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Effects of taurochenodeoxycholic acid on Ca^{2+}/CaM signalling mediated by the TGR5 signalling pathway

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Taurochenodeoxycholic acid (TCDCA), a natural bioactive substance in animal bile, has anti-inflammatory and immunoregulatory effects. This study evaluates the effects of TCDCA on calcium/calmodulin (Ca^{2+}/CaM) signalling mediated by G Protein Coupled Bile Acid Receptor 1 (TGR5) to provide preliminary information on the mechanism of TCDCA in immune regulation and also to benefit future research. After treatment of NR8383 and high TGR5 expression cell (TGR5-NR8383) with TCDCA (10^{-6} mol/L, 10^{-5} mol/L, and 10^{-4} mol/L) for 1 h, we measured TGR5 and CaM gene and protein levels by quantitative reverse transcription-polymerase chain reaction (qPCR) and western blotting, respectively. The inositol triphosphate (IP_3) concentration was measured by Enzyme-linked Immunosorbent Assay (ELISA), and the Ca^{2+} concentration was measured by calcium fluorescent probe (Fluo-3 AM). The present study showed that the expression levels of IP_3, Ca^{2+}, and CaM in NR8383 cells were increased by TCDCA at concentrations ranging from 10^{-6}mol/L to 10^{-4} mol/L. TCDCA (10^{-4} mol/L) increased both the gene and protein expression of IP_3 and CaM through TGR5. TCDCA (10^{-4} mol/L or 10^{-5} mol/L) also increased the Ca^{2+} concentration via the TGR5 receptor. Our data suggest TCDCA activates Ca^{2+}/CaM signalling via the TGR5 signalling pathway.

1. Introduction

Taurochenodeoxycholic acid (TCDCA), which is a natural bioactive substance in animal bile, has anti-inflammatory and immunoregulatory effects (Liu et al. 2011). The G protein-coupled receptor TGR5 has been identified as an important component of the bile acid signalling network that mediates the effects of bile acid on energy balance, inflammation, and digestion (Jensen et al. 2013; Pols et al. 2011). Previous studies have shown that the activation of TGR5 in bile acid suppresses proinflammatory cytokine production and macrophage phagocytosis (Kida et al. 2013). However, the mechanisms and spatiotemporal control of TGR5 signalling remain poorly understood. We investigated TGR5 signalling associated with trafficking in transfected NR8383 cells. Previous studies have shown that some bile acids can increase the intracellular Ca^{2+} concentration and induce calmodulin (CaM) production through IP_3 (inositol 1,4,5-triphosphate) receptors (DEVOR et al. 1993; Moser et al. 1998). IP_3 signalling occurs through G protein-coupled receptors at the plasma membrane, and the information is transmitted inside the cell, leading to the hydrolysis of phosphatidylinositol biphosphate (PIP2) to form IP_3 and diacylglycerol (DAG). IP_3 then binds to the IP_3 receptor (IP_3Rs), which has been demonstrated to release Ca^{2+} from non-mitochondrial stores (Clapham 2007; Mikoshiba 2015). Ca^{2+} acts as an intracellular messenger and plays a critical role in cells of the immune system that participate in the regulation of cell differentiation, gene transcription and effector functions (Berridge et al. 1998; Feske 2007). CaM, a primary Ca^{2+}-binding protein, mediates various processes, including inflammation, metabolism, apoptosis and the immune response (Kumar et al. 2013). To assess the functional roles of TCDCA and TGR5, we used the NR8383 and TGR5-NR8383 cell to examine the differential effects of TCDCA on the Ca^{2+}-CaM levels in cells with and without TGR5. The results of this study will increase the preliminary understanding of the mechanism of TCDCA in immunoregulation through TGR5 and will serve as a foundation for further research.

2. Investigations and results

2.1. Expression of TGR5 in NR8383 cells

The expression of TGR5 in NR8383 cells was determined using qPCR (Fig. 1A) and western blotting (Fig. 1B).

2.2. Effects of TCDCA on IP_3 expression

The IP_3 levels in NR8383 and TGR5-NR8383 supernatants were assessed by ELISA after treatment with TCDCA. High levels of IP_3 were observed following treatments with different concentrations of TCDCA in both NR8383 and TGR5-NR8383cells (Fig. 2). Before TCDCA treatment, no difference was observed between NR8383 and TGR5-NR8383 cells. In addition, 10^{-4}mol/L TCDCA had a more significant effect on TGR5-NR8383 cells than on NR8383 cells.

