites). Various kinds of stimuli cause the GJIC function to be reduced or suppressed, which can result in apoptosis, necrosis and carcinogenesis [7–9]. Cx gene expression and gap junction channel gating are two major mechanisms of GJIC control [10]. Gap junction channels are key in the control of hepatic homeostasis, as these structures are frequently affected upon impairment of this critical balance, which occurs during liver toxicity [11]. Previous studies have revealed that Cd disrupts gap junction function in hepatocytes both in vitro and in vivo [12,13].

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http://dx.doi.org/10.1016/j.bbrc.2015.03.027
0006-291X/

Autophagy is an evolutionarily conserved process implicated in degradation and recycling of cytoplasmic proteins, macromolecules, and organelles [14]. Microtubule associated protein light chain 3 (LC3) and autophagy related genes (ATGs) such as Beclin-1, Atg-5, Atg-7, and Atg-12 are involved in the process of autophagy. Autophagy and apoptosis are important and interconnected stress-response mechanisms. Furthermore, autophagy is involved in GJIC regulation via Cx degradation [15]. Autophagy is critically important for cellular fate, as it removes damaged organelles and harmful substances to maintain cellular homeostasis. However, massive and persistent autophagy can degrade normal organelles and consume energy which results autophagic cell death [16]. Previous studies have shown Cd-induced autophagy and apoptosis in various types of cells, yet the role that autophagy plays in Cd-induced cell death is still unclear [17].

Our previous studies indicated that Cd exposure induces apoptosis in BRL 3A rat liver cells. This study aimed to investigate the mechanism of Cd-induced apoptosis involving autophagy and GJIC in BRL 3A cells with cytobiology and molecular biology methods.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). PrimeScript™ RT Reagent kit and SYBR® Premix Ex Taq were obtained from TaKaRa (Dalian, China). Oligonucleotide primers were synthesized by Invitrogen (Shanghai, China). The enhanced chemiluminescence (ECL) detection kit was purchased from Millipore (Burlington, MA, USA). Other reagents were from Sigma (Shanghai, China).

2.2. Cell culture

BRL 3A rat liver cells were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and then incubated at 37 °C in 5% CO_2 atmosphere.

2.3. Hoechst 33258 staining

After treatment, cells were washed twice in phosphate-buffered saline (PBS) and fixed in 4% formaldehyde for 10 min at room temperature. The fixed cells were stained with 5 μg/mL Hoechst 33258 according to the manufacturer's instructions (Beyotime, Jiangsu, China). Cell nuclear morphology was observed under a camera-equipped fluorescence light microscope using a filter of 450 nm–490 nm.

2.4. Real time analysis of cytotoxicity

Cell proliferation and cytotoxicity was monitored using the xCELLigence real-time cell analysis (RTCA; Roche Applied Science, Basel, Switzerland). The protocol was operated according to the xCELLigence system manufacturer's instructions [18]. BRL 3A cells were then seeded at a density of 10,000 cells/well in 100 μL medium aliquots in quadruplicate. After incubating for 30 min, cells were analyzed every 15 min. During the phase of rapid CI increase, cells were treated according to the experimental design. The results were normalized at the time of treatment.

2.5. Gap junction activity analysis

Gap junction activity was analyzed using the scrape-loading/dye-transfer method (SL/DT) as previously reported [19]. Briefly, following treatment the cells were cut with a surgical scalpel in the presence of Lucifer yellow (LY) (0.5 mg/mL) and rhodamine dextran (RD) (2.5 mg/mL), followed by incubation for 3 min at room temperature, then fixed with 4% paraformaldehyde. The level of gap junction activity was quantified as the average distance traveled (μm) by the LY dye from the designated cut to the neighboring cells from six different sites in each sample, measured using a fluorescent microscope (Leica DMI 3000B, Solms, Germany).

2.6. Western blot analysis

Immediately after treatment, Cells were harvested, lysed in ice-cold RIPA lysis buffer, and then sonicated. The protein content was determined using a BCA protein assay kit (Beyotime, Jiang Su, China). Equal amounts of protein were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membrane was incubated with blocking solution containing 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) before incubation with primary antibodies against Cx43, P-Cx43, Cx32, LC3 (1:1000) or β-actin (1:5000) overnight at 4 °C, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000) at room temperature for 2 h. The membranes were then washed with TBST and the protein bands were visualized using ECL reagents. The results were analyzed using Image Lab software (Bio-Rad, Hercules, CA, USA) and determined by standard scanning densitometry with normalization of densitometry measures to β-actin.

