

•Research article•

Taurochenodeoxycholic acid mediates cAMP-PKA-CREB signaling pathway

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[ABSTRACT] Taurochenodeoxycholic acid (TCDCA) is one of the main effective components of bile acid, playing critical roles in apoptosis and immune responses through the TGR5 receptor. In this study, we reveal the interaction between TCDCA and TGR5 receptor in TGR5-knockdown H1299 cells and the regulation of inflammation via the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA)-cAMP response element binding (CREB) signal pathway in NR8383 macrophages. In TGR5-knockdown H1299 cells, TCDCA significantly activated cAMP level via TGR5 receptor, indicating TCDCA can bind to TGR5; in NR8383 macrophages TCDCA increased cAMP content compared to treatment with the adenylate cyclase (AC) inhibitor SQ22536. Moreover, activated cAMP can significantly enhance gene expression and protein levels of its downstream proteins PKA and CREB compared with groups of inhibitors. Additionally, TCDCA decreased tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, IL-8 and IL-12 through nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) activity. PKA and CREB are primary regulators of anti-inflammation and immune response. Our results thus demonstrate TCDCA plays an essential anti-inflammatory role via the signaling pathway of cAMP-PKA-CREB induced by TGR5 receptor.

[KEY WORDS] Taurochenodeoxycholic acid; TGR5 receptor; Cyclic adenosine monophosphate; Protein kinase A; cAMP response element binding; Anti-inflammation

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Introduction

Bile acids (BAs), a valuable amphipathic molecule, are derived from cholesterol through a series of enzymatic reactions primarily taking place in hepatocytes of the liver [1, 2]. The bile acid family has a lot of members, synthesized via two pathways of classic and acidic, such as cholic acid (CA), chenodeoxycholic acid (CDCA) and TCDCA [3, 4]. Bile acids are also the main effective component of bile, playing important roles to help the liver to reduce poison, resist inflammation and enhance immunity [5].

TCDCA is synthesized by taurine and chenodeoxycholic

in the liver in combination [6]. TCDCA, as a star of bile acids, has been researched in many fields. TCDCA can induce cells apoptosis via activating caspase systems and the protein kinase C (PKC)/c-Jun N-terminal kinase (JNK) signaling pathway [7, 8]. In adjuvant arthritis rats, TCDCA can repress by regulating the gene expression to reduce the release of TNF- α , IL-1 β , IL-6 and IL-10 [9]. It was also found that in response to immune regulation TCDCA could increase the ratio of CD4⁺ and CD8⁺ cells compared with taurocholic acid (TCA) did [10]. These mentioned outcomes are probably mediated by a special G-protein-coupled receptor (GPCR) of TGR5, also known as bile acids receptor, activated by BAs and lipopolysaccharides (LPS) [11]. It has been elucidated that TCDCA can bind to TGR5 in computing biology [12]. However, it is unknown how TCDCA can down-regulate the expression of cytokines and which signaling pathways play key roles.

cAMP-PKA-CREB is known to be a suitable cell signaling pathway in anti-inflammation. It was reported that extra-

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cellular visfatin can induce expression of gluconeogenic enzymes in HepG2 cells through the PKA-CREB pathway [13]. Bile acids enter NR8383 cells by binding to the TGR5 receptor, further inducing expression of subunit proteins Gi and Gq of TGR5. Activated Gi and Gq proteins induce AC and cAMP [14]. cAMP induces activation of its downstream protein PKA, typically *via* activation site Thr197; then PKA activates its relative protein CREB *via* its activation site Ser 133; activated CREB enters the nucleus from the cytoplasm by nuclear translocation and induces expression of cytokines [15]. However, some other molecules can also play roles in this signaling pathway in the inflammatory response to the cells. In this study, we aimed to unveil the anti-inflammatory role of TCDCA and whether it has a relationship with cAMP-PKA-CREB signaling pathway induced by TGR5 receptor.

Materials and Methods

Chemicals and Reagents

TCDCA (purity $\geq 98\%$), TLCA (purity $\geq 95\%$), LPS, SQ22536, H89 and G418 (Sigma-Aldrich, USA), Dabrafenib (Selleck, USA), LipofectamineTM 3000 transfection reagent were purchased from Invitrogen (USA), SDS-PAGE gels (Beyotime Biotechnology, China), Anti-TGR5 antibody and Anti-PKA (phosphor T197) antibody (Abcam, USA), control siRNA (B2213), TGR5 siRNA (B2513) (Santa, USA), CCK-8 (EV738) (Tongren Institute of Chemistry, Japan), anti-I κ B α antibody and anti-CREB (phosphor Ser133) antibody (CST, USA), BCA Protein Assay (Pierce, USA), SYBR[®] Premix Ex TagTM, TripTM RNA reagent and PrimeScriptTM 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), cAMP ELISA kit (Cayman, USA), TNF- α , IL-1 β , IL-6, IL-8 and IL-12 ELISA kits (Invitrogen, USA) were purchased from China biotechnology company.