2.3. TCDCA increased the concentrations of Ca^{2+} in cells

The effects of TCDCA on the Ca^{2+} concentrations in NR8383 and TGR5-NR8383cells were detected by Fluo-3 AM. In both NR8383 and TGR5-NR8383 cells, different concentrations of TCDCA increased the production of Ca^{2+} compared with control cells. Significant differences in the Ca^{2+} concentrations following treatment with 10^{-4}mol/L and 10^{-5}mol/L TCDCA were observed in TGR5-NR8383 and NR8383 cells compared with control cells (Fig. 3).

2.4. Effect of TCDCA on CaM mRNA expression

CaM mRNA levels in NR8383 and TGR5-NR8383cells were determined by qPCR after treatment with TCDCA. The results showed that CaM mRNA expression was induced by TCDCA at concentrations ranging from 10^{-6} to 10^{-4}mol/L. Before TCDCA treatment, no difference was observed between NR8383 and TGR5-NR8383 cells. However, 10^{-4}mol/L TCDCA significantly increased CaM mRNA expression in TGR5-NR8383 cells compared with NR8383 cells (Fig. 4A).

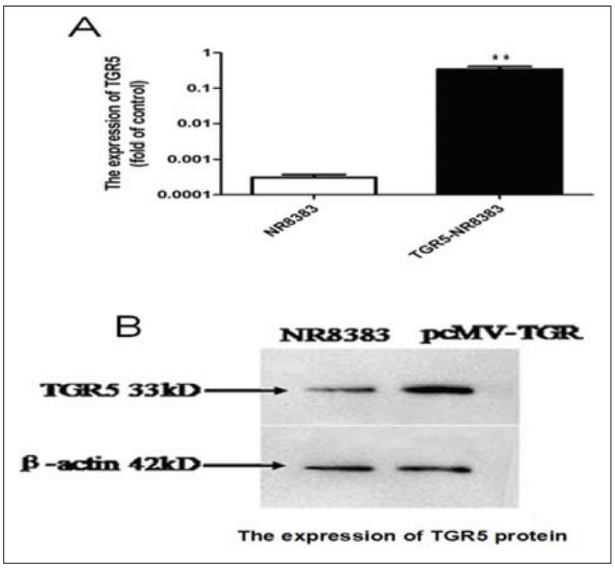

Fig. 1: Expression of TGR5 in NR8383 cells. A. The expression of TGR5 mRNA. B. The expression of TGR5protein. *P<0.05 and **P<0.01 vs. control.

To further investigate the role of the IP_3 receptor in NR8383 and TGR5-NR8383 cells, we examined the effect of an IP_3 receptor antagonist, 2-APB (Tamashiro and Yoshino 2014), on the cells. After treatment with 2-APB, both the NR8383 control and TGR5-NR8383 control cells showed no differences compared with the normal control cells. However, the levels of CaM mRNA were suppressed by 2-APB in the NR8383 and TGR5-NR8383 cells treated with 10^{-4}mol/LTCDCA, although they were still increased significantly compared with the respective controls. No difference in CaM mRNA expression was observed between NR8383 and TGR5-NR8383cells treated with TCDCA at concentrations ranging from 10^{-6} to10^{-4}mol/L.

2.5. Effects of TCDCA on CaM protein expression in cells

The effects of TCDCA on CaM protein levels in NR8383 and TGR5-NR8383 cells were detected by western blotting. The results showed that different concentrations of TCDCA increased CaM protein expression in NR8383 and TGR5-NR8383 cells. Upon treatment with 2-APB, a significant decrease in the production of CaM was observed in NR8383 and TGR5-NR8383 cells compared with the respective untreated cells. However, CaM expression did not differ between NR8383 and TGR5-NR8383 cells following incubation with 2-APB. These results indicated that TCDCA exerted identical effects on the CaM protein and mRNA levels.

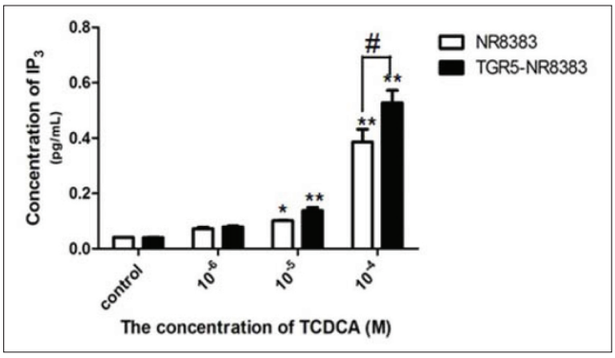

Fig. 2: Effect of TCDCA on the concentration of IP3 in NR8383 and TGR5-NR8383. Untreated cells were used as a negative control. *P<0.05 and **P<0.01 vs. control. # P<0.05 and ## P<0.01 vs.TGR5-NR8383 or NR8383.

