Original Article
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Gap junction blockage promotes cadmium-induced apoptosis in BRL 3A derived from Buffalo rat liver cells

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Gap junctions mediate direct communication between cells; however, toxicological cascade triggered by nonessential metals can abrogate cellular signaling mediated by gap junctions. Although cadmium (Cd) is known to induce apoptosis in organs and tissues, the mechanisms that underlie gap junction activity in Cd-induced apoptosis in BRL 3A rat liver cells has yet to be established. In this study, we showed that Cd treatment decreased the cell index (a measure of cellular electrical impedance) in BRL 3A cells. Mechanistically, we found that Cd exposure decreased expression of connexin 43 (Cx43), increased expression of p-Cx43 and elevated intracellular free Ca^{2+} concentration, corresponding to a decrease in gap junctional intercellular communication. Gap junction blockage pretreatment with 18β-glycyrrhizic acid (GA) promoted Cd-induced apoptosis, involving changes in expression of Bax, Bcl-2, caspase-3 and the mitochondrial transmembrane electrical potential ($\Delta\psi m$). Additionally, GA was found to enhance ERK and p38 activation during Cd-induced activation of mitogen-activated protein kinases, but had no significant effect on JNK activation. Our results indicated the apoptosis-related proteins and the ERK and p38 signaling pathways may participate in gap junction blockage promoting Cd-induced apoptosis in BRL 3A cells.

Keywords: BRL 3A cells, apoptosis, cadmium, gap junction blockage

Introduction

Gap junction (GJ) is a channel-forming structure connecting membranes of adjacent cells. GJ affords the passive intercellular diffusion of small and hydrophilic substances, such as Ca^{2+} and inositol 1,4,5-triphosphate (IP_3) [15]. Direct communication between cells through GJ, called gap junctional intercellular communication (GJIC), is considered a key mechanism in control of the homeostatic balance in cell differentiation, proliferation, transformation and apoptosis [6]. The proteins that form the GJ are called connexins. Kameritsch *et al*. [11] reported that connexin expression enhances apoptosis induction in a connexin-type-dependent manner. Among different isoforms of connexin molecules, connexin 43 (Cx43) has been extensively investigated because of its ubiquitous expression in a variety of cell types. Huang *et al*. [8] suggested that regulation of apoptosis by Cx43 is mediated by down-regulation of the apoptosis inhibitor protein Bcl-2 in human glioblastoma cells. Nakase *et al*. [20] demonstrated that rats that lack Cx43 show severe apoptosis with significantly increased caspase-3 levels. Upon progression of cell death, GJIC appears to decrease, as evidenced by the absence of communication between apoptotic bodies [35]. Investigations have shown that GJ blockage limits intercellular spreading of astrocytic apoptosis [22].

Cadmium (Cd) is an important contaminant because its use is widespread in industrial processes and it cannot be degraded in the environment [31]. Cd is known to be toxic to many systems and organs. When considering Cd-induced toxicity, the liver is the most important target organ because Cd primarily accumulates in it [28,29]. Acute or chronic exposure to Cd causes hepatic damage that manifests as cell proliferation, nodular hyperplasia, and apoptosis [10,13]. The calcium overload and mitogen-activated protein kinase (MAPK) pathway tightly regulate Cd-induced apoptosis [1,30]. As a nonessential metal, Cd inhibits GJ function in hepatocytes *in vivo* and *in vitro* [2,32]. Jeong *et al*. [9] reported that Cd inhibited GJIC in the liver by

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decreasing the expression of Cx32 and Cx26. In recent years, studies have shown that Cx43 hemichannels may contribute to Cd-induced cell injury [4] in LLC-PK1 cells.

However, it is still unclear how GJ influences Cd-induced apoptosis. Therefore, in this study, we selected BRL 3A rat liver cells as a hepatic model. An established GJ blocking agent, 18β-glycyrrhizic acid (GA), was employed to investigate which signal pathways were involved in Cd-induced apoptosis when GJ was blocked. Here, we present data correlating GJ and Cd-induced apoptotic pathways in BRL 3A cells.