Cell culture

NR8383 cells were purchased from the Shanghai Institute of Cellular Biology of Chinese Academy of Sciences. Both H1299 cells and TGR5-knockdown-H1299 cells were obtained from PhD Wei Mao. TGR5 overexpressed NR8383 cells (called TGR5 cells) and pCDNA 3.1 NR8383 cells (called vector cells) were constructed by Qian Liu from Peifeng Li's lab. 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) and 200 mg·L⁻¹ of G418. Cells were cultured at constant 37 °C under humidified conditions with 5% CO₂. When the confluence of cells reached approximately at 70%–80%, the non-adherent cells were discarded and the adherent cells were trypsinized with 0.25% trypsin. Afterwards, they were divided into 3 or 4 parts and incubated in the same medium.

TGR5 gene silencing in NR8383 macrophages

NR8383 macrophages were seeded into the 12-well

plates at a concentration of 1×10^5 , containing DMEM culture medium with 1 mL of FBS. According to manufacturing protocol of Lip3000; 2 μ L of 10 μ mol·L⁻¹ siRNA was added into the 50 μ mol·L⁻¹ opti-DMEM, and 3 μ L Lip3000 was added into the 50 μ mol·L⁻¹ opti-DMEM, respectively; and then mixed them completely and incubated them for 30 min at room temperature. Mixed Lip3000 reagents were gently added into the NR8383 macrophages, incubated for 7 h at 37 °C and 5% of CO₂. To obtain RNA and protein from NR8383 macrophages after culturing 24 h. cAMP content was measured in NR8383 macrophages and TGR5 siRNA NR8383 macrophages using ELISA reagent of cAMP.

Proliferation-toxic effects

100 μ L of suspension of NR8383 macrophages were added into 96-well plates, and then added 10 μ L of different concentration of TCDCA (0–1000 μ mol·L⁻¹) into 96-well plates after incubating 24 h, continually incubating for 24 h. Next, 96-well plates were added 10 μ L of CCK-8 buffer, incubating it for 0.5 h; at last, 96-well plates were measured at 450 nm by multiple microplate reader.

cAMP assays

H1299 cells and TGR5-knockdown-H1299 cells were cultured in 12-well plates at a concentration of 1×10^5 for 24 h and then treated with TCDCA at different concentrations of 10 100 and 1000 μ mol·L⁻¹ for 20 min; another 12-well plates of H1299 cells and TGR5-knockdown-H1299 cells were treated with 100 μ mol·L⁻¹ TCDCA at different time of 5, 10, 15, 20, 25 and 30 min. cAMP content was measured according to the cAMP-ELISA assay protocol.

Vector Cells and TGR5 cells were cultured in 12-well plates at a concentration of 1×10^5 for 24 h. The vector and TGR5 NR8383 cells were treated with 50 μ mol·L⁻¹ TLCA and 1, 10 and 100 μ mol·L⁻¹ TCDCA for 20 min at 37 °C, then cAMP content was analyzed using cAMP ELISA kit, using TLCA for positive control. In addition, for further investigation whether TCDCA regulates cAMP *via* AC, an up-stream protein of cAMP, we used an AC specific inhibitor SQ22536 to treat NR8383 cells for 1 h prior to the treatment with 50 μ mol·L⁻¹ TLCA and 1, 10 and 100 μ mol·L⁻¹ TCDCA for 20 min at 37 °C; similarly, cAMP ELISA kit was used to measure the cAMP content.

RNA isolation and qPCR assays

Quantitative real-time PCR (qPCR) was carried out to analyze the mRNA levels of different cytokines. NR8383 cells were cultured in 24-well plates overnight for attachment. Thereafter, cells were pre-treated with different inhibitors for 1 h before co-treatment with TCDCA for another hour. The total cellular mRNA was extracted from 24-well plates using TripTM RNA reagents. The quality of mRNA was determined by agarose gel electrophoresis and the ratio of OD_{260/280}. Synthesis of cDNA was carried out using PrimeScriptTM RT Master Mix kit following the manufacturer's protocol. The cDNA was amplified using the ViiATM 7 system (ABI Biosystems[®], USA) with the SYBR[®] Premix Ex TagTM kit. In brief, a total of 25 μ L reaction mixture includ-

ing 2 μL of cDNA, 12.5 μL of $2 \times \text{SYBR}^{\text{®}}$ Premix Ex TagTM, 1 μL of specific target primers ($10 \mu\text{mol}\cdot\text{L}^{-1}$) forward and reverse and 8.5 μL of ddH₂O. The qPCR thermal cycling settings were 30s at 95 °C, followed by 39 cycles of 5s at 95 °C, and 30s at T_m, and then 15s at 95 °C. The qPCR was carried out with the specific primers (Table 1). All data were calcu-

lated based on the comparative Ct formula and each sample was normalized to β -actin. Relative mRNA expressions were analyzed according to the Ct values, based on the equation: $2^{-\Delta\text{Ct}}$ [$\Delta\text{Ct} = \text{Ct (TGR5, PKA, CREB, NF-}\kappa\text{B, TNF-}\alpha, \text{IL-1}\beta, \text{IL-6, IL-8, IL-12)} - \text{Ct } (\beta\text{-actin})$]. The melting curves guaranteed the purity of each reaction.

