Taurochenodeoxycholic acid induces NR8383 cells apoptosis via PKC/JNK-dependent pathway

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Abstract

Our former studies have suggested that taurochenodeoxycholic acid (TCDCA) as a signaling molecule shows obvious anti-inflammatory and immune regulation properties. In this research, we tentatively explored the potential effects and the possible mechanism that involve in the apoptotic process in NR8383 cells induced by TCDCA. Using flow cytometry analysis, we evaluated the apoptosis rate. Gene expression levels were determined by qPCR. The expressions of protein kinase C (PKC), Jun N-terminal kinase (JNK) and their phosphorylation were measured by Western Blot. We observed the activities of caspase-3 and caspase-8 with Caspase-Glo[®] reagent. The results demonstrated that TCDCA dramatically improved the apoptosis rate of NR8383 cells in a concentration-dependent manner. In the meantime, PKC mRNA levels and activities were significantly augmented by TCDCA treatments. In addition, JNK, caspase-3 and caspase-8 mRNA expression levels and activities were increased by TCDCA, while they were markedly decreased by specific

inhibitors. We conclude that TCDCA contributes to the apoptosis through the activation of the caspase cascade in NR8383 cells, and the PKC/JNK signaling pathway may be involved in this process. These results indicate that TCDCA may be a latent effective pharmaceutical product for apoptosis-related diseases.

Key Words: TCDCA; Apoptosis; PKC; JNK; Caspase; Macrophage

1. Introduction

Apoptotic cell death is viewed as programmed cell suicide and it is a strictly mediated process. Apoptosis not merely plays a prominent part in immune regulation and tissue homeostasis, but it also effects pathological and treatment settings. On one hand, deficient apoptosis can lead to persistent infections, neoplasia and autoimmune diseases. On the other hand, an excess of apoptosis contributes to T-cell depletion, degeneration of liver cells or neurodegenerative diseases (Daniel, 2000). The apoptotic process may be mediated by multiple molecular signaling pathways. However, the intrinsic pathway is the best portrayed and most dominated pathway, which involves mitochondria (Shi, 2002). As well, the extrinsic pathway induced apoptosis occurs through the activation of death receptors at the cytomembrane (Ashkenazi, 2002). Though these two classic apoptosis pathways act independently, there are some mutual pivotal proteins to both pathways.

Bile acid is present in humans and most animal species. As detergent molecules, they synthesized mainly from the cholesterol metabolic pathway, which involves a sophisticated series of chemical reactions in the liver (Russell and Setchell, 1992; Amaral et al., 2009). These amphiphilic molecules are also essential factors to activate certain pancreatic enzymes. Because of the amphiphilic property of bile acids,

they have the ability to form mixed micelles with lipid soluble vitamins, phospholipids, cholesterol, triglycerides and fatty acids in the gallbladder and intestines. These features contribute to solubilization and absorption (Vlahcevic et al., 1991). However, recent studies indicate that bile acids also act as signaling molecules. Lithocholic acid, for example, activates mitochondrial/caspase-9 pathway that is initiated by caspase-8 leading to apoptosis in colon cancer cell lines (Katona et al., 2009). Caspase-3 and caspase-8 are the primary initiator caspases of death-receptors. Caspase-8 induces apoptosis through direct activation of effector caspases in type I cells (Peter and Krammer, 2003). Several cellular signaling pathways are involved in bile acids, such as the translocation and activation of PKC, calcium mobilization and the synthesis of cyclic AMP (cAMP) (Nguyen and Bouscarel, 2008). In the Kupffer cells (specialized stellate macrophages located in the liver), bile acids promote the secretion of proinflammatory factors. For instance, tumor necrosis factor α (TNF α) and interleukin-1 β (IL-1 β) can activate TNF receptor signaling and the mitogen-activated protein kinase (MAPK)/JNK pathway (Miyake et al., 2000; Gutierrez et al., 2006).