Materials and Methods

Reagents

Cadmium acetate ($CdAC_2$), GA, Lucifer yellow (LY) dilithium salt, rhodamine-labeled dextran (RD), Fluo-4/AM and Hoechst 33258 were purchased from Sigma-Aldrich (USA). An annexin V-FITC Fluorescence Microscopy Kit was obtained from BD Biosciences Pharmingen (USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco Laboratories (USA). Cx43, p-Cx43, Bax, Bcl-2, caspase-3, ERK, p-ERK, JNK, p-JNK, p-38, p-p38 and β-actin were purchased from Cell Signaling Technology (USA). All other chemicals and reagents used were of analytical grade and acquired locally.

Cell culture

BRL 3A-immortalized rat hepatocytes were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology (China) and cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone) at 37°C under 5% CO_2.

Measurement of cell proliferation by real-time cell system

Cell-based cytotoxicity was quantified by the xCELLigence real-time cell analysis (RTCA) system (Roche Applied Science, Switzerland), which detects cellular impedance as an index of attachment and proliferation [24]. Cell growth was recorded as the cell index (CI), which corresponds to the electrical impedance of a well. The normalized CI relative to a specified reference time point was determined by the RTCA software.

Changes in BRL 3A cell proliferation were analyzed by seeding 1×10^4 cells/well in the E-plate and then culturing them for 14 h at 37°C under 5% CO_2 to allow the cells to adhere and reach the proliferative phase. Cells were treated with Cd (0, 2.5, 5, 10 and 20 μM), GA (5 μM) or pretreated with GA (5 μM) for 30 min followed by Cd (10 μM) for the experiment.

Scrape-loading dye transfer assay

GJIC was assessed by the scrape-loading/dye transfer method. LY (457 Da) permeates GJ channels, whereas RD (1,000 kDa) does not cross GJ channels and instead enters the cytosol of cells with disrupted plasma membranes. Briefly, cells were treated with Cd (0 and 10 μM) or GA (5 μM) alone or GA (5 μM) plus Cd (10 μM) for 9 h. Several scrape lines were made on the cell monolayer with a surgical blade. After a period of 3 min for diffusion of fluorescent dye mixture (0.5 mg/mL LY and 2.5 mg/mL RD), cells were fixed with 4% paraformaldehyde for 15 min. Fluorescent signals were then evaluated using fluorescence microscopy.

Flow cytometry

Culture medium was removed after the cells were treated with Cd (0 and 10 μM) alone, GA (5 μM) or pretreated with GA (5 μM) for 30 min followed by incubation with Cd (10 μM) for 9 h. Cells were then collected and washed twice with phosphate-buffered saline (PBS).

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was detected using Fluo-4/AM as an intracellular Ca^{2+} fluorescent probe. After treatment, cells were collected by trypsinization and incubated with Fluo-4/AM (5 mM) in the dark for 30 min at 37°C. Stained cells were washed with PBS and analyzed by flow cytometry (Becton, Dickinson and Company, USA).

To measure the apoptosis rate, cells were stained with 5 μL annexin V−FITC and 5 μL propidium iodide (PI) for 15 min according to the protocol provided by the manufacturer (BD Biosciences Pharmingen, USA), after which cells were analyzed by flow cytometry. Total apoptotic proportion included the early apoptotic (Annexin V+/PI-) and late apoptotic (Annexin V+/PI+) fractions.

The mitochondrial membrane potential ($\Delta\psi m$) was detected by using a JC-1 Mitochondrial Membrane Potential Assay Kit (Beyotime, China), which has been widely applied to detect mitochondrial depolarization during apoptosis [26]. Harvested cells were stained with JC-1 in the dark for 20 min at 37°C. Cells were washed with 1 × JC-1 buffer, resuspended in 1 mL 1 × JC-1 buffer, and analyzed by flow cytometry.