Table 1 Primer, temperature, amplified productions size and reference in the study

Primer	Temperature	Amplified products size	Sequence (5'-3')
TGR5	60.1 °C	439 bp	Forward: CCTGGCAAGCCTCATCATCA Reverse: TTCGAGGTACAGGTAGGGGG
PKA	59.2 °C	262 bp	Forward: GCGAGATTTCAGATCCAGACTC Reverse: ATAAGACAGAAGGCCGGCAG
CREB	55.9 °C	285 bp	Forward: AGTTGTGTGTTCAAGCTGCTCTG Reverse: TCCTTAAGTGCTTTAGCTCCTCA
NF- κ B	57.6 °C	220 bp	Forward: CCACCTACGATGGGACGAC Reverse: AGTATGTCAAATACCTGCCAGT
TNF- α	58.2 °C	360 bp	Forward: ACCCCCAACCTATGAAGAAA Reverse: TCCACGCAAAACGGAATGAA
IL-1 β	56.5 °C	377 bp	Forward: TTGTGGCTGTGGAGAAGCTG Reverse: GCCGTCTTCATACACAGG
IL-6	59.8 °C	210 bp	Forward: TAG AGTCACAGAAGGAGTGG Reverse: GCC AGTCTTCGTAGAGA
IL-8	52.38 °C	170 bp	Forward: TGATCATGGAAGGTCGTCTCAG Reverse: CATCACTCCGATTGCAGCAG
IL-12	59.2 °C	210 bp	Forward: CAGGGGTCTGACGCAATAC Reverse: GGGAAATCTGCACCTCAACG
β -actin	60.0 °C	150 bp	Forward: GGAGATTACTGCCCTGGCTCCTA Reverse: GACTCATCGTACTCCTGCTTGCTG

Whole-cells protein extraction

Vector Cells and TGR5 Cells were processed as described by qPCR assay. The whole-cell protein was extracted from cells using Nuclear Extraction kit following 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 contained phosphatase inhibitors. The supernatant was removed and the precipitate was resuspended in 1 mL PBS contained phosphatase inhibitors, and centrifuged at 200 g for 5 min in a pre-cooled centrifuge at 4 °C. The supernatant was discarded and the precipitate was resuspended in 300 μL complete lysis buffer, and then left on ice for 20 min on a shaking table set at 150 rpm. Then the solution was vortexed and centrifuged at 14 000 g for 20 min at 4 °C. The supernatant containing whole-cell extracts was collected and stored at -80 °C.

Statistical analysis

Statistical analysis was applied using SPASS 17.0 software. Data were expressed as the means \pm standard deviation (SD). Significance of the differences between controls and experimental groups was determined by one-way ANOVA analysis. $P < 0.05$ was considered significant.

Results

Proliferation-toxic effects of TCDCA on NR8383 macrophages

TCDCA has a proliferative effect on NR8383 cells at concentrations of 10^{-7} and $10^{-3} \text{ mol}\cdot\text{L}^{-1}$; growth of NR8383 cells was inhibited by 10^{-8} , 10^{-6} , 10^{-5} and $10^{-4} \text{ mol}\cdot\text{L}^{-1}$ of TCDCA, inhabitation rate of TCDCA on NR83783 cells is 10%–20%, and it is not significantly toxic to NR8383 cells

(Fig. 1). Thus, we used $10^{-4} \text{ mol}\cdot\text{L}^{-1}$ of TCDCA for the next experiments.

TCDCA can bind to TGR5

To investigate whether TCDCA binds to TGR5 receptor to activate downstream membrane protein G α s and induce the secondary message cAMP production under AC, we assayed cAMP levels in H1299 cells and TGR5-knockdown-H1299 cells (Figs. 2A, 2B) which were both treated with TCDCA for 15 min. There was $100 \mu\text{mol}\cdot\text{L}^{-1}$ TCDCA significantly increases cAMP content ($P < 0.001$) in H1299 cells than that in TGR5-knockdown-H1299 cells (Fig. 2C), indicating TCDCA can bind to TGR5 increasing cAMP content, and cAMP level was dramatically increased in H1299 cells treated with TCDCA for 15 min (Fig. 2D) compared to TGR5-knockdown-H1299 cells ($P < 0.001$).

