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Deoxycholic acid inhibited proliferation and induced apoptosis and necrosis by regulating the activity of transcription factors in rat pancreatic acinar cell line AR42J

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Abstract The objective of this study is to investigate the effect of deoxycholic acid (DCA) on rat pancreatic acinar cell line AR42J and the functional mechanisms of DCA on AR42J cells. AR42J cells were treated with various concentrations of DCA for 24 h and also treated with 0.4 mmol/L DCA for multiple times, and then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to detect the AR42J cell survival rate. Flow cytometric was used to detect the cell apoptosis and necrosis in AR42J cells treated with 0.4 mmol/L and 0.8 mmol/L DCA. The cells treated with phosphate buffer saline (PBS) were served as control. In addition, the DNA-binding activity assays of transcription factors (TFs) in nuclear proteins of cells treated with DCA were determined using Panomics Procarta Transcription Factor Assay Kit. The relative survival rates were markedly decreased ($P<0.05$) in a dose- and time-dependent manner. Compared with control group, the cell apoptosis and necrosis ratio were both significantly elevated in 0.4 mmol/L DCA and 0.8 mmol/L DCA groups ($P<0.01$). A significant increase ($P<0.05$) in the activity of transcription factor 2 (ATF2), interferon-stimulated response element (ISRE), NKX-2.5, androgen receptor (AR), p53, and hypoxia-inducible factor-1 (HIF-1) was observed, and the activity of peroxisome proliferator-activated receptor (PPAR), activator protein 1 (AP1), and E2F1 was reduced ($P<0.05$). In conclusion, DCA inhibited proliferation and induced apoptosis and necrosis in AR42J cells. The expression changes of related genes regulated by TFs might be the molecular mechanism of AR42J cell injury.

Keywords Rat pancreatic acinar cell · Deoxycholic acid · Cell injury · Transcription factors

Introduction

Acute pancreatitis (AP) is a sudden and formidable necroinflammatory disease, which is triggered by numerous factors (Frossard *et al.* 2008). The most common cause of AP is the presence of gallstones or obstruction within the distal common bile duct, and this etiology of AP is known as acute biliary pancreatitis (ABP) (van Geenen *et al.* 2010). ABP is associated with significant morbidity and mortality and accounts for 30–50% of AP cases in both children and adults (Bai *et al.* 2011). Thus, it is an urgent task to explore the underlying molecular mechanisms responsible for ABP progression.

A proposed mechanism for ABP is considered to be that the obstruction blocks the efflux of pancreatic zymogens, provokes elevated pressure in the pancreas, and leads to abnormal reflux of bile acids into the pancreatic duct, eventually causes

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function and structural damage of pancreatic acinar cells (Opie and Meakins 1909; Jones *et al.* 1987). Acinar cell injury is caused by bile acids through inducing various cellular changes, including decrease of ATP levels (Voronina *et al.* 2010), reduced mitochondrial membrane potential (Voronina *et al.* 2004), and increased production of reactive oxygen species (Booth *et al.* 2011).

Bile acids consist of a variety of organic and inorganic solutes, and the major component of bile is the amphipathic bile acids (Fisher and Yousef 1973; Weinman and Jalil 2009). Deoxycholic acid (DCA), as a secondary bile acid (Boyer 2013), induces nonspecific cell lysis, as well as diminishes membrane integrity and cell viability (Schuller-Petrovic *et al.* 2008). An in vitro study by Klein et al. has also shown that DCA reduces 3T3-L1 adipocyte viability even at low doses (Klein *et al.* 2009). Additionally, various cells, including primary human subcutaneous adipocytes, human dermal fibroblasts, human melanoma cell, and human keratinocytes, expose to high concentrations of DCA leading to rapid cell death due to membrane disruption (Schuller-Petrovic *et al.* 2008; Thuangtong *et al.* 2010). Recently, a case report of Gregory et al. has found that a patient with recurrent AP has a marked elevation of fecal DCA levels (Plotnikoff 2014). Nevertheless, the role of DCA in pancreatic acinar cells and its mechanisms remain unclear and need to be further elucidated.