TCDCA is one of the primary bioactive compounds of animals' bile acid, which taurine and chenodeoxycholic acid are joined by dehydration synthesis in organism (Hofmann, 1984). In our previous study, TCDCA was shown to significantly suppress both acute and chronic inflammation (Liu et al., 2011a). However, it is still uncertain the relationship between anti-inflammatory and immune regulation effect of TCDCA and its influence on apoptosis of immune cells. In this regard, we chose NR8383 cells (alveolar macrophages of rat) as the research objects. In order to targeted understanding the anti-inflammatory and immune regulation effect after treatment of TCDCA on immune cells. The aim of this study focused mainly on whether TCDCA

has influence on apoptosis of macrophages and the potential molecular mechanism involved in this process.

2. Materials and Methods

2.1. Reagents

Sodium taurochenodeoxycholate (TCDCA, purity $\geq 97\%$), SP600125 and Gö 6983 (Sigma-Aldrich, USA), SDS-PAGE gels (Beyotime Biotechnology, China), FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA), Anti-PKC alpha antibody [Y124], Anti-PKC alpha (phospho S657+Y658) antibody, Anti-JNK1 antibody and Anti-JNK1(phospho T183+Y185) antibody (Abcam, USA), Caspase-Glo[®] 3/7 Assay and Caspase-Glo[®] 8 Assay (Promega, USA), BCA Protein Assay (Pierce, USA), SYBR[®] Premix Ex Tag[™], TripRe[™] RNA reagent and PrimeScript[™] RT Master Mix (TaKaRa Bio, Japan), Goat anti-Mouse IgG and Goat anti-Rabbit IgG (Gaithersburg, USA), Nuclear Extract Kit (Active Motif, USA), anti-actin (Proteintech Group, USA), Super Signal[®] West Femto (Thermo, USA).

2.2. Cell culture

NR8383 cells were purchased from the Shanghai Institute of Cellular Biology of Chinese Academy of Sciences. Cells were cultured in 25 ml plastic flasks (Corning, USA) and maintained in DMEM (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (ExCell Bio, Australia). Cells were cultured at constant 37°C under a humidified condition with 5% CO₂. When the confluence of cells reached approximately 70–80%, the non-adherent cells were discarded and the adherent cells

were trypsinized with 0.25% trypsin, then divided into 3 or 4 parts and incubate in the same medium.

2.3. Flow cytometry analysis

FITC Annexin V and Proidium Iodide (PI) binding was used to identify the existence of apoptosis. Cells were treated with different concentrations of TCDCA (100 μ M, 10 μ M, 1 μ M) for 48 h, while control NR8383 cells were incubated in DMEM alone. Then, all steps were performed on the basis of the manufacturer's protocol. In brief, the NR8383 cells were washed 2 to 3 times with pre-cooled PBS, centrifuged and resuspended at a concentration of 1×10^6 cells/ml with $1 \times$ Binding Buffer. Then 100 μ l of the solution namely 1×10^5 cells were transferred to 1.5 ml centrifuge tubes and FITC-Annexin V (final concentration 5 μ l/100 μ l) and PI (final concentration 5 μ g/ml) were added. After incubation at 25°C in the dark for 20 min, apoptosis was instantly detected using a BD FACS Aria™ flow cytometer (BD Biosciences, USA). About 1×10^4 cells were collected and analyzed with Cell Lab Quanta™ SC Analysis software (BECKMAN COULTER®, USA) per sample.

2.4. RNA isolation and qPCR assays

Quantitative real-time PCR (qPCR) was carried out to analyze the mRNA level of various cytokines. NR8383 cells were added into 24-well plates and cultured overnight for attachment. Thereafter, cells were pre-treated with different inhibitors for 1 h before being co-treated with TCDCA for another hour. The total cellular mRNA was extracted from 24-well plates using Tripure™ RNA reagent. The quality of

mRNA was determined by agarose gel electrophoresis and the ratio of OD_{260/280}. Synthesis of cDNA was carried out using PrimeScript™ RT Master Mix kit on the basis of the manufacturer's protocol. The cDNA was amplified on ViiA™ 7 system (ABI Biosystems®, USA) using SYBR® Premix Ex Tag™ kit. In brief, a total of 25 µl reaction mixture including 2 µl of cDNA, 12.5 µl of 2×SYBR® Premix Ex Tag™, 1 µl of specific target primers (10 µM) forward and reverse and 8.5 µl of ddH₂O. The qPCR thermal cycling settings were 30 s at 95°C, followed by 39 cycles of 5 s at 95°C, and 30 s at T_m, and 15 s at 95°C. The qPCR was carried out with the specific primers (Tab. 1). All data were calculated based on the comparative Ct formula (Liu et al., 2011b) and each sample was normalized by β-actin. Relative mRNA expressions were analyzed according to the Ct values, based on the equation: $2^{-\Delta Ct} [\Delta Ct = Ct (PKC, JNK, caspase-3, caspase-8) - Ct (\beta\text{-actin})]$. The melting curves were guaranteed the purity of each reaction.