2.7. Assessment of autophagy

To assess autophagy, cells were stained with monodansylcadaverine (MDC), which is a specific autophagosome marker. Briefly, after treatment, cells on cover slips were stained with 50 μM MDC for 45 min at room temperature, washed three times with PBS and then fixed with 4% paraformaldehyde. The stained cells were then observed by fluorescent microscopy with a source using 335 nm excitation and 512 nm emission.

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

Total RNA was extracted from cultured cells and liver tissue using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 900 ng total RNA using a PrimeScript™ RT Reagent kit with gDNA Eraser (Takara, Japan). The primers were designed using Primer Premier 5 as follows: β-actin: Forward 5'–CGTTGACATCCGTAAAGACCTC-3'

Reverse 5'–TAGGAGCCAGGGCAGTAATCT-3'

Atg-5: Forward 5'–AGGCTCAGTGGAGGCAACAG-3'

Reverse 5'–CCCTATCTCCCATGGAATCTTCT-3' Atg-7: Forward 5'–GCTGGTCTCCTTGCTCAAC-3'

Reverse: 5'–CAGGGTGCTGGGTTAGGTTA-3'

Beclin-1: Forward 5'–TTCAAGATCCTGGACCGAGTGAC-3'

Reverse 5'–AGACACCATCCTGGCGAGTTTC-3'

Expression levels of all genes were measured using a real-time PCR system (Applied Biosystems 7500, USA) and the reactions were performed with a SYBR® Premix Taq™ II kit (Takara, Japan) according the manufacturer's instructions. Annealing temperatures were 60 °C for all primers. The analyses of relative mRNA levels was carried out using the $\Delta\Delta C_T$ method. The values were

normalized using β-actin as an endogenous housekeeping control gene.

2.9. Statistical analysis

Results are presented as the mean ± standard deviation (SD). Statistical data comparisons among groups were performed using a non-parametric, one way analysis of variance (ANOVA) with $P < 0.05$ considered statistically significant. Each experiment was performed at least in triplicate.

3. Results

3.1. Cd triggers apoptosis in BRL 3A cells

Apoptotic morphological changes induced by Cd in BRL 3A cells were observed with Hoechst 33258 staining (Fig. 1A). Control cells displayed round and homogenous nuclei, with evenly dispersed chromatin structures. After exposure to Cd (2.5, 5 and 10 μM) for 6 h, cells showed typical apoptotic nuclear morphological changes: the nuclei of apoptotic cells shrunk, deformed, contained nuclear fragments which were smaller than the nuclei of untreated cells and the nuclear chromatin became condensed. Cd-induced cytotoxicity in BRL 3A cells was monitored in real-time by the xCELLigence system. As shown in Fig. 1B, After exposure to Cd, the CI value decreased in a time- and concentration-dependent manner in comparison to control cells.

3.2. Cd induces GJIC inhibition in BRL 3A cells

Gap junction activity was determined in BRL 3A cells following treatment with Cd. As shown in Fig. 2A and B, treatment with Cd for 6 h resulted in GJIC inhibition in a concentration-dependent manner. Furthermore, the phosphorylation level of P-Cx43 was significantly increased ($P < 0.05$) in a concentration-dependent manner after Cd treatment for 6 h, while Cx43 expression was downregulated (Fig. 2C and D). Culturing with 50 μM the prototypical gap junction blocker CBX did not affect CI, whereas CBX combined with 2.5 μM Cd caused significant cytotoxicity compared with the control group (Fig. 2E). These results suggest that Cd-induced apoptosis in BRL 3A cells might be associated with its induction of GJIC inhibition.