Rat pancreatic acinar cell line AR42J with most functions of pancreatic acinar cell is served as the standard cell line in in vitro experiments of AP (Folch-Puy *et al.* 2006; Long *et al.* 2009). In this study, we examined the effect of DCA on the AR42J cell proliferation, apoptosis, and necrosis and then further explored the DNA-binding activity of transcription factors (TFs) in AR42J cells.

Materials and Methods

Cell culture. Rat pancreatic acinar cell line AR42J was purchased from Shanghai Cell Bank of Chinese Academy of Sciences. The cells were cultured in Ham's F12K medium (Sigma, Louis, MO) supplemented with 20% fetal bovine serum (FBS, Gibco, Carlsbad, CA) and 100 U/mL penicillin/streptomycin (Harbin Pharmaceutical Group, Harbin, China) in 37°C incubator with a humid atmosphere of 5% CO_2. The medium was changed every 3–4 d.

MTT assay. The 2×10^4 cells in logarithmic phase were seeded into each well of a 96-well plate (Corning, Steuben County, New York, NY). At the second day, the culture medium was changed. On the one hand, the confluent cells were maintained with medium containing various concentrations of deoxycholic acid (DCA, Alfa Aesar, Ward Hill, MA) (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mmol/L) for 24 h. Medium without DCA was used as the negative group (0 mmol/L). On the other hand, the confluent cells were treated with 0.4 mmol/L DCA for 0 (negative control), 12, 24, 36, 48, and 60 h. The blank group (medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO) was set up. Then, 10 μL MTT (Sigma) in phosphate buffer saline (PBS) with a concentration of 5 g/L was added into each well for 4 h at 37°C. The culture medium was removed, and dimethyl sulfoxide (DMSO, 100 μL, Sigma) was later added into each well to solubilize the formazan crystals. The absorbances were read at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The relative cell survival rate was calculated as optical density (OD) $_{\text{test group}-\text{blank group}}/\text{OD}_{\text{negative group}-\text{blank group}}$. All determinations were carried out in triplicate.

Flow cytometric detection. Annexin V/PI Apoptosis Detection kit (Centre Bio, Beijing, China) was used to detect the cell apoptosis and necrosis. The 1×10^6 cells in logarithmic phase were seeded into each well of a six-well plate. After 24 h, the culture medium was removed and the cells were treated with 0.4 mmol/L and 0.8 mmol/L DCA for 24 h, respectively. The cells treated with phosphate buffer saline (PBS) were served as control. Then, the cells were digested with 0.125% trypsin + 0.02% EDTA and collected by centrifugation at 1500 rpm for 6 min and then washed with PBS for one time. Cells were slightly resuspended with 1× binding buffer and then added FITC–annexin V and PI in the dark for 15 min at 25°C. Cells were then added 400 μL 1× binding buffer and detected using flow cytometer (Gallios, Beckman Coulter Inc, Brea, CA).

Nuclear protein extraction. The 1×10^6 cells in logarithmic phase were seeded into each well of a six-well plate. After 24 h, the culture medium was removed and the cells were treated without DCA (control group) and with DCA (0.4 mmol/L) (treat group) for 30 min, 1 h, and 4 h, respectively. Cells were collected, and then, nuclear protein was extracted with Procarta TF Nuclear Extraction Kit (Fremont, CA), according to the manufacturer's instructions. In brief, the cells were washed twice with PBS, added 1 mL buffer A working reagent (1 mL buffer A, 10 μL dithiothreitol (DTT), 10 μL protease inhibitor, 10 μL phosphotase inhibitor I, and 10 μL phosphotase inhibitor II), and then placed on ice for 10 min in a shaker with 200 rpm. The cells were collected in 1.5-mL tubes, and supernatants were removed by centrifugation at 14,000 rpm for 3 min at 4°C. Sequentially, 150 μL buffer B working reagent (1 mL buffer B, 10 μL DTT, 10 μL protease inhibitor, 10 μL phosphotase inhibitor I, and 10 μL phosphotase inhibitor II) was added in 1.5-mL tubes containing the precipitation. The tubes were placed on ice for 2 h in a shaker with 200 rpm, and supernatants (nuclear protein) were acquired by centrifugation at 14,000 rpm for 5 min at 4°C. The protein concentration was detected by the BCA Protein Assay