Table.1 Primers sequence

Primer	Sequence (5'→3')
β-actin	Forward: GGAGATTACTGCCCTGGCTCCTA
	Reverse: GACTCATCGTACTCCTGCTTGCTG
PKC	Forward: TCCAAATGGGCTTTCGGAT
	Reverse: TGGATCGGATTTTGGTT
JNK	Forward: ACAGTGAGCAGAGCAGGCATA
	Reverse: CTTGTCAGGAGCACCATTC
caspase-3	Forward: GAGACAGACAGTGGAAGTACGATG
	Reverse: GGCGCAAAGTGACTGGATGA

2.5. Whole-Cell Protein Extraction

Cells were processed as described in the qPCR assay. The whole-cell protein was extracted from cells using Nuclear Extract kit on the basis of the manufacturer's protocol. In brief, cells (3×10^6 cells/per flask in 25 ml cell culture flasks) were washed 3 times with pre-cooled PBS/Phosphatase Inhibitors. The supernate was removed and the precipitate was resuspended in 1 ml PBS/Phosphatase Inhibitors, and centrifuged at $200 \times g$ for 5 min in a pre-cooled centrifuge at 4°C . The supernate was discarded and the precipitate was resuspended in 300 μl complete lysis buffer, left on ice for 20 min on a shaking table set at 150 rpm. Then the solution was vortexed and centrifuged at $14,000 \times g$ for 20 min at 4°C . The supernate that contained whole-cell extraction was collected and stored at -80°C .

2.6. Western Blot

BCA Protein Assay was performed to ensure the quality of total protein of lysates. Equal amounts of protein (40-60 μg) were loaded into 8-12% SDS-PAGE gels, and then transferred to PVDF membranes (Millipore Corporation, USA). The PKC and JNK activities were determined using the primary anti-bodies: anti-PKC alpha, anti-JNK1 antibodies and anti-phospho-PKC alpha, anti-phospho-JNK1 antibodies. The secondary antibodies conjugated to HRP were used as indicated. The bands were visualized by maximum sensitivity substrate. Chemiluminescence signals were measured using the ECL system (ImageQuant LAS 4000, GE Healthcare).

2.7. Caspase-3 and caspase-8 activities assays

The enzymatic activities of caspase-3 and caspase-8 were detected using Caspase-Glo[®] 3/7 and Caspase-Glo[®] 8 assay kits on the basis of the manufacturer's protocol. In brief, NR8383 cells were added into white 96-well plates (Nunc, USA) at the density of 6000 cells/well in triplicate wells and cultured overnight for attachment. Then cells were pre-treated with JNK inhibitor for 1 h before being co-treated with TCDCA for another hour. Thereafter same volume of Caspase-Glo[®] reagent was added into each well. Samples were incubated at 25°C and luminescence was detected after 1 h using a plate-reading luminometer (Synergy 4, Bio-tek, USA).

2.8. Statistical analysis

All data were executed using SPSS 17.0 software. Data were expressed as the means $\pm$ standard deviation (S.D.). Differences between control and experimental groups were compared using one-way ANOVA. $P < 0.05$ was considered significant.

3. Results

3.1. TCDCA induced NR8383 cells apoptosis

We first examined if TCDCA has an apoptotic effect on NR8383 cells. After cells were treated with different concentrations of TCDCA, we used flow cytometry to determine the apoptosis percentage of NR8383 cells. Based on FITC Annexin V

and PI double staining, the results demonstrated that TCDCA treatment enhanced the apoptosis rate of NR8383 cells. We found that the apoptosis percentage of NR8383 cells was augmented in the 10 μ M TCDCA treatment group, and even higher in the 100 μ M TCDCA treatment group. Therefore, TCDCA induced NR8383 cells apoptosis in a concentration-dependent manner (Fig. 1).