3.3. Cd triggers autophagy in BRL 3A cells

Cells treated with 2.5 μM Cd for 12 h were stained with MDC, which is incorporated into the autophagosome and emits blue fluorescence under ultraviolet excitation. Control cells displayed diffuse staining, whereas Cd treatment increased MDC fluorescent intensity in a concentration-dependent manner (Fig. 3A). The

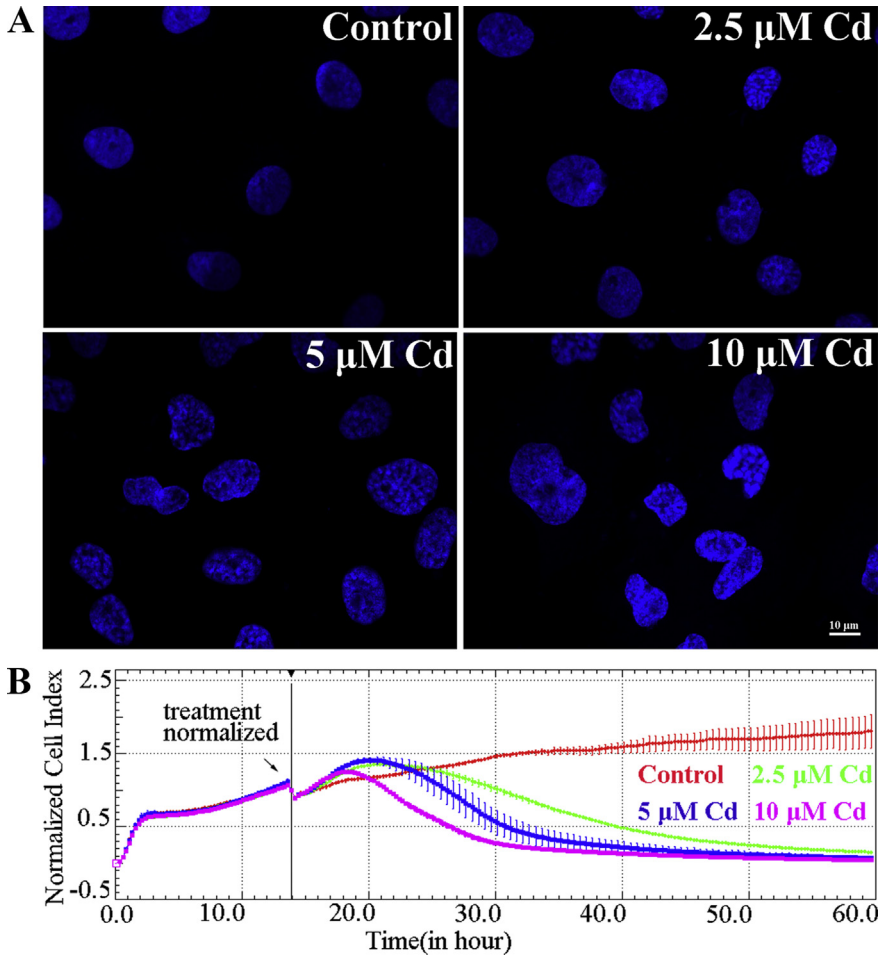

Fig. 1. Cd triggers apoptosis in BRL 3A cells. (A) Cd-induced apoptotic morphological changes in BRL 3A cells. BRL 3A cells were incubated with Cd (0, 2.5, 5, and 10 μM) for 6 h and stained with Hoechst 33258. Scale bar = 10 μm. (B) The effect of Cd on cell index. Data were normalized at the time of Cd treatment. All curves were plotted as an average of quadruplicate treatments. Error bars show SD (n = 4).

autophagosomal marker LC3-II was also detected in Cd treated cells. As shown in Fig. 3B and C, the expression of LC3-II significantly increased ($P < 0.01$) in a concentration-dependent manner after Cd treatment for 6 h. Correspondingly the mRNA expression of autophagy-related proteins Atg-5, Atg-7, Beclin-1 was increased (Fig. 3D). The autophagic inducer RAP prevented the CI decrease induced by Cd, while the autophagic inhibitor CQ caused a further reduction in CI (Fig. 3E and F). These results demonstrate that autophagy has a protective effect on Cd-induced cytotoxicity in BRL 3A cells.