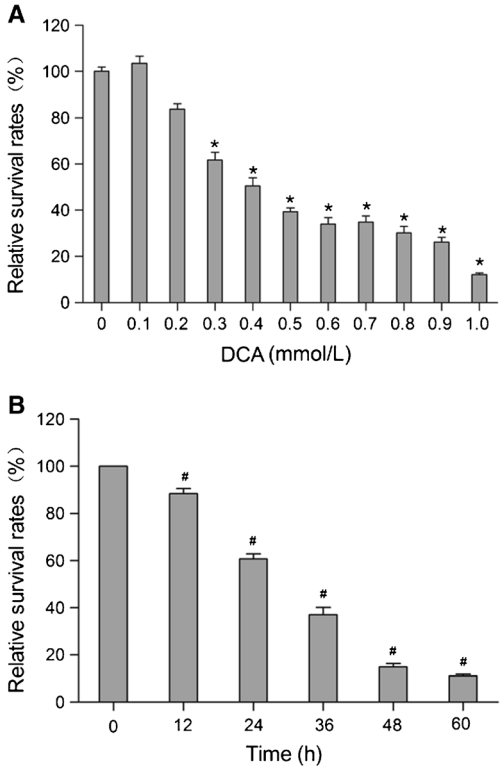

Figure 1. DCA inhibited AR42J cell proliferation in a dose- and time-dependent manner. **A** the relative survival rates were markedly decreased with the doses of DCA exceeding 0.3 mmol/L. **B** The relative survival rates were markedly decreased with the effect time exceeding 12 h in the presence of DCA (0.4 mmol/L). *P<0.05 versus 0 mmol/L group, #P<0.05 versus 0-h group.

Kit (Pierce, IL). Nuclear protein was stored at −80°C for the analysis of the DNA-binding activity of 40 TFs.

Analysis of the DNA-binding activity of 40 TFs. DNA-binding activity assays for 40 TFs in nuclear proteins were determined using Panomics Procarta Transcription Factor Assay Kit (40-plex Panel 1; Affymetrix, Santa Clara, CA) based on Luminex xMAP technology as previous study (Jiang *et al.* 2006; Ramadas *et al.* 2011). Briefly, nuclear protein was incubated with a mixture of biotin-labeled cis-element probes to form protein/DNA complexes. The complexes were bound to a filter, and unbound probes were then removed. Bound probes were denatured by heat and hybridized to Luminex microbeads with TF-specific anti-sense sequence. Probe-bound microbeads were detected with streptavidin–phycoerythrin and analyzed using the Luminex 100-IS instrument (Luminex, TX). The experiment was repeated three times with the same independent experimental samples.

Statistical analysis. Statistical analysis was performed by SPSS 12.0 statistical analysis software (SPSS Inc., Chicago, IL). Data were expressed as the mean±SD and analyzed by one-way ANOVA and independent samples t test. A value of P<0.05 was considered significant, and P<0.01 was considered highly significant.

Results

Effect of DCA on cell proliferation in AR42J. We performed MTT analysis to observe the cell proliferation, and the results showed that the relative survival rates were markedly decreased with the doses of DCA exceeding 0.3 mmol/L (P<0.05) (Fig. 1*A*), as well as with the effect time exceeding 12 h in the presence of DCA (0.4 mmol/L) (P<0.05) (Fig. 1*B*), indicating that DCA inhibited AR42J cell proliferation in a dose- and time-dependent manner.