Hoechst 33258 staining

After treatment, cells were fixed in 4% paraformaldehyde at 4°C for 20 min, stained with 5 μg/mL Hoechst 33258 at room temperature for 15 min, then examined by fluorescence microscopy to observe the cellular and nuclear morphology.

Western blot analysis

After treatment, cells were washed 3 times with cold PBS, then collected and extracted into RIPA lysis buffer on ice for 30 min, then sonicated at 3W for 15 s and centrifuged at 1.2×10^4 g for 10 min at 4°C. Protein content was determined using a BCA protein assay kit. Equivalent amounts of protein from each treatment were separated on 10 to 15% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated at room temperature for 2 h with 5% non-fat milk, then incubated with primary antibodies: rabbit anti-rat antibody Cx43, p-Cx43, Bax, Bcl-2, procaspase-3, cleaved

caspase-3, ERK, p-ERK, JNK, p-JNK, p38 and p-p38 (1 : 1,000 dilution) or β-actin (1 : 5,000 dilution), followed by HRP conjugated IgG antibodies (1 : 5,000 dilution) for 2 h. Finally, membranes were visualized using an ECL detection kit.

Statistical analysis

All results are presented as the means ± SD. Significance was assessed by one-way ANOVA and statistical analysis was performed using the SPSS software (ver. 18.0; SPSS, USA). *P* values < 0.05 and < 0.01 were considered significant and highly significant, respectively.

Results

Effects of Cd and GA on the growth of BRL 3A cells

RTCA was used to assess the effects of Cd on the growth of BRL 3A cells. Cells were treated with a series of concentrations of Cd (0, 2.5, 5, 10 and 20 μM), which resulted in a dose-dependent decrease in the CI value (panel A in Fig. 1). Cells were pretreated with GA (5 μM) for 30 min followed by Cd (10 μM), and the Cd-induced reduction in CI value was enhanced by GA (panel B in Fig. 1).

Cd and GA inhibited GJIC between BRL 3A cells

Functional analysis of GJIC was quantified by measurement of LY dye from the scraped edge to neighboring cells. Cd down-regulated GJIC, while GA almost completely inhibited GJIC when administered either alone or with Cd (panel A in Fig. 2). Cd decreased Cx43 expression and increased p-Cx43 level, whereas pretreatment with GA did not significantly alter the effect that Cd exerted on expression levels of Cx43 and p-Cx43 (panels B and C in Fig. 2). Cd exposure led to a significant increase in $[Ca^{2+}]_i$ compared to control groups, and pretreatment with GA was associated with a higher concentration of $[Ca^{2+}]_i$ compared to the Cd-treated group (panel D in Fig. 2).

GA promotes Cd-induced apoptosis in BRL 3A cells

Control cells exhibited no significant changes in cell nuclei, whereas Cd-treated cells demonstrated chromatin condensation and nuclear fragmentation (panel A in Fig. 3). Cd increased apoptotic rates from 7.6% to 14.6%; pretreatment with GA enhanced the Cd-induced apoptotic rate from 14.6% to 21.4% (panel B in Fig. 3). In addition, Cd increased the ratios of Bax/Bcl-2 and cleaved caspase-3/ procaspase-3 (panels C and D in Fig. 3), while it significantly decreased Δψm (panel E in Fig. 3). Pretreatment with GA enhanced Cd-induced changes in Bax, Bcl-2, caspase-3 and Δψm (panels C and E in Fig. 3). Taken together, these results suggest that pretreatment with GA promoted Cd-induced apoptosis by regulating apoptosis-related proteins in BRL 3A cells.

Effects of Cd and GA on expression of MAPK proteins in BRL 3A cells

Cd apparently increased phosphorylation levels of ERK, JNK and p38. Surprisingly, pretreatment with GA significantly enhanced the phosphorylation levels of ERK and p38 activation, but had no significant effects on phosphorylation of JNK activation (Fig. 4). These results suggest that the ERK and p38 signaling pathways played important roles in GJ blockage promoting Cd-induced apoptosis in BRL 3A cells.