3.4. The interaction between GJIC and autophagy in Cd-induced cytotoxicity

To assess the effect of GJIC on autophagy in Cd-induced cytotoxicity, the prototypical gap junction blocker CBX (50 μM) was added to BRL 3A cells. As shown in Fig. 4A, CBX further promoted Cd-induced autophagy, with MDC fluorescence intensity increasing in the co-treatment group. Correspondingly, the mRNA expression of autophagy-related genes Atg-5, Atg-7, Beclin-1 was significantly increased ($P < 0.01$) in the co-treatment group, and LC3-II expression was also increased, while CBX significantly increased ($P < 0.05$) the expression of Beclin-1 mRNA (Fig. 4B–D).

The effect of autophagy on GJIC in Cd-induced cytotoxicity was also investigated. Co-treatment with CQ significantly blocked ($P < 0.01$) the Cd-induced decrease in Cx43 expression, whereas RAP further reduced Cx43 expression. In addition, RAP significantly decreased ($P < 0.05$) Cx43 expression (Fig. 4E and F). Correspondingly, alterations in GJIC function were found to be in accordance with Cx43 expression (Fig. 4G and H).

4. Discussion

Accumulating evidence demonstrates that Cd can induce damage in various organs and cause apoptosis in a variety of cells [20–22]. Liver is one of the major targets of Cd-mediated toxicity [23], yet the underlying mechanism for Cd-induced hepatotoxicity remains unclear. The results of this study demonstrate that Cd induced an obvious apoptotic morphological change in nuclear chromatin. The real-time cell analysis (RTCA) results indicate that Cd induced cytotoxicity in a time- and concentration-dependent manner in BRL 3A cells. These results correlate with data collected using conventional techniques in our previous study [24]. In addition, GJIC and autophagy were found to be involved in Cd-induced hepatotoxicity in BRL 3A cells.

In multicellular organisms, the global interplay between extra-, intra- and inter-cellular communication controls the maintenance of homeostatic balance. Much toxicological research focuses on single cells, ignoring the influence that adjacent cells have on cell survival or death. It has been well described that functional loss of gap junctions can result in apoptosis, necrosis and carcinogenesis [7–9]. Besides allowing the diffusion of small and hydrophilic substances, GJIC can spatially extend apoptosis through the communication of cell death signals from apoptotic cells to healthy cells [25]. When cells are exposed to hazardous stimulants, GJIC downregulation occurs, which causes injured cells to lose rescue signals (such as glucose, ATP and ascorbic acid) provided by healthy cells, which results in a loss of normal growth regulation by the surrounding cells and growth independence [26]. Previous studies have indicated that Cd inhibits GJIC in mouse liver and the hepatic WIF-B9 cell line, and this inhibition is correlated with parallel Cx