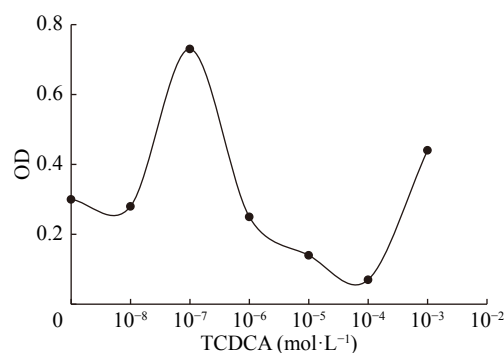


Fig. 1 Proliferation-toxic effects of TCDCA on NR8383 cells 10^{-7} and $10^{-3} \text{ mol}\cdot\text{L}^{-1}$ of TCDCA promoted proliferation of NR8383 cells; however, 10^{-8} , 10^{-6} , 10^{-5} and $10^{-4} \text{ mol}\cdot\text{L}^{-1}$ of TCDCA inhibited growth of NR8383 cells, $10^{-4} \text{ mol}\cdot\text{L}^{-1}$ of TCDCA had best effect of inhabitation on NR8383 cells

TGR5 gene was successfully edited by RNAi from NR8383 macrophages, and the knockdown rate of TGR5 gene and protein almost reached to 80% in TGR5 siRNA NR8383 macrophages after detecting using qPCR and western blot (Figs. 2E, 2F). cAMP contents were assayed in NR8383 macrophages and TGR5 siRNA NR8383 macro-

phages, we found that cAMP contents all decreased in TGR5 siRNA NR8383 macrophages respectively treated with 10 $\mu\text{mol}\cdot\text{L}^{-1}$, 100 $\mu\text{mol}\cdot\text{L}^{-1}$ and 1000 $\mu\text{mol}\cdot\text{L}^{-1}$ of TCDCA compared to NR8383 macrophages (Fig. 2G). The result is similar with that in H1299 cells, thus, we confirm that TCDCA could bind to TGR5.