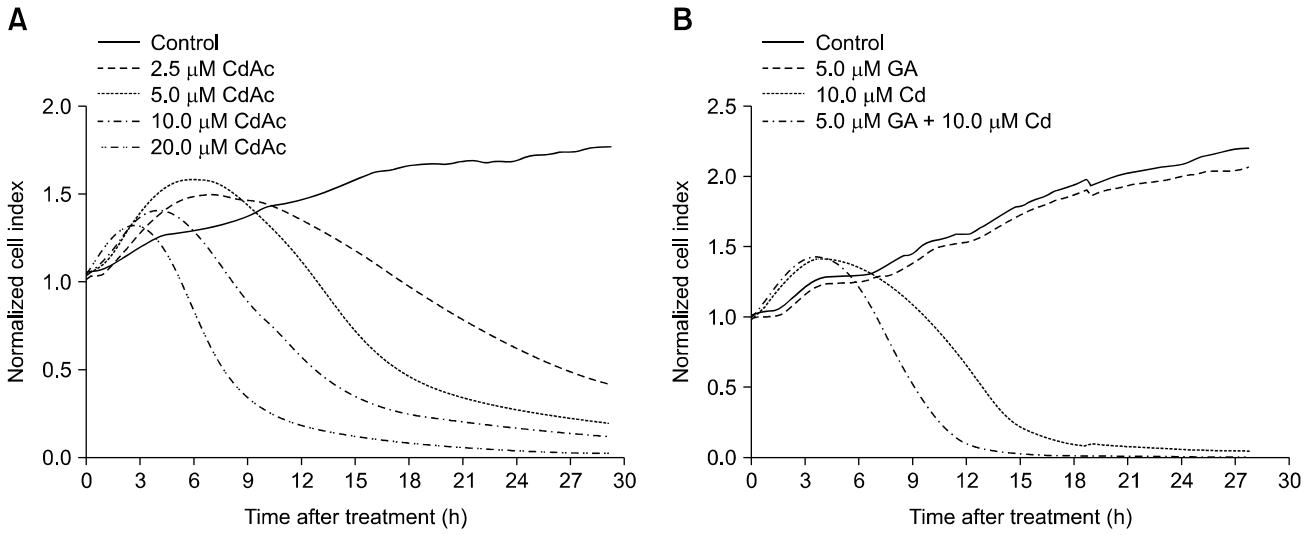

Fig. 1. Effects of 18β-glycyrrhizic acid (GA) on cadmium (Cd)-induced cytotoxicity in BRL 3A rat liver cells. (A) BRL 3A cells treated with different Cd concentrations (0, 2.5, 5, 10 and 20 μM) showed dose-dependent decreases in cell index (CI) values. (B) Treatment of BRL 3A cells with Cd (10 μM) and GA (5 μM) for 9 h, either alone or together, revealed that GA enhanced Cd-inhibited CI values. Data were normalized at the time that the growth medium was replaced.