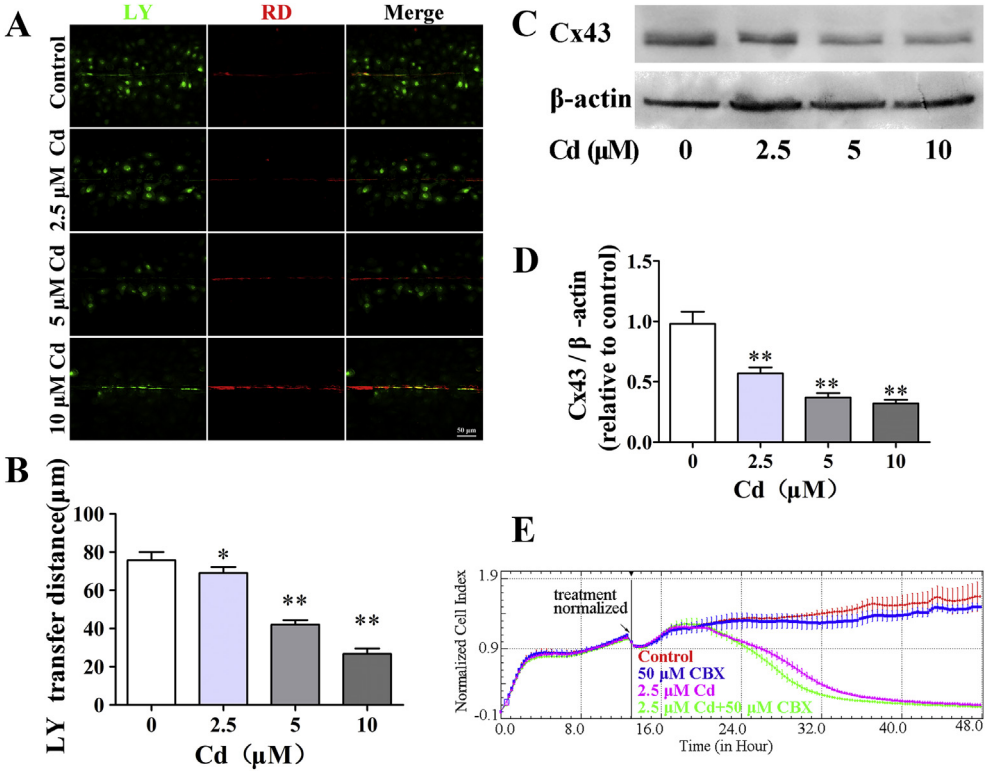

Fig. 2. Cd induces GJIC inhibition in BRL 3A cells. (A) Cd-induced downregulation of GJIC in BRL 3A cells. LY (green) transferred to adjacent cells via open gap junctions, whereas RD (red) remained at the nick site. Scale bar = 50 μm. (B) The average distance of LY spread from the side of the scraped edge from six different sites in each sample was obtained for quantification. (C, D) Western blot analysis of Cx43 after Cd treatment (n = 3). Values represent the mean ± S.D. *$P < 0.05$, **$P < 0.01$ versus the control group. (E) Cell index for cells treated with 2.5 μM Cd and 50 μM CBX. Results were normalized at the time of treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reduction [12,27]. In this study, we demonstrate that Cd inhibits GJIC in BRL 3A cells, in addition to causing downregulation of the gap junctional channel protein Cx43. These results confirm that GJIC is involved in Cd-induced apoptosis which is consistent with our previous study [28]. Cd inhibited the function of gap junctions in BRL 3A cells and further inhibited GJIC by the prototypical gap junction blocker CBX. Overall, these results demonstrate that GJIC may play an important role in Cd-induced apoptosis in BRL 3A cells.

Autophagy is a catabolic process of subcellular degradation that involves numerous autophagy related genes (ATGs) such as Beclin-1, Atg5, Atg7 and Atg12 [29]. In addition, LC3 was found to be a specific biochemical marker for autophagy, and is required for autophagosome formation via its conversion from cytosolic LC3-I to membrane-bound LC3-II [30]. Autophagy is activated in response to various kinds of stress [31]. Acidic vesicular organelles are formed in the process of autophagy, which can be observed using vital staining with MDC. In this study, Cd-induced autophagy was demonstrated by MDC staining, and the level of Atg-5, Atg-7, and Beclin-1 mRNA expression, as well as LC3-II, were increased in a concentration-dependent manner following treatment with Cd. These results indicate that Cd triggers autophagy in BRL 3A cells, with the conversion of a fraction of LC3-I into LC3-II and upregulation of Atg-5, Atg-7, and Beclin-1 mRNA. Furthermore, when CQ and RAP were used to detect the role of autophagy in Cd-induced cytotoxicity in BRL 3A cells, CQ was found to significantly enhance Cd-induced CI decrease while RAP had the opposite effect. This indicates that Cd-induced autophagy has a protective effect by delaying Cd induced cytotoxicity in BRL 3A cells. Similar findings have been reported in PC-12 cells [32], however Wang et al. found that autophagy has no significant effect on Cd-induced apoptosis in mesangial cells [33].

There is a close relationship between autophagy and gap junctions. The regulation of Cx protein levels and trafficking are crucial to the function of gap junctions, with Cx having a short half-life of 1.5–5 h [34]. Recent studies have shown that autophagy related the degradation and localization of Cx [35,36]. In this study, GJIC inhibition was found to promote Cd-induced autophagy in BRL 3A cells via the use of CBX. In addition, autophagy triggered by Cd negatively regulated the function of gap junctions in BRL 3A cells, with downregulation of Cx43 expression. These results allowed us to infer that Cd induces organelle damage, then promotes autophagy to degrade damaged or superfluous proteins and organelles. It disrupts cellular homeostasis and Cx degradation and localization, which results in GJIC downregulation. It appears that autophagy plays a rescue role in Cd induced cytotoxicity at an early stage, and previous studies shows a high level of autophagy leads to cell death via excessive degradation of essential molecules or organelles [37,38]. GJIC plays an important role in autophagy induced cell death or survival.