3.2. TCDCA effected PKC gene expression and activity

NR8383 cells were treated with different concentrations of TCDCA (100 μ M, 10 μ M, 1 μ M) for 1 h, while control NR8383 cells were incubated in DMEM alone. The gene expression level of PKC was detected by qPCR, and the activity of PKC was observed using Western Blot analysis with anti-PKC alpha and anti-phospho-PKC alpha antibodies. The results revealed that mRNA expression level of PKC was markedly augmented by TCDCA (100 μ M, 10 μ M and 1 μ M) treatments (Fig. 2). Treatment with 100 μ M and 1 μ M TCDCA remarkably enhanced PKC- α expression level compared with control group (Fig. 3B). Besides, phosphorylation of PKC- α was considerably increased by TCDCA (100 μ M and 10 μ M) treatments (Fig. 3C).

3.3. TCDCA effected JNK gene expression and activity

Gö 6983, which is a specific inhibitor of PKC, was used to testify whether the apoptotic process induced by TCDCA via PKC/JNK signaling pathway. For single treatments, NR8383 cells were treated with 100 μ M, 10 μ M and 1 μ M of TCDCA or Gö 6983 (10 μ M) for 1 h. For combined-treatments, NR8383 cells were pretreated with Gö 6983 for 1 h before being co-treated with TCDCA (100 μ M, 10 μ M and 1

μM) for another hour. The gene expression level of JNK was detected by qPCR, and the activity of JNK was observed by Western Blot analysis with anti-JNK1 and anti-phospho-JNK1 antibodies. The results showed that treatments with 100 μM , 10 μM and 1 μM of TCDCA significantly increased JNK mRNA levels. Besides, PKC specific inhibitor markedly reduced the mRNA expression level of JNK compared with control group. Thus, this is supporting a role for PKC as critical signal for the JNK gene expression. While TCDCA (100 μM , 10 μM and 1 μM) / Gö 6983 co-treatment markedly increased the mRNA expression level of JNK compared with Gö 6983 single treatment (Fig. 4). JNK expression levels were significantly augmented by TCDCA (100 μM , 10 μM and 1 μM). However Gö 6983 single treatment markedly suppressed JNK protein expression compared with control group (Fig. 5B). Furthermore, the expression of phosphorylated JNK was remarkably increased by 100 μM and 10 μM TCDCA. Meanwhile, PKC specific inhibitor significantly prevented JNK phosphorylation expression, suggesting that JNK was a downstream target of PKC activation in NR8383 cells (Fig. 5C).

3.4. TCDCA effected caspase-3 and caspase-8 gene expression and activities

The hypothesis that TCDCA induced NR8383 cells apoptosis through activation of JNK was tested using SP600125, which is a specific inhibitor of JNK. JNK performs a catalytic mechanism that activates the caspase cascade. For single treatments, NR8383 cells were treated with 100 μM , 10 μM and 1 μM of TCDCA or SP600125 (10 μM) for 1 h. For combined-treatments, NR8383 cells were pretreated with SP600125 for 1 h before being co-treated with TCDCA (100 μM , 10 μM and 1 μM) for another hour. Caspase-3 and caspase-8 mRNA expression levels were

examined using qPCR. We found that TCDCA (100 μ M, 10 μ M and 1 μ M) significantly increased the mRNA expression levels of caspase-3 and caspase-8 in a concentration-dependent manner, while significantly reduced by SP600125 compared with control group. Meanwhile, caspase-3 and caspase-8 mRNA levels were remarkably increased by TCDCA (100 μ M, 10 μ M and 1 μ M)/ SP600125 co-treatments compared with single treatment with SP600125 (Fig. 6).

The luminescent caspase enzymatic activity assay was performed in order to evaluate the activities of caspase-3 and caspase-8 with Caspase-Glo[®] reagent. The luminescent signals of the caspases represented caspase-3 and caspase-8 activities. The lower activities of caspase-3 and caspase-8 were found in the 1 μ M TCDCA group compared with control group, but they were remarkably augmented in the 100 μ M TCDCA treatment group. In addition, the activities of both caspase-3 and caspase-8 were markedly reduced by SP600125 treatment. Furthermore, caspase-3 activity was significantly increased by 100 μ M TCDCA/ SP600125 co-treatment and caspase-8 activity was markedly augmented by TCDCA (100 μ M, 10 μ M and 1 μ M)/ SP600125 in a concentration-dependent manner (Fig. 7).