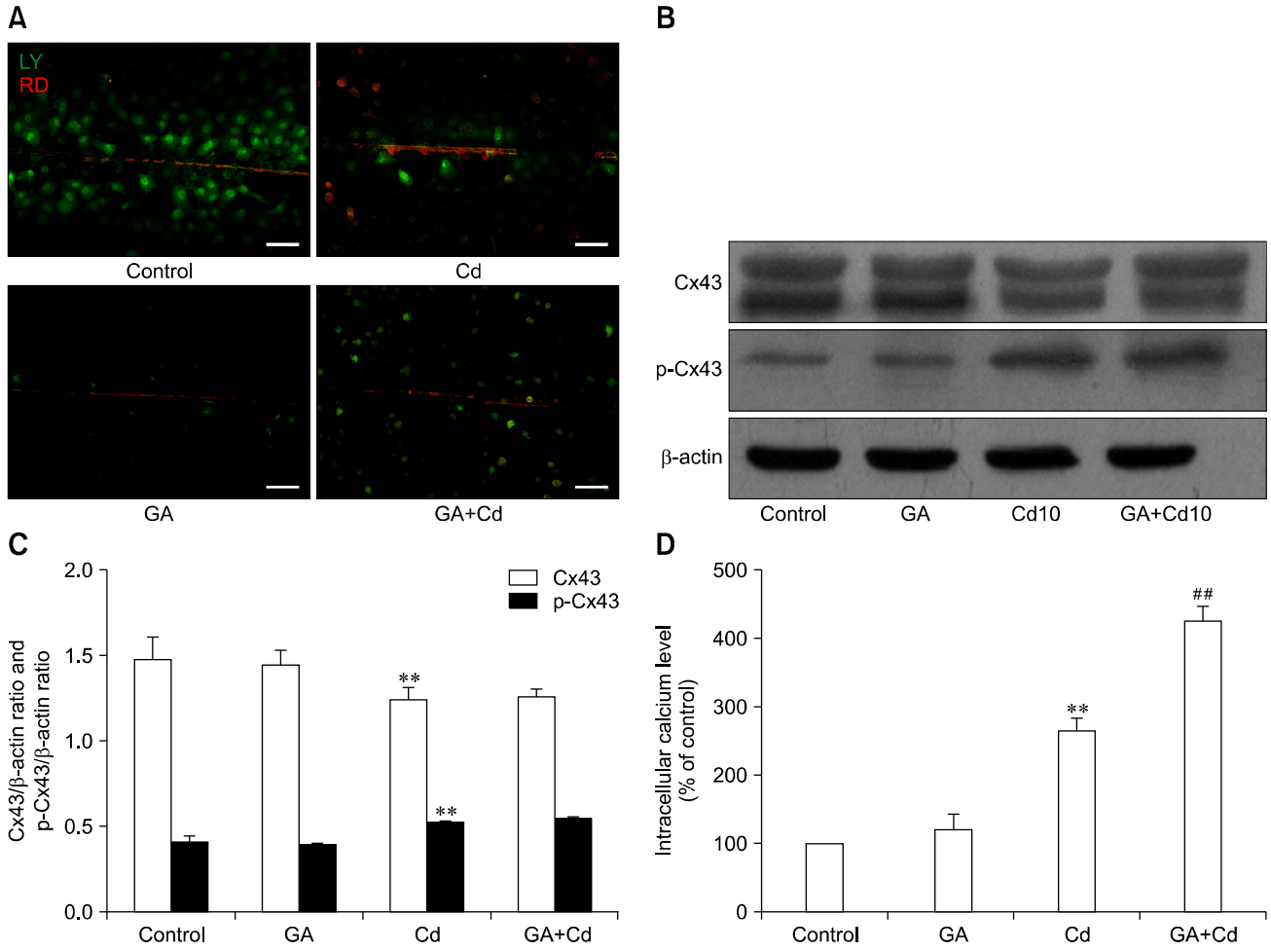

Fig. 2. Effects of Cd and GA on gap junctional intercellular communication (GJIC), the expression of connexin 43 (Cx43) and p-Cx43, and the release of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). (A) Effect of GA on Cd-induced down-regulation of GJIC in BRL 3A cells. GJIC was assessed using the scrape-loading/dye transfer method under an inverted fluorescence microscope. (B) Western blot analysis was performed using Cx43 and p-Cx43 antibodies. All experiments were performed in triplicate. (C) Blots for Cx43 and p-Cx43 were semi-quantified using Image Lab software. The values show the ratio of Cx43/β-actin and the ratio of p-Cx43/β-actin. Data are expressed as the means ± SD (n = 3). (D) $[Ca^{2+}]_i$ was detected with Fluo4/AM by flow cytometry. LY, Lucifer yellow; RD, rhodamine-labeled dextran. $^{*}p < 0.05$ or $^{**}p < 0.01$ *vs*. control group, $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ *vs*. Cd-treated group.