Effect of DCA on cell apoptosis and necrosis in AR42J. The apoptosis and necrosis ratio were quantitatively analyzed by flow cytometry, and the results showed that compared with control group, the cell apoptosis and necrosis ratio were both significantly elevated in 0.4 mmol/L DCA and 0.8 mmol/L DCA groups (P<0.01). In addition, cell apoptosis ratio was decreased (P<0.01) and cell necrosis ratio was increased (P<0.01) in 0.4 mmol/L DCA group compared with 0.8 mmol/L DCA group (Fig. 2, Table 1).

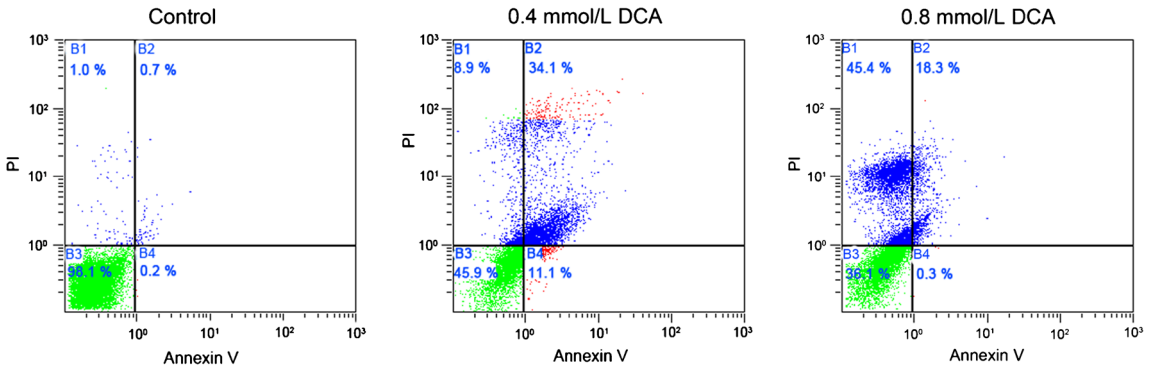

Figure 2. DCA induced AR42J cell apoptosis and necrosis. Compared with control group, the cell apoptosis and necrosis ratio were both significantly elevated in 0.4 mmol/L DCA and 0.8 mmol/L DCA groups.

Table 1. Cell apoptosis and necrosis ratio in the presence of DCA

Group	Cell apoptosis ratio (%)	Cell necrosis ratio (%)
Control	0.97±0.31	0.93±0.21
0.4 mmol/L DCA	45.5±1.08**	8.9±0.3**
0.8 mmol/L DCA	18±0.78**##	45.8±1.06**##

DCA deoxycholic acid

**$P<0.01$ versus control group, ##$P<0.01$ versus 0.4 mmol/L DCA group

Effect of DCA on the DNA-binding activity of TF in AR42J cells. We analyzed the ability of DCA to activate 40 different TFs using the Procarta Transcription Factor Assay Kit. After 1 h of DCA treatment, a significant increase ($P<0.05$) in the DNA-binding activity of transcription factor 2 (ATF2), NKX-2.5, androgen receptor (AR), p53, and hypoxia-inducible factor-1 (HIF-1) was observed and the DNA-binding activity of peroxisome proliferator-activated receptor (PPAR), activator protein 1 (AP1), E2F1, and interferon-stimulated response element (ISRE) was reduced ($P<0.05$) (Fig. 3). DCA-induced activity change of these TFs was disappeared after 4 h of DCA treatment (Fig. 3).

Discussion

The present study demonstrated that DCA inhibited AR42J cell proliferation in a dose- and time-dependent manner and 0.4 mmol/L DCA induced AR42J cell apoptosis and necrosis;