4. Discussion

Cell apoptosis not only influences a wide range of tissues, such as kidney, liver, central nervous system, and especially heart, but also plays a vital role in many pathologic conditions and biological processes (Amaral et al., 2009). Consequently, it is not surprising that there is a dramatic growing interest in the molecular targets of apoptosis process. What's more, there is an urgency in finding novel alternative

therapies for apoptosis-associated disorders, like neurological disorders such as Huntington's diseases, Alzheimer's and Parkinson's. Accordingly, at both basic science and clinical levels, this field will continue to remain active.

We explored the connections between TCDCA and apoptosis in NR8383 cells. Furthermore, we would like to develop this active component of traditional Chinese medicine and make it become a new effective therapeutic agent based on its regulation effects of the apoptosis process. TCDCA was separated and purified from animal bile. Because of it is low-cost and safe, TCDCA was considered more convenient for many patients. The flow cytometry analysis results indicated that, TCDCA at high concentration (100 μ M) could significantly enhanced the apoptosis percentage in NR8383 cells, in a dose-dependent manner. This observation is in line with our previous report showing that TCDCA could effectively induce apoptosis in adjuvant arthritis fibroblast-like synoviocytes (FLS) (Li et al., 2013). If the apoptosis system was damaged, it either contributed to prolonged survival of cells or an excess of cells removal. Accordingly, the etiopathogenesis in many immune disorders were involved in dysregulation (S. Leena. et al., 2015). Our study provides evidence of TCDCA induces apoptosis in macrophages. Furthermore, we tentatively explored the potential mechanism that TCDCA inducing apoptotic process of NR8383 cells via the activation of the caspase cascade in NR8383 cells, and the PKC/JNK signaling pathway may be involved in this process. This provides theoretical foundation for further comprehension of the molecular mechanism of TCDCA inducing apoptotic process.

PKC involves many cellular regulation processes such as mediating immune responses, cell death and tumor promotion, and to a lesser extent, growth control and differentiation. PKC is classified into various isotypes, according to the cell types and

the expression of various kinds of isotypes, each exerting an important action in modulating cell cycle and endocellular signaling pathway. PKC plays a biological effect through regulating other protein's phosphorylation (Newton, 1997). For instance, PKC- α and PKC- β work as mitogenetic or anti-apoptosis kinases in mammary epithelial cells, while PKC- δ behaves like an inhibitor of proliferation or a trigger of apoptosis (Li and Weinstein, 2006; Ali et al., 2009). In this study the activity of PKC- α was detected using anti-phospho-PKC-alpha antibody, and its phosphorylation site is serine/tyrosine (Ser/ Tyr). We revealed that TCDCA enhanced the mRNA expression and activity of PKC- α , therefore hypothesizing that TCDCA induced the apoptotic process of NR8383 cells by upregulating phosphorylation of PKC- α .

JNK is one of the MAPK family members and it is regarded as a serine/threonine (Ser/Thr) protein kinase (Davis, 2000). Generally, UV stimulation, environmental stress and proinflammatory cytokines are the active factors for JNK pathway (Karin et al., 2005; Dong et al., 2002). JNK regulates the downstream target gene expression level and protein activity through its phosphorylation which leads to apoptosis (Dhanasekaran and Reddy, 2008). Our results demonstrated that TCDCA increased both mRNA expression and activity of JNK. Meanwhile, by using the PKC specific inhibitor Gö 6983, we demonstrated that PKC/JNK signaling pathway is involved in the apoptosis promoting effect of TCDCA in NR8383 cells.