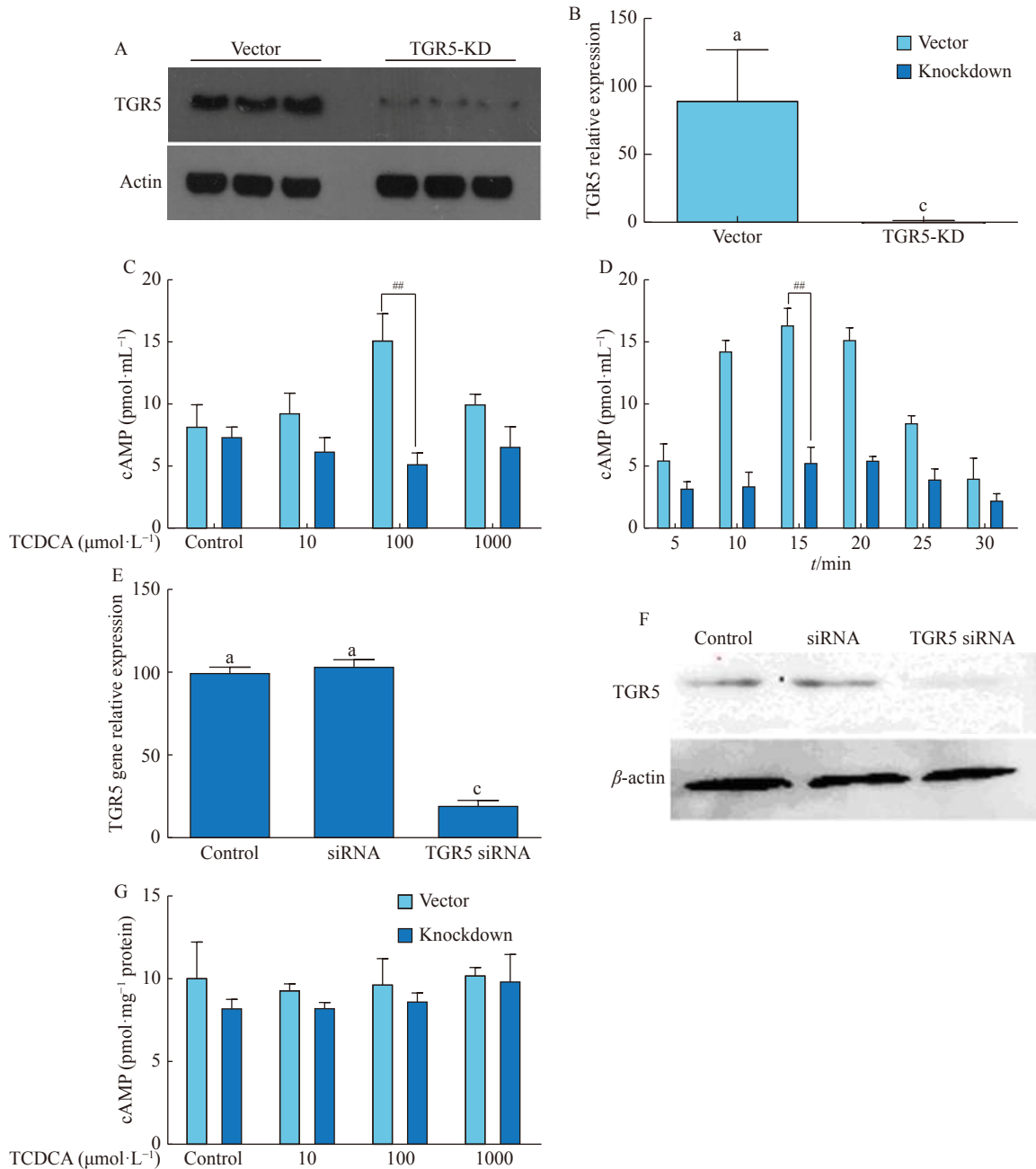


Fig. 2 TCDCA can bind to TGR5 TGR5 expression in mRNA and protein levels were assayed by qPCR and western blot in H1299 cells and TGR5-knockdown-H1299 cells (A, B). cAMP content was measured by ELISA in H1299 cells and TGR5-knockdown-H1299 cells treated with TCDCA at different concentrations for 15 min (C). cAMP content was measured by ELISA in H1299 cells and TGR5-knockdown-H1299 cells treated with 100 $\mu\text{mol}\cdot\text{L}^{-1}$ TCDCA at different times (D). The knockdown rate of TGR5 gene and protein almost reached to 80% in TGR5 siRNA NR8383 macrophages (E, F); cAMP contents all decreased in TGR5 siRNA NR8383 macrophages respectively treated with 10, 100 and 1000 $\mu\text{mol}\cdot\text{L}^{-1}$ of TCDCA compared to NR8383 macrophages (G). Representative of three independent experiments with similar results are shown. Data are presented as mean \pm SD. The interphase letters indicate that the differences is extremely significant ($P < 0.01$); ## indicates that differences is extremely significant compared with the adjacent group ($P < 0.01$)

TCDCA increased cAMP content via AC

AC, also known as a phosphorylation protein kinase, is located upstream of cAMP, directly regulating cAMP content. To determine whether TCDCA increased content of the second messenger cAMP via TGR5, we first examined the cAMP content in the vector cells and TGR5 cells upon TCDCA stimulation. cAMP content was also determined in normal NR8383 cells pre-treated with SQ22586, using TLCA as a positive control. We found that 50 $\mu\text{mol}\cdot\text{L}^{-1}$ TLCA increased the expression of cAMP significantly in the TGR5 cells compared to vector cells. Moreover, 1, 10 and 100 $\mu\text{mol}\cdot\text{L}^{-1}$ TCDCA can also increase the expression of cAMP compared to the control, but 100 $\mu\text{mol}\cdot\text{L}^{-1}$ of TCDCA led to a remarkable increase in cAMP content than other concentrations of TCDCA in TGR5 cells (Fig. 3A). In normal NR8383 cells, the increased cAMP content in response to TCDCA was reduced by SQ, 22536 compared to control. Similarly, TLCA increased cAMP content more than the SQ,22536 group (Figs. 3B, 3C). This finding reveals that TCDCA can increase the cAMP content via TGR5.

TCDCA activated PKA expression via cAMP

To determine whether TCDCA induces expression of PKA via cAMP, TCDCA and SQ22586 were used to treat vector cells and TGR5 cells. The PKA expression in the gene level and the protein level was examined by qPCR and western blot. Our results showed that TCDCA can significantly increase PKA expression in the TGR5 cells and vector cells, but the PKA expression was higher in the TGR5 cells than in the vector cells. Moreover, under SQ22536 treatment, PKA expression was decreased in the TGR5 cells and vector cells which were both treated with TCDCA. Our findings suggest that TCDCA can induce the expression of PKA via cAMP (Fig. 4).

TCDCA increased CREB expression via PKA

In these experiments, we used the PKA specific inhibitor

or H89, and the Raf specific inhibitor Dabrafenib. In vector cells and TGR5 cells, TCDCA significantly increased the expression of CREB compared with control. However, using inhibitors H89 and Dabrafenib decreased the expression of CREB in the TCDCA-stimulated vector cells and TGR5 cells at the protein level, but not at the mRNA level. Thus, we clearly show that TCDCA can activate the expression of CREB via PKA (fig. 5).

TCDCA decreased cytokine production via activation of NF- κ B

In order to interrogate the effects of TCDCA on cytokines, vector cells and TGR5 cells were incubated with 100 $\mu\text{mol}\cdot\text{L}^{-1}$ of TCDCA for 1 h, using TLCA as a positive control. Inhibitor of κ B (I κ B α), TNF- α , IL-1 β , IL-6, IL-8, and IL-12 expression at the mRNA and protein levels was determined using q-PCR and western blot. To highlight that TCDCA influences cytokine expression via NF- κ B in two kinds of cells, PKA was targeted as a specific upstream protein of NF- κ B using the specific inhibitor H89 for 1h. We found that constitutive I κ B α protein expression was significantly higher in the TGR5 cells than vector cells. In response to the stimulation from LPS, TCDCA dramatically decreased I κ B α expression in the TGR5 cells and vector cells, but H89 had no effect on I κ B α expression. As regard to cytokines, LPS dramatically increased expression of TNF- α , IL-1 β , IL-6, IL-8, and IL-12 in the TGR5 cells compared to the vector cells. Surprisingly, in the TGR5 cells we found that TCDCA significantly decreased TNF- α , IL-1 β , IL-6, IL-8 and IL-12 expression compared to vector cells. Under H89 inhibition of PKA, TCDCA effectively inhibited cytokines production in TGR5 cells compared to vector cells. These results show that LPS can markedly increase cytokine production and I κ B α expression. However, TCDCA significantly decreased cytokines, including TNF- α , IL-1 β , IL-6, IL-8, and IL-12, and I κ B α expression related to TGR5 receptor (Fig. 6).