Discussion

Acute or chronic exposure to Cd induces hepatocyte apoptosis *in vitro* [37] and *in vivo* [34]. Apoptosis was characterized morphologically by chromatin condensation observed using Hoechst 33258 staining, and early and late apoptotic cells were detected using Annexin V and PI, respectively [18]. In this study, we demonstrated that Cd induced BRL 3A cells apoptosis, as indicated by CI values, apoptotic morphological changes and apoptosis rates. During apoptosis in mammalian cells, Bcl-2 interacts with the mitochondrial plasma membrane and prevents mitochondrial membrane pores from opening, thereby blocking apoptotic signals, including Bax [25]. The Bax/Bcl-2 ratio is a decisive factor in the regulation of apoptosis [14]. Caspase-3 is responsible for the proteolytic cleavage of many proteins and considered one of the key agents of apoptosis [12]. The current study showed Cd increased Bax expression, while it decreased Bcl-2 expression and $\Delta\psi m$, which triggered apoptosis in BRL 3A cells. This scenario suggests that modulation of Bcl-2 members might be essential to Cd induction of BRL 3A cells. Moreover, our previous study indicated that MAPKs were involved in Cd-induced apoptosis in BRL 3A cells [37].

GJIC activity is considered a key regulatory mechanism in homeostatic control [5]. Phosphorylation or expression of the connexin subunits, pH and $[Ca^{2+}]_i$ voltage have been reported to regulate GJIC [33]. In the current study, we determined that Cd down-regulated GJIC between BRL 3A cells in conjunction with decreased Cx43 expression, increased Cx43 phosphorylation