Caspases are a family of cysteine proteases that are main executors of the apoptotic process. They were classified according to their various functions, the inflammatory caspases 1, 4, 5, 11, 12, 13, 14, the effector caspases 3, 6 and the initiator caspases 2, 8, 9, 10, (Singh, 2007). The apoptotic process that induced by mitochondrial pathway is triggered through intracellular stresses mainly in the

mitochondria. This could result in membrane permeabilization, mitochondrial membrane potential disruption and apoptogenic proteins release (Kroemer et al., 2007). These changes ultimately activate caspases, which play essential biological parts in both apoptosis and inflammation (Garcia et al., 1999; Nicholson and Thornberry, 1997). Caspase 8 activates downstream effector caspases inducing apoptosis damage, and each effector caspases targets a limited set of cellular proteins (Wallach et al., 1999). With the presence of cytochrome *c* and dATP, the N-terminal prodomain of caspase-8 interacts with Apaf-1 leading to the cleavage and activation of caspase 9. Then caspase 9 has a further effect on caspases 3, 6, and 7. The execution phase of apoptosis needs effector caspases. Many of those effector caspases lead to morphology changes and cellular injury during cell death (Los et al., 1999). Our study showed that the gene expression level of caspase-3 and caspase-8 were significantly augmented by the treatment with TCDCA (100 μ M, 10 μ M and 1 μ M), while markedly reduced by SP600125. Furthermore, high concentration of TCDCA (100 μ M) dramatically enhanced caspase-3 and caspase-8 activities. However, they were significantly reduced by SP600125 treatment, indicating that the caspase cascade was a downstream target of JNK in NR8383 cells.

5. Conclusions

Our study showed that TCDCA induced the apoptosis process through the activation of caspase cascade in macrophages, and this process may be involved in PKC/ JNK signaling pathway. These findings may emphasize the apoptosis effects of TCDCA. All this information may be useful in the development of TCDCA as a latent

and effective active component of traditional Chinese medicine for apoptosis-related diseases.

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Figure captions

Fig. 1. Apoptosis percentage was determined using flow cytometry analysis. Negative control was untreated with TCDCA. Treatment groups were treated with indicated concentrations of TCDCA for 24 h, then double staining with FITC-Annexin V and PI. Data represents of means $\pm$ S.D. of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control.

Fig. 2. Gene expression levels of PKC induced by TCDCA. Negative control was untreated with TCDCA. PKC expression levels were expressed as fold-change compared with control groups. Data represents of means $\pm$ S.D. of 3 independent experiments. ** $P < 0.01$ vs. control.

Fig. 3. PKC- α and phosphorylated PKC- α were visualized by Western Blot analysis as described in Materials and Methods. The arrows indicate approximately 80-kDa band corresponding to PKC- α and phosphorylated PKC- α , respectively. The protein expression levels were expressed as fold-change compared with control groups. Data represents of means $\pm$ S.D. of 3 independent experiments. ** $P < 0.01$ vs. control.

Fig. 4. Gene expression levels of JNK induced by TCDCA. Negative control was untreated with TCDCA. JNK expression levels were expressed as fold-change compared with control groups. Data represents of means $\pm$ S.D. of 3 independent experiments. $**P < 0.01$ vs. control; $\#P < 0.05$, $## P < 0.01$ vs. Gö 6983.

Fig. 5. JNK and phosphorylated JNK were visualized by Western Blot analysis as described in Materials and Methods. The arrows indicate approximately 48-kDa band corresponding to JNK and phosphorylated JNK, respectively. The protein expression levels were expressed as fold-change compared with control groups. Data represents of means $\pm$ S.D. of 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control; $## P < 0.01$ vs. Gö 6983.

Fig. 6. Gene expression levels of caspases induced by TCDCA. Negative control was untreated with TCDCA. Caspase-3 and caspase-8 mRNA expression levels were expressed as fold-change compared with control groups. Data represents of means $\pm$ S.D. of 3 independent experiments. $**P < 0.01$ vs. control. $## P < 0.01$ vs. SP600125.

Fig. 7. The activities of caspases induced by TCDCA. Negative control was untreated with TCDCA. Treatment groups were treated with indicated concentrations of TCDCA for 24 h, luminescent signals were detected that represent of caspase-3 and caspase-8 enzymatic activities. Data represents of means $\pm$ S.D. of 3 independent experiments. $**P < 0.01$ vs. control. $\# P < 0.05$, $## P < 0.01$ vs. SP600125.

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