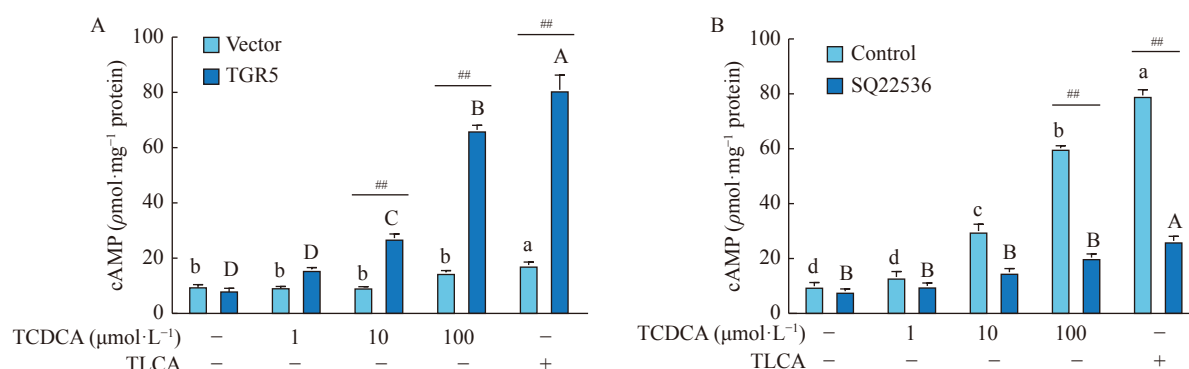


Fig. 3 TCDCA increased cAMP content via TGR5 TLCA was used as a positive control. (A) Effects of three different doses of TCDCA on the cAMP content was measured by ELISA, 100 $\mu\text{mol}\cdot\text{L}^{-1}$ TCDCA significantly increased cAMP content compared to other doses. (B) Effects of three different concentrations of TCDCA on the cAMP content was measured by ELISA. Representative of three independent experiments with similar results are shown. Data are presented as mean \pm SD. The adjacent lowercase letters indicate significant differences in NR8383 cells ($P < 0.05$), the interphase lowercase letters indicate that differences are extremely significant in NR8383 cells ($P < 0.01$); The adjacent uppercase letters indicate significant differences in NR8383-TGR5 cells and NR8383 cells treated with inhibitor ($P < 0.05$), the interphase uppercase letters indicate that differences are extremely significant in NR8383-TGR5 cells and NR8383 cells treated with inhibitor ($P < 0.01$); ## indicates that differences is extremely significant compared with the adjacent group ($P < 0.01$)

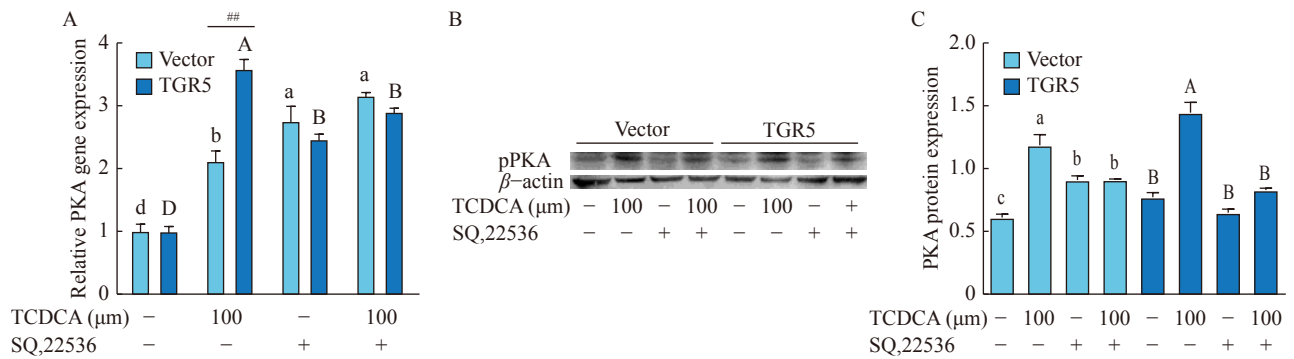


Fig. 4 Effects of TCDCA on PKA expression by TGR5. SQ22536 was used as a special inhibitor AC. NR8383 cells either with vector or TGR5 expression stimulated with TCDCA, and SQ22536. (A) PKA gene expression was detected by qPCR with TCDCA and SQ22536 treatment. (b, c) PKA phosphorylation level was detected by western blot with TCDCA and SQ22536 treatment. Representative of three independent experiments with similar results are shown. Data are presented as mean \pm SD. The adjacent lowercase letters indicate significant differences in NR8383 cells ($P < 0.05$), the interphase lowercase letters indicate that differences are extremely significant in NR8383 cells ($P < 0.01$); The adjacent uppercase letters indicate significant differences in NR8383-TGR5 cells ($P < 0.05$), the interphase uppercase letters indicate that differences are extremely significant in NR8383-TGR5 cells ($P < 0.01$); # indicates that significant differences compared with the adjacent group ($P < 0.05$)

Discussion

In recent years, the concepts that bile acids have effects on anti-inflammation and immunity has been widely accepted. TGR5 was identified as the receptor for BAs, after research found that it is a member of the GPCR family [16], while it has been reported that TGR5 is widely expression in tissues and organs of human and animals, including lung, liver, spleen, placenta, stomach and so on; expression of TGR5 receptor in the cells varied in these tissues and organs [17]. As TGR5 receptor is higher expression in the lung cancer cells of H1299 and H1975 being easily edited, so H1299 cell line which could be used in the study [18].