3. Discussion

Research interest in animal bile acids is dramatically increasing, especially with regard to therapy for autoimmune diseases. TCDCA, which is a natural bioactive substance in animal bile, has been shown to have notable immunoregulatory effects in mice (Wang et al. 2013). In a previous study, TCDCA was demonstrated to inhibit non-specific immunity and cellular immunity but to enhance humoral immunity. Thus, it has various regulatory effects on immune function in animals (Li et al. 2013). However, as the effects of TCDCA on Ca^{2+} and CaM expression through TGR5 remain unknown, we investigated this in the present study.

TGR5-NR8383 cells were obtained by the transfection of NR8383 cells with pcMV-TGR5.TGR5 is a G-protein-coupled receptor (GPCR) that contains seven trans membrane domains and transduces extracellular signals through heterotrimeric G proteins (Wang et al. 2011). As shown in this study, TGR5 is involved in both the formation of cAMP and inhibition of the inflammatory signalling pathway (Bunnett 2014). Indeed, TGR5 may act as a critical receptor that is activated by micromolar concentrations of TCDCA, elevations in the intracellular IP_3 and Ca^{2+} levels, and the generation of CaM.

The ubiquitously expressed IP_3 receptor is mainly localised to the endoplasmic reticulum membrane (Putney and Bird 1993), where it functions as an ion channel to release stored Ca^{2+} into the cytoplasm to increase the cytoplasmic free Ca^{2+} concentration following activation by its physiological ligand, IP_3, which is generated in the cytoplasm as part of a signalling cascade resulting from activation of specific plasma membrane receptors by various extracellular stimuli (Berridge; 1993 Mak and Foskett 2015). There is accumulating evidence that the IP_3 receptor plays an important role in cellular functions; specifically, IP_3R forms an important macro-molecular complex that works as a signalling hub by associating with various molecules and determines the direction of cellular signalling pathways (Mikoshiba 2015). In this study, we assessed IP_3 levels by ELISA after treatment with TCDCA and found that 10^{-4} mol/L TCDCA significantly increased the concentration of IP_3 through the TGR5 receptor.

One of the most versatile and universal signalling agents in the human body is the calcium ion (Berridge et al. 1998). $Ca^{2+}$is an important intracellular second messenger in such processes as growth factor and hormone signalling, cell cycle regulation, gene expression, and apoptosis, among others (Hook and Means 2001). Calcium signals are ubiquitous, and their versatility relies on a variety of spatio-temporal behaviours exhibited by intracellular Ca^{2+}. IP_3R-mediated Ca^{2+} signals are a key component of the Ca^{2+} signalling toolkit (Choe and Ehrlich 2006). However, ryanodine receptors, sister receptors of IP_3, are also critical in Ca^{2+} signalling, and these receptors also contribute to bile acid-induced calcium signaling (Husain et al. 2012). This activity may explain the effects of TCDCA on plasma membrane Ca^{2+}/CaM channels in NR8383

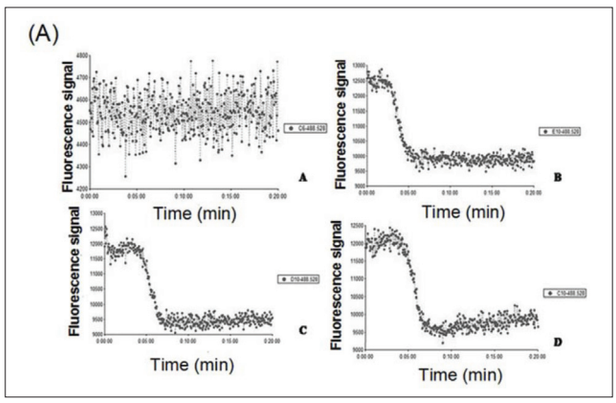

Fig. 3 (A) A: Effect of DMEM on Ca2+ in NR8383; B: Effect of TCDCA (10-6 mol/L) on Ca2+ in NR8383; C: Effect of TCDCA (10-5mol/L) on Ca2+ in NR8383; D: Effect of TCDCA (10-4 mol/L) on Ca2+ in NR838;

cells. Whether NR8383 cells have ryanodine receptors and whether these receptors drive Ca^{2+} signalling are our next targets.
Calmodulin, a 16.7 kDa protein found in all eukaryotic cells, has been extensively studied as a primary Ca^{2+}-binding protein (Fok et al. 2008). CaM mediates various processes, including inflammation, metabolism, apoptosis, cell motility, growth, and proliferation and the immune response (Fok et al. 2008). Our results showed that the expression of CaM was induced by TCDCA at concentrations ranging from 10-6 to10-4 mol/L. In addition, 2-APB exhibited a suppressive effect on TCDCA, stimulating the production of CaM. Our study demonstrates that TCDCA has a stimulatory effect on Ca^{2+}/CaM and that this process is mediated by the TGR5 signalling pathway. These findings may promote the investigation of TCDCA as a novel therapeutic agent for immunological treatment, which will be beneficial not only for increasing the added value and utilisation of animal bile but also for promoting advances in medicine and animal husbandry.