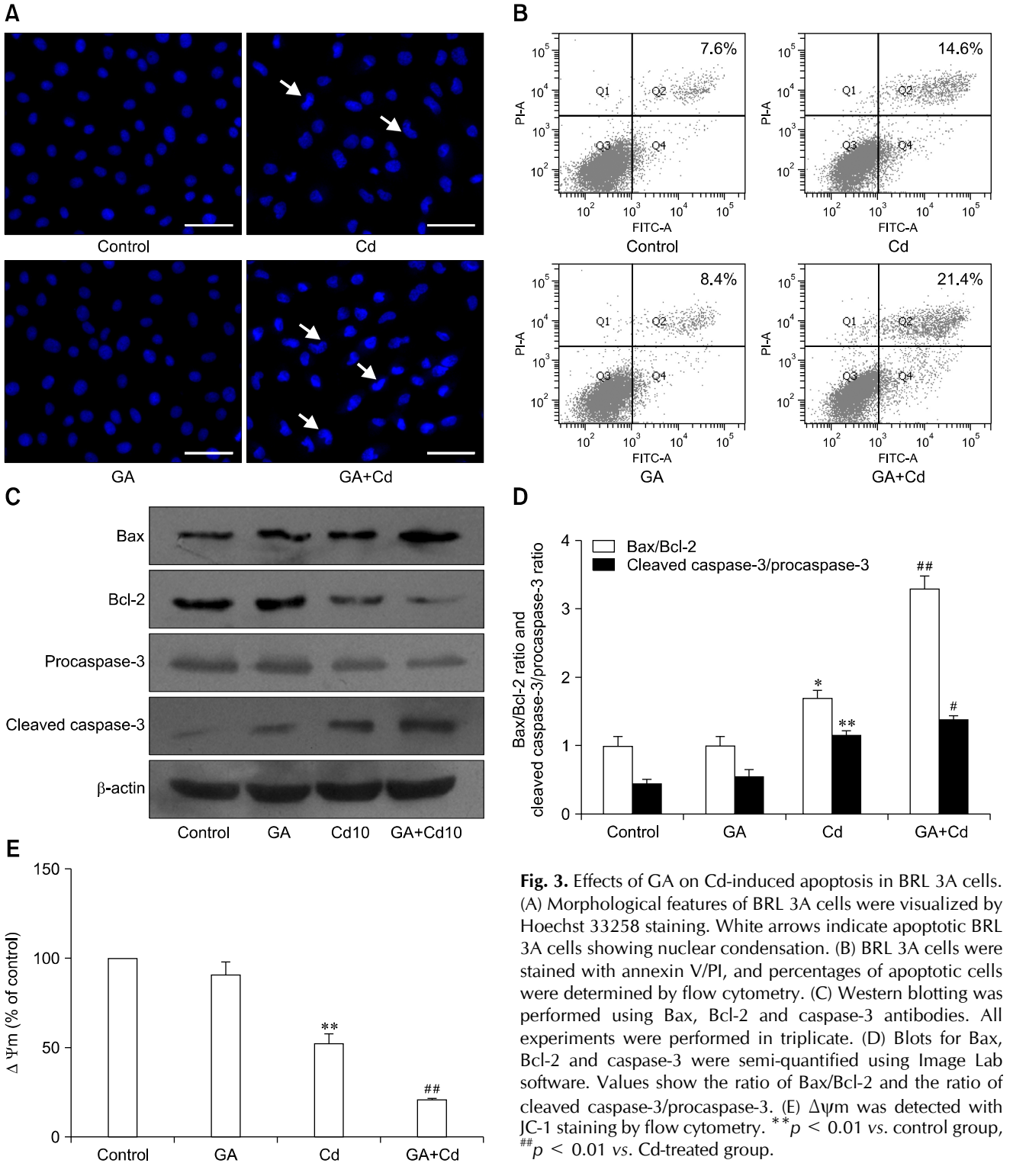

Fig. 3. Effects of GA on Cd-induced apoptosis in BRL 3A cells. (A) Morphological features of BRL 3A cells were visualized by Hoechst 33258 staining. White arrows indicate apoptotic BRL 3A cells showing nuclear condensation. (B) BRL 3A cells were stained with annexin V/PI, and percentages of apoptotic cells were determined by flow cytometry. (C) Western blotting was performed using Bax, Bcl-2 and caspase-3 antibodies. All experiments were performed in triplicate. (D) Blots for Bax, Bcl-2 and caspase-3 were semi-quantified using Image Lab software. Values show the ratio of Bax/Bcl-2 and the ratio of cleaved caspase-3/procaspase-3. (E) Δψm was detected with JC-1 staining by flow cytometry. $^{**}p < 0.01$ *vs*. control group, $^{\#\#}p < 0.01$ *vs*. Cd-treated group.

and elevated $[Ca^{2+}]_i$. Similarly, Cx43 expression was decreased in the diabetic rat heart, while Cx43 phosphorylation was increased [17]. $[Ca^{2+}]_i$ plays a vital role in protecting healthy cells from damaged cells by reducing the flux of toxic metabolites through GJ [3]. Our previous report demonstrated that Cd inhibited GJIC by $[Ca^{2+}]_i$ release [38].

Previous studies have shown that so-called death signals and cell survival factors are transferred via GJIC [36]. To elucidate