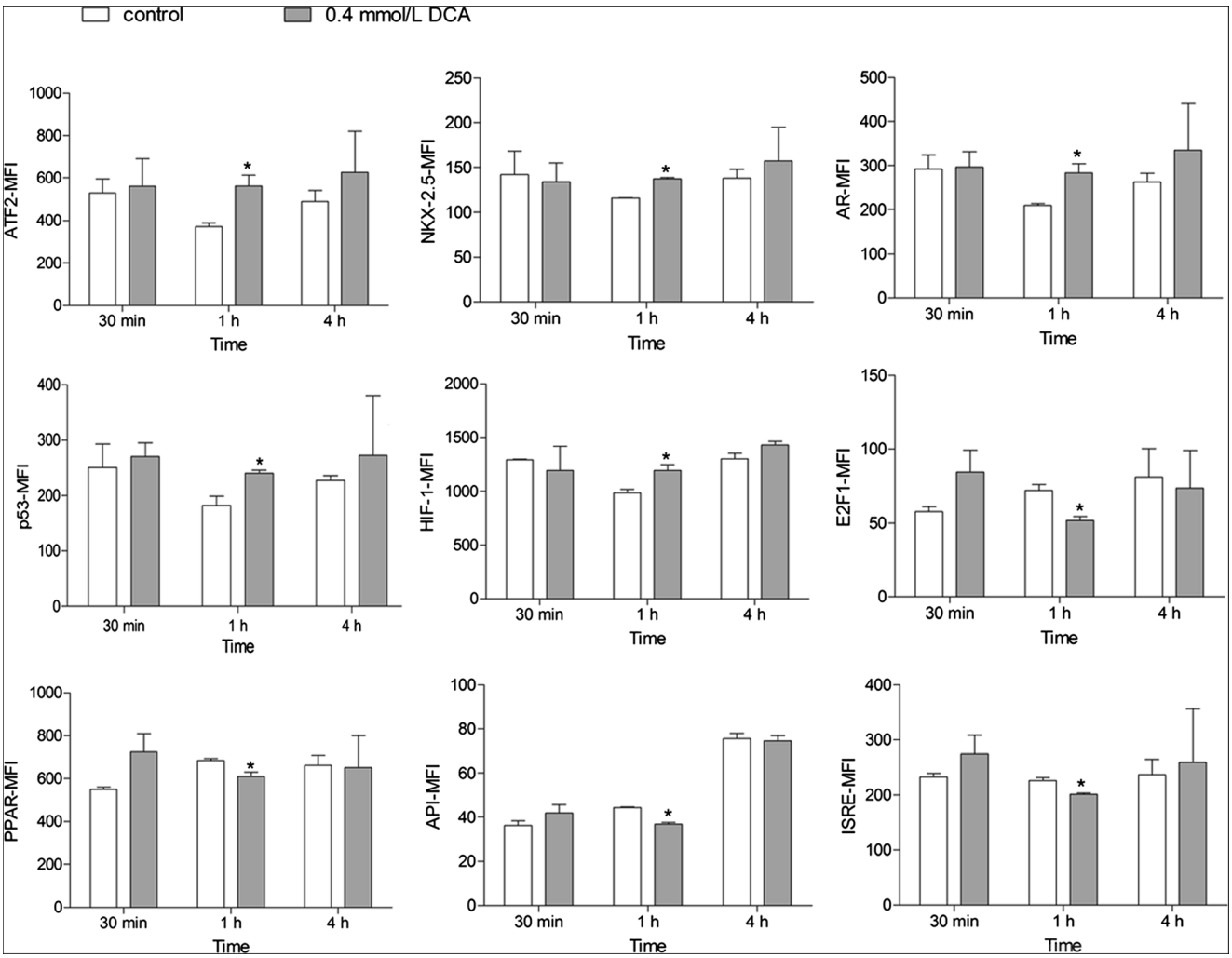

Figure 3. DCA changed the activity of certain transcription factors in AR42J cells. After 1 h of DCA treatment, a significant increase in the DNA-binding activity of ATF2, NKX-2.5, AR, p53, and HIF-1 was observed and the DNA-binding activity of PPAR, AP1, E2F1, and ISRE was reduced. *$P<0.05$ versus control. *MFI* median fluorescence intensity, *ATF2* transcription factor 2, *AR* androgen receptor, *HIF-1* hypoxia-inducible factor-1, *PPAR* peroxisome proliferator-activated receptor, *AP1* activator protein 1, *ISRE* interferon-stimulated response element.

however, 0.8 mmol/L DCA mainly induced AR42J cell necrosis. In addition, the DNA-binding activity of some TFs, including ATF-2, NKX-2.5, AR, p53, HIF-1, PPAR, API, E2F1, and ISRE, was changed after AR42J cells were treated with 0.4 mmol/L DCA, indicating that the expression changes of related genes regulated by these TFs might be the molecular mechanism of AR42J cell injury.