4. Experimental

4.1. Reagents

TCDCA and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Sigma (USA). TCDCA was dissolved in DMEM. The NR8383 cell line was purchased from the Shanghai Cell Bank of the Chinese Academy of Science. Fetal bovine serum (FBS) was purchased from ExCell Bio (Shanghai, China). Lipofectamine TW 2000 was obtained from Invitrogen (USA). SYBR Prime Ex Taq TW II, Prime Script RT Master Mix and RNAiso Plus were acquired from TaKaRa (Dalian, China). Rabbit ant-rat TGR5 IgG and rabbit ant-rat CaM IgG antibodies were purchased from Abcam (USA); goat anti-rabbit IgG and goat anti-mouse IgG (Gaitherburg, USA); and PCMV-TGR5 was constructed in our laboratory. ELISA kits for inositol 1,4,5-triphosphate were purchased from Blue Gene (Shanghai, China). Fluo-3 AM was obtained from Beyotime Institute of Biotechnology (China).

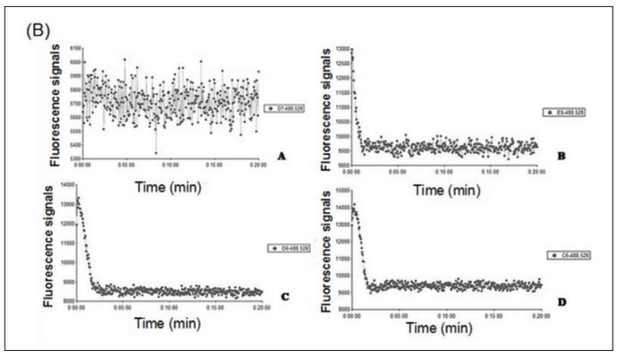

Fig. 3 (B) A: Effect of DMEM on Ca2+ inTGR5-NR8383; B: Effect of TCDCA (10-6 mol/L) on Ca 2+ in TGR5-NR8383; C: The effect of TCDCA (10-5 mol/L) on Ca2+ in TGR5- NR8383; D: The effect of TCDCA (10-4 mol/L) on Ca2+ in TGR5-NR8383;

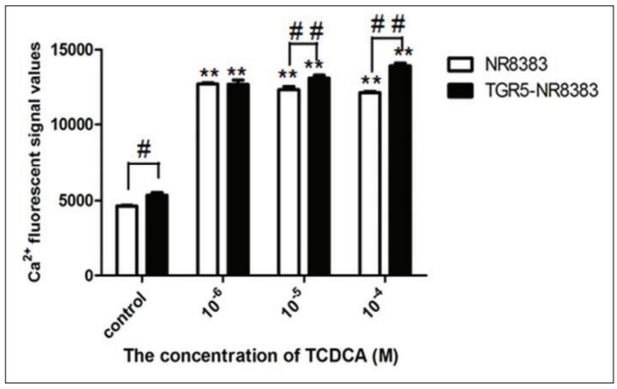

Fig. 3(C) The effect of TCDCA on Ca2+ production in NR8383 and TGR5-NR8383. Untreated cells were used as negative control. *P<0.05 and **P<0.01 vs. control.# P<0.05 and ## P<0.01 vs.TGR5-NR8383 or NR8383.

4.2. Cell culture and transfection

NR8383 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified atmosphere with 5% CO^2 at 37 °C (Chen et al. 2013; Kerecman et al. 2008). Cells were transiently transfected with PCMV-TGR5 (Lin-kai et al. 2014) using Lipofectamine TW 2000 and were examined after 48 h (Lane et al. 1998).

4.3. qPCR assay for TGR5 and CaM gene expression in cells

qPCR analysis was performed to assess TGR5 and CaM gene expression in the cells (Keitel et al. 2007; Levy et al. 2004). Total cellular RNA was extracted using TRNAiso Plus, and OD260/280ratios were calculated and agarose gel electrophoresis was performed to ensure that the RNA quality was adequate. cDNA synthesis was performed using PrimeScript RT MasterMix. All primers were synthesised by TaKaRa Biotechnology (China). The primer sequences are shown in the Table. Relative gene expression levels corrected for β-actin expression were calculated based on the Ct values according to the following equation: 2 −ΔCt [ΔCt=Ct (TGR5 or CaM)-Ct (β-actin)].