In summary, the present work demonstrates that Cd induces GJIC inhibition in addition to autophagy and apoptosis. Autophagy serves an important protective function in Cd-induced cytotoxicity

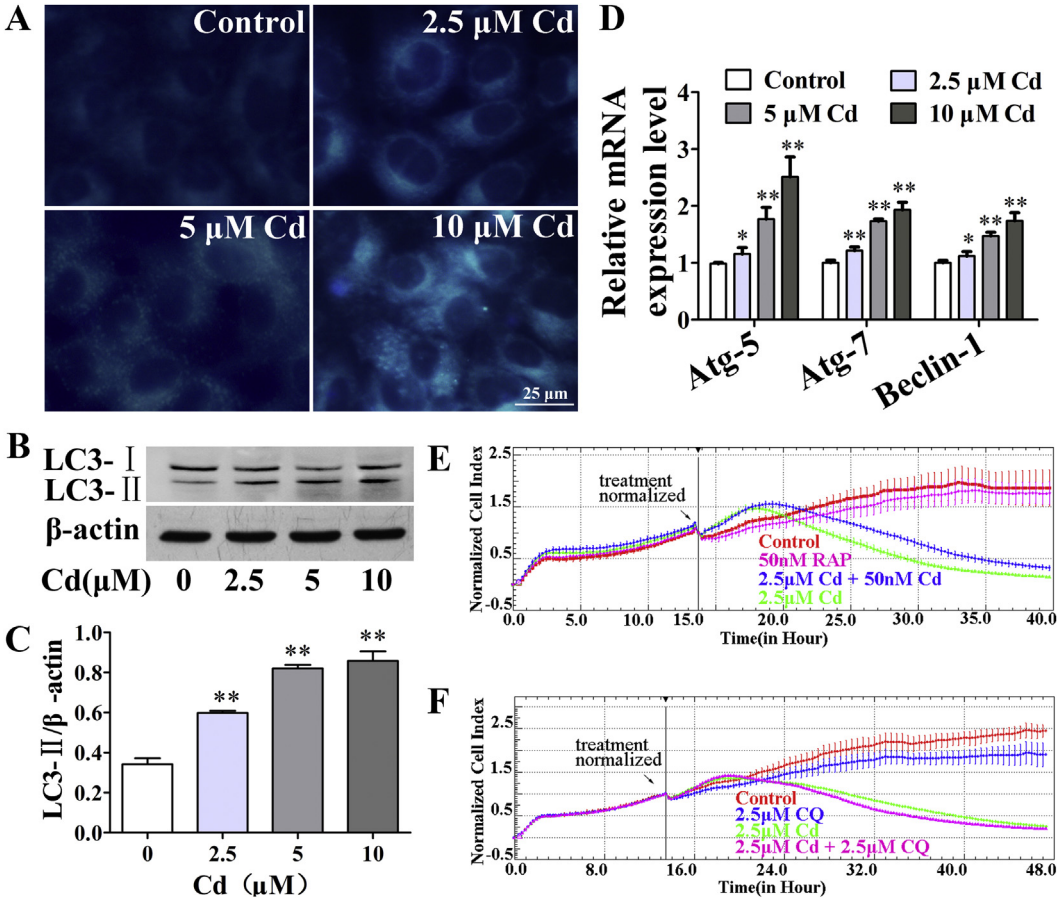

Fig. 3. Cd triggers autophagy in BRL 3A cells. (A) BRL 3A cells were treated with Cd for 6 h then stained with MDC to visualize autophagolysosome formation. Scale bar = 25 μm (B, C) Western blot analysis of LC3-II after Cd treatment (n = 3). (D) Effect of Cd on Atg-5, Atg-7 and Beclin-1 mRNA expression (n = 9). Values represent the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group. (E) Effects of RAP on Cd-induced cytotoxicity. Results were normalized at the time of treatment. (F) Effects of CQ on Cd-induced cytotoxicity. Results were normalized at the time of treatment.