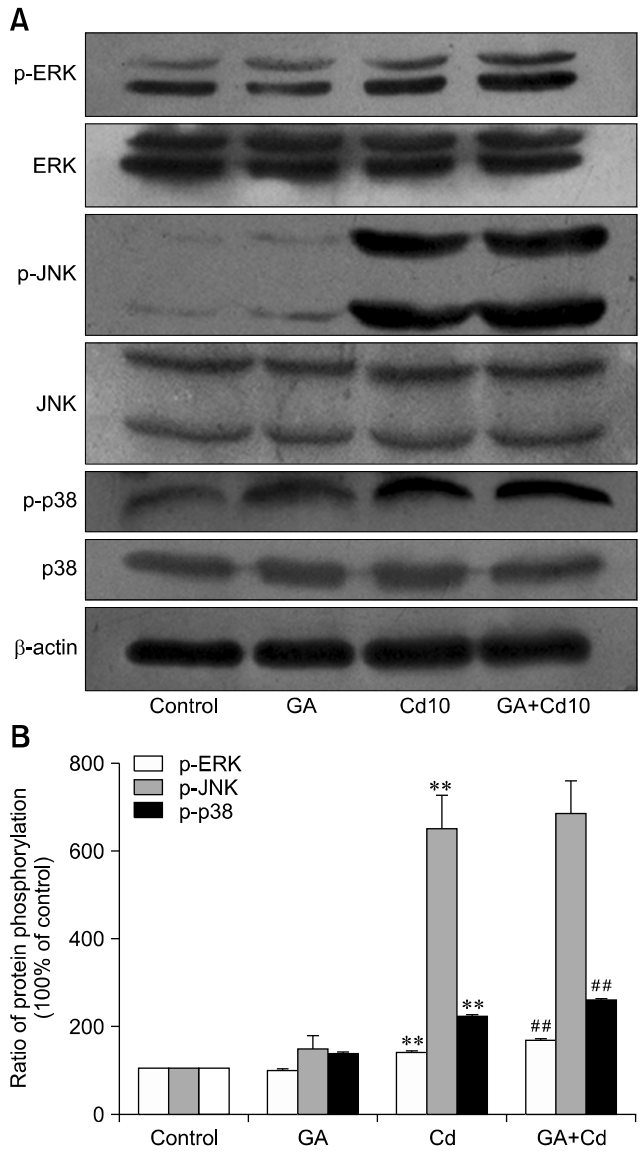

Fig. 4. Effects of Cd and GA on MAPK pathways. (A) ERK, JNK and p38 expression and phosphorylation were detected by western blot analysis with corresponding antibodies. All experiments were performed in triplicate. (B) Blots for ERK, JNK and p38 phosphorylation were semi-quantified using Image Lab software. Data are expressed as the means ± SD (n = 3) *vs*. the control group.

whether GJ coupling was involved in Cd-induced apoptotic reactions, BRL 3A cells were treated with GA. It is known that GA rapidly and reversibly inhibits GJ coupling [7]. In the present study, GA inhibited GJIC and did not directly interfere with apoptotic signaling processes since the rate of apoptosis in GA-treated cells was unaffected. We observed that GA promoted Cd-induced apoptosis in BRL 3A cells. Similarly, GJ blockage has been reported to be involved in the promotion of apoptotic processes [21]. In contrast, GJ blockage may be involved in C1B1 peptides protecting cells from apoptosis [16] and GJ promotes apoptosis in HeLa cells in a connexin-type-dependent manner [11]. It is believed that long-term inhibition of GJIC caused $[Ca^{2+}]_i$ overload, resulting in a series of cascade reactions that may eventually lead to apoptosis [33]. In the current study, $[Ca^{2+}]_i$ released by Cd was enhanced by GA. Our results also showed that GA enhanced Cd-induced changes in Bax, Bcl-2, caspase-3 and $\Delta\psi m$. These findings suggest that GA promotes Cd-induced apoptosis and influences expression levels of apoptosis-related proteins.

Three major mammalian MAPKs, ERK, JNK and p38 kinase, link extracellular signals to the machinery that controls cellular processes such as growth, proliferation and apoptosis [23]. Pretreatment with GA was found to significantly enhance phosphorylation levels of ERK and p38 activated by Cd. The ERK pathway plays an important role in cell proliferation, acting as a negative regulator, and inducing apoptosis when its activity is highly elevated. The p38 pathway is involved in apoptosis by promoting cell death [27]. MAPK activity is regulated by distinct signal transduction pathways that control many aspects of mammalian cellular physiology [19]. The results of the present study suggest that transmission of certain extracellular signals was influenced by GJ blockage, resulting in augmented Cd-induced apoptosis through ERK and p38 signaling pathways.

In conclusion, Cd induced apoptosis and down-regulated GJIC in BRL 3A cells. GJ blockage promoted Cd-induced apoptosis in BRL 3A cells via changes in expression levels of apoptosis-related proteins, ERK and p38 signaling pathways. This study partly elucidated the effects of gap junctions on Cd-induced apoptosis in BRL 3A cells. Overall, the findings presented herein provide insight into the hepatotoxic mechanism of *in vitro* exposure to Cd.

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Conflict of Interest

There is no conflict of interest.

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