4.4. Measurement of TGR5 and CaM protein expression in cells

The TGR5 and CaM protein expression levels in the cells were measured by western blotting (Poole et al. 2010). Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose filter membranes (Amersham, USA). The membranes were washed and incubated for 1 h at room temperature with blocking buffer and were then probed with a TGR5 rabbit anti-rat TGR5 IgG or rabbit anti-rat CaM IgG antibody overnight at 4 °C. Then, the membranes were incubated for 1 h with secondary antibodies. Protein loading was normalised using β-actin staining. Bands were visualised using ECL.

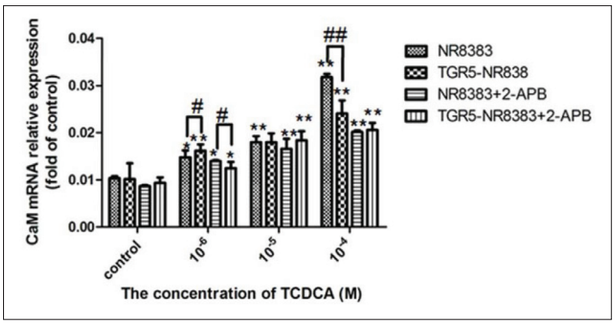

Fig. 4(A): Effect of TCDCA on CaM mRNA expression in NR8383 and TGR5-NR8383;

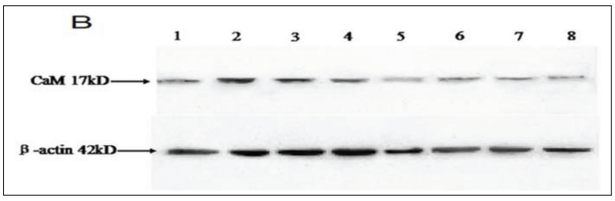

Fig. 4(B): Effect of TCDCA on the protein production of CaM in NR8383. 1. control, 2. 10-4 mol/L TCDCA; 3.10-5 mol/L TCDCA; 4. 10-6 mol/L TCDCA; 5. 2-APB control; 6. 2-APB+10-4 mol/L TCDCA; 7. 2-APB+10-5 mol/L
TCDCA; 8. 2-APB+10-6 mol/L TCDCA

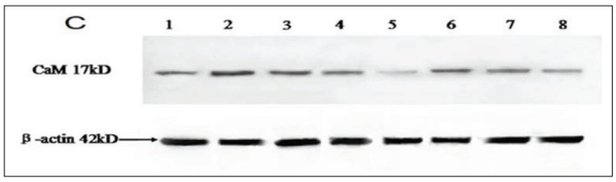

Fig. 4(C): Effect of TCDCA on the protein production of CaM in TGR5-NR8383, 2. 10-4 mol/L CDCA; 3. 10-5 mol/L TCDCA; 4. 10-6mol/L TCDCA; 5. 2-APB control;6. 2-APB+10-4 mol/L TCDCA; 7. 2-APB+10-5 mol/L TCDCA; 8.2-APB+10-6 mol/L TCDCA.

Table: The sequence of primer

Primer	Amplified Products size	Sequence
β-Actin	150 bp	Sense: 5'-GGAGATTACTGCCCTGGCTCCTA-'3
		Antisense: 5'-GACTCATCGTACTCCTGCTTGCTG-'3
TGR5	92 bp	Sense: 5′- AAAGGTGGCTACAAGTGCTTC-3′
		Antisense: 5′- TTCAAGTCCAAGTCGTGCTG-3′
CaM	84 bp	Sense: 5'- AGCGAGTCGAGTGGTTGTCTGTT-3'
		Antisense: 5'-CGATCTGCTCTTCAGTCAGTTGGT-3'

4.5. Measurement of IP_3 concentrations in cells

The concentrations of IP_3 in NR8383 and TGR5-NR8383 cells were measured by ELISA according to the manufacturer's instructions (Di Tomo et al. 2013).

4.6. Measurement of Ca^{2+} concentrations in cells

The concentrations of Ca^{2+} in NR8383 and TGR5-NR8383 cells were measured by Fluo-3 AM according to the manufacturer's instructions.

4.7. Statistical analysis

Statistical analysis was performed using SPSS software. The significance of differences between the control and experimental groups was determined by one-way ANOVA analysis. $P< 0.01$ was considered highly significant, and $P< 0.05$ was considered significant.

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