Previous study has shown that a Ca^{2+}-dependent activation of calcineurin induced by bile acids led to acinar cell injury (Muili *et al.* 2013). Also, bile acids have been shown to involve in regulating cell proliferation, apoptosis, and differentiation by activating protein kinase C (PKC) signaling pathway (Clemens *et al.* 1992; Huang *et al.* 1992). In this study, our results showed that DCA inhibited AR42J cell proliferation and induced AR42J cell apoptosis and necrosis, suggesting that DCA had a cytotoxic effect on AR42J cells. Our results were in agreement with some scholars, who had demonstrated that the cytotoxicity of DCA was caused by both apoptosis and necrosis in colon cancer cells, while the majority of the cells were undergoing apoptosis (Martinez *et al.* 1998; Glinghammar *et al.* 2002). In addition, we also found that a high concentration of DCA mainly induced AR42J cell necrosis rather than cell apoptosis compared to a low concentration of DCA, suggesting that induced AR42J cell necrosis might be an important reason for DCA enhancing the cell injury and the severity of ABP.

To investigate the mechanism of the cytotoxicity of DCA on AR42J cells, we analyzed the ability of DCA to activate TFs. Our study showed that DCA activated the DNA-binding activity of ATF-2, NKX-2.5, AR, p53, and HIF-1 and inhibited the DNA-binding activity of PPAR, AP1, E2F1, and ISRE. These TFs regulated target gene transcription either positively or negatively by binding to specific sequences termed response elements. ATF2 has previously been implicated in the regulation of a wide series of genes that involved in the regulation of cell growth, apoptosis, differentiation, and immune response, including c-Jun (Van Dam *et al.* 1995), E-selectin (Kaszubska *et al.* 1993), transforming growth factor β (TGF-β) (Kim *et al.* 1992), tumor necrosis factor alpha (TNF-α) (Tsai *et al.* 1996), and cyclin A (Shimizu *et al.* 1998). Ivanov and Ronai had shown that ATF2 contributed to UVC-induced apoptosis by reducing the activation of the TNF-α promoter and decreasing expression of TNF-α in melanoma cells (Ivanov and Ronai 1999). It was well known that p53 played a decisive role in cell apoptosis, cell cycle arrest, senescence, and other physiological processes (Vousden and Prives 2009). As a TF, p53 promoted the expression of several proapoptotic genes, including Bax, Puma, Noxa, and Bid and also repressed the transcription of certain anti-apoptotic genes, including Bcl-2, Bcl-xL, and survivin (Laptenko and Prives 2006; Vousden and Prives 2009). HIF-1 was also demonstrated to have a proapoptotic function by regulating its target genes, including Nip3 (Bruick 2000) and RTP801 (Shoshani *et al.* 2002). In addition, PPARα could suppress hepatocyte apoptosis induced by peroxisome proliferators (Roberts *et al.* 1998). Collectively, the activity changes of these TFs might be related to the cytotoxicity of DCA on AR42J cells. However, further experiments are still needed to confirm our result.

In conclusion, DCA inhibited AR42J cell proliferation and induced AR42J cell apoptosis and necrosis. The expression changes of related genes regulated by TFs might be the molecular mechanism of AR42J cell injury. DCA might be a novel therapeutic target for ABP.

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Authors' contributions GZ participated in the design of this study, and they both performed the statistical analysis. HC carried out the study, together with JZ, collected important background information, and drafted the manuscript. DS and BQ conceived of this study and participated in the design and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest All authors declare that they have no conflict of interest to state.

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