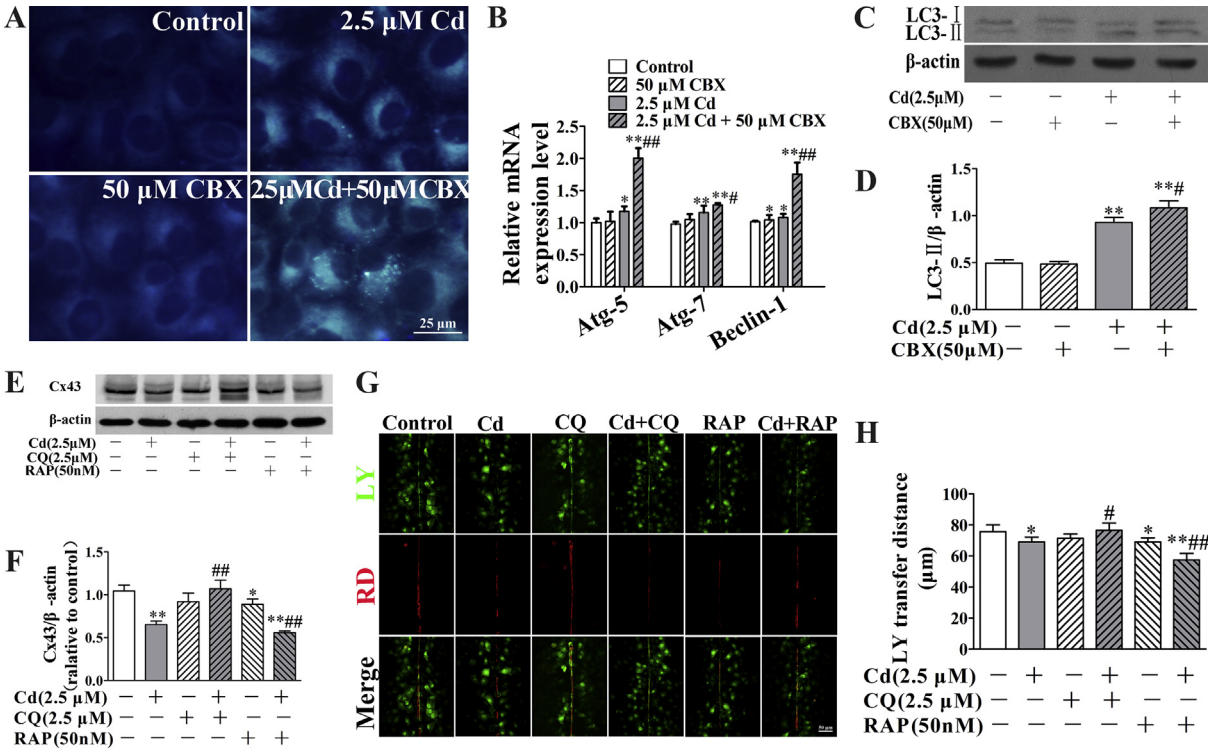

Fig. 4. The interaction between GJIC and autophagy in Cd-induced cytotoxicity. (A) BRL 3A cells were treated with 2.5 μM Cd and 50 μM CBX for 6 h then stained with MDC to visualize autophagolysosome formation. Scale bar = 25 μm. (B) Effect of 2.5 μM Cd and 50 μM CBX on Atg-5, Atg-7 and Beclin-1 mRNA expression in BRL 3A cells (n = 9). (C, D) Western blot analysis of LC3-II after Cd and CBX treatment (n = 3). (E, F) Western blot analysis of Cx43 expression after Cd, CQ and RAP treatment (n = 3). (G) Effect of CQ and RAP on Cd-induced downregulation of GJIC in BRL 3A cells. Scale bar = 50 μm. (H) The average distance of LY spread from the side of the scraped edge was obtained for quantification (n = 6). Values represent the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the 2.5 μM Cd group.

in BRL 3A cells at an early stage, meanwhile, autophagy exacerbates Cd-induced GJIC inhibition. Further studies will focus on the mechanism of the interaction between GJIC and autophagy, and the role of autophagy in the process of Cd-induced hepatotoxicity.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31101866, No. 31372495, No. 31302058) and a Project Funded by Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.027.

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