Emerging evidence has indicated the anti-inflammatory role of TCDCA in various mammalian immune cells, as demonstrated with rat alveolar macrophages and mouse alveolar macrophages [18, 19]. However, the interaction between TCDCA and the TGR5 receptor in immune cells remains largely unclear. Herein we report that TCDCA plays a key anti-inflammatory role *via* inhibiting cytokine production of TNF- α , IL-1 β , IL-6 and IL-12 induced by the TGR5 receptor in TGR5 overexpressing NR8383 cells. Moreover, our data showed that TCDCA suppressed cytokine expression via the classical cell signaling pathway of cAMP-PKA-CREB, suggesting that TCDCA might play a critical role in connecting inflammation with TGR5 receptor (Fig. 7).

Some research papers have reported that the TGR5 receptor is highly expressed in the liver and lung [18]. However, we found that TGR5 expression in gene and protein levels by qPCR and western blot was low. This result is not consistent with previously reported results. Therefore, according to Chinese laws of health and safety, our lab constructed a stable NR8383 cell line over-expressing TGR5, as determined by qPCR, western blot, and immunofluorescence. As expected, we certificated TCDCA activated TGR5 to induce

cAMP expression. In further study, our data showed that TCDCA increased the expression of PKA and CREB, thereby activating CREB to enter the nucleus from the cytoplasm to suppress NF- κ B expression. Suppression of NF- κ B inhibited secretion of cytokines, decreasing inflammation in the NR8383 cells. This is the first study to evaluate TCDCA mediated cAMP-PKA-CREB activation through the TGR5 receptor.

TCDCA is a member of the bile acid family affects many processes, including anti-inflammation, immune activities, apoptosis, and fat metabolism [2]. These activities might be due to the activation of specific receptors on the surface of the cell. After reviewing a lot of research papers, we found that TCDCA and other bile acids can regulate lipid metabolism: they may induce farnesoid-X receptor (FXR) to induce lipid changes such as triglyceride and fatty acid. Previous research has shown that there are many receptors capable of activating the cAMP-PKA-CREB pathway in immune cells, such as glucocorticoid receptor (GRs) [20, 21]. It would be of interest in the future to determine whether TCDCA may bind to other GPCR receptors, such as GRs, Toll like receptors (TLRs), or FXR, to further induce the cAMP-PKA-CREB cell signaling pathway. Further studies are ongoing to address these questions associated with TCDCA in our lab.

Conclusion

TCDCA could bind to TGR5 in H1299 cells; meanwhile, TCDCA decreases cytokine production *via* the classic inflammatory signaling pathway cAMP-PKA-CREB in NR8383 macrophages. To provide an important way of analyzing other bile acids to support further research into TCDCA in clinical settings.

Abbreviations

TCDCA: Taurochenodeoxycholic acid; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; CREB:

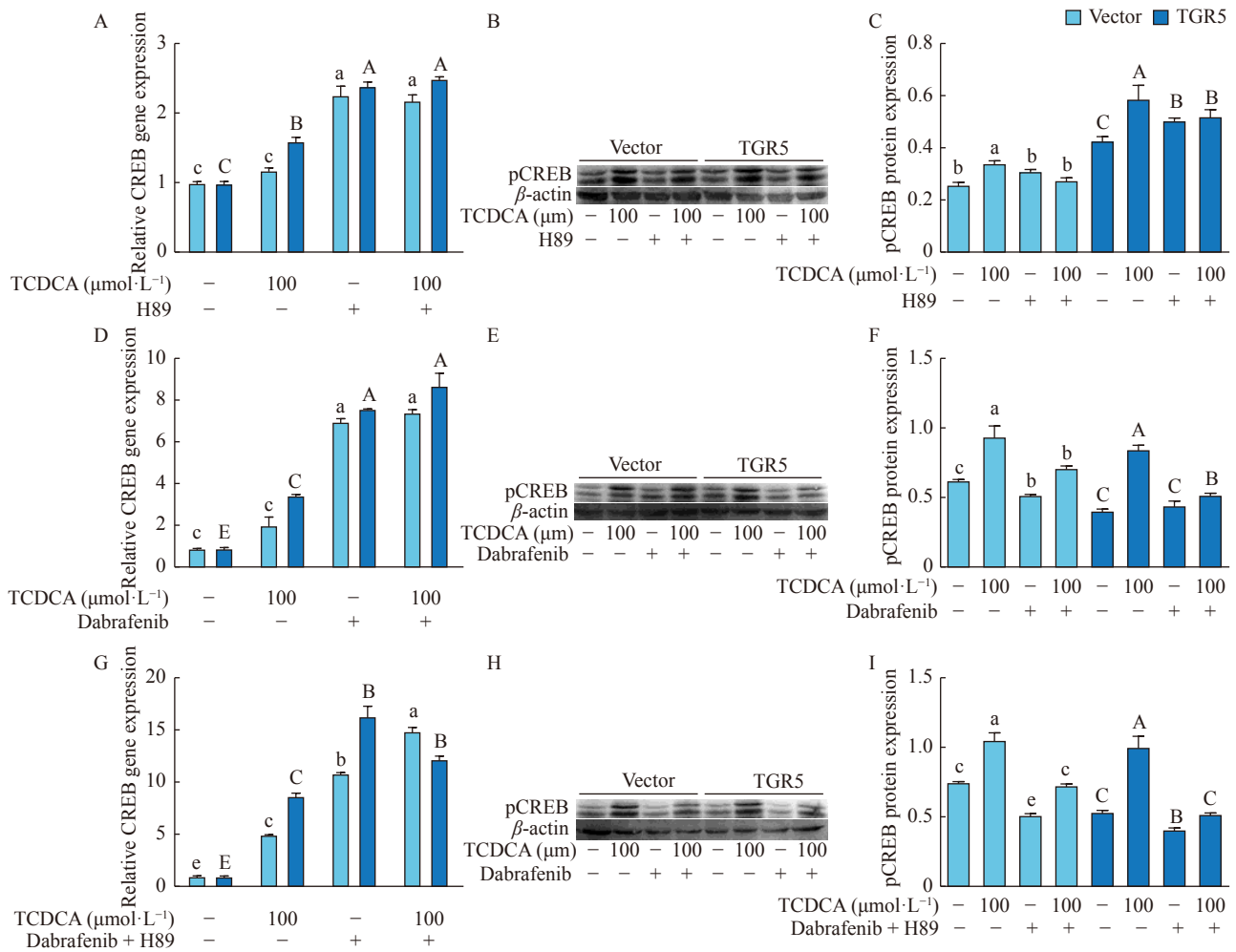
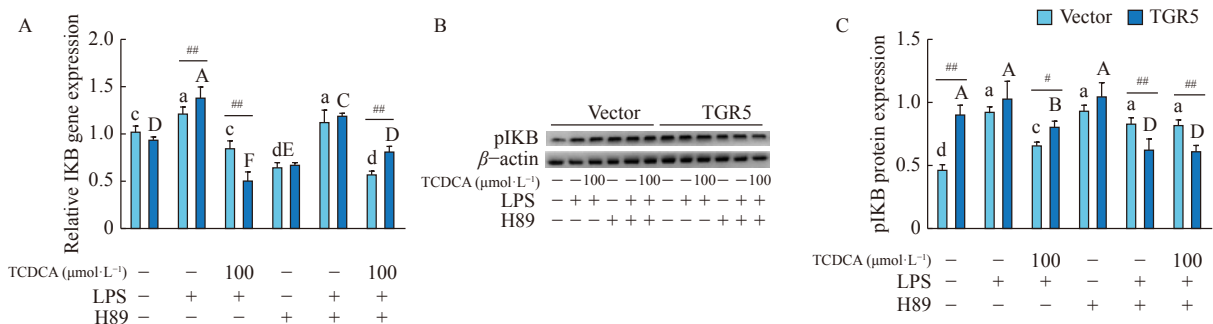


Fig. 5 Effects of TCDCA on CREB expression by TGR5. H89 was used as a special inhibitor of the PKA, Dabrafenib as a special inhibitor for the Raf. NR8383 cells either with vector or TGR5 expression stimulated with TCDCA, H89, and Dabrafenib. (A) CREB gene expression was detected by qPCR with TCDCA and H89 treatment. (B, C) CREB phosphorylation level was detected by western blot with TCDCA and H89 treatment. (D) CREB gene expression was detected by qPCR with TCDCA and Dabrafenib treatment. (E, F) CREB phosphorylation level was detected by western blot with TCDCA and Dabrafenib treatment. (G) CREB gene expression was detected by qPCR with TCDCA, H89 and Dabrafenib treatment. (H, I) CREB phosphorylation level was detected by western blot with TCDCA, H89 and Dabrafenib treatment. Representative of three independent experiments with similar results are shown. Data are presented as mean \pm SD. The adjacent lowercase letters indicate significant differences in NR8383 cells ($P < 0.05$), the interphase lowercase letters indicate that differences are extremely significant in NR8383 cells ($P < 0.01$); The adjacent uppercase letters indicate significant differences in NR8383-TGR5 cells ($P < 0.05$), the interphase uppercase letters indicate that differences are extremely significant in NR8383-TGR5 cells ($P < 0.01$); # indicates that significant differences compared with the adjacent group ($P < 0.05$), ## indicates that differences are extremely significant compared with the adjacent group ($P < 0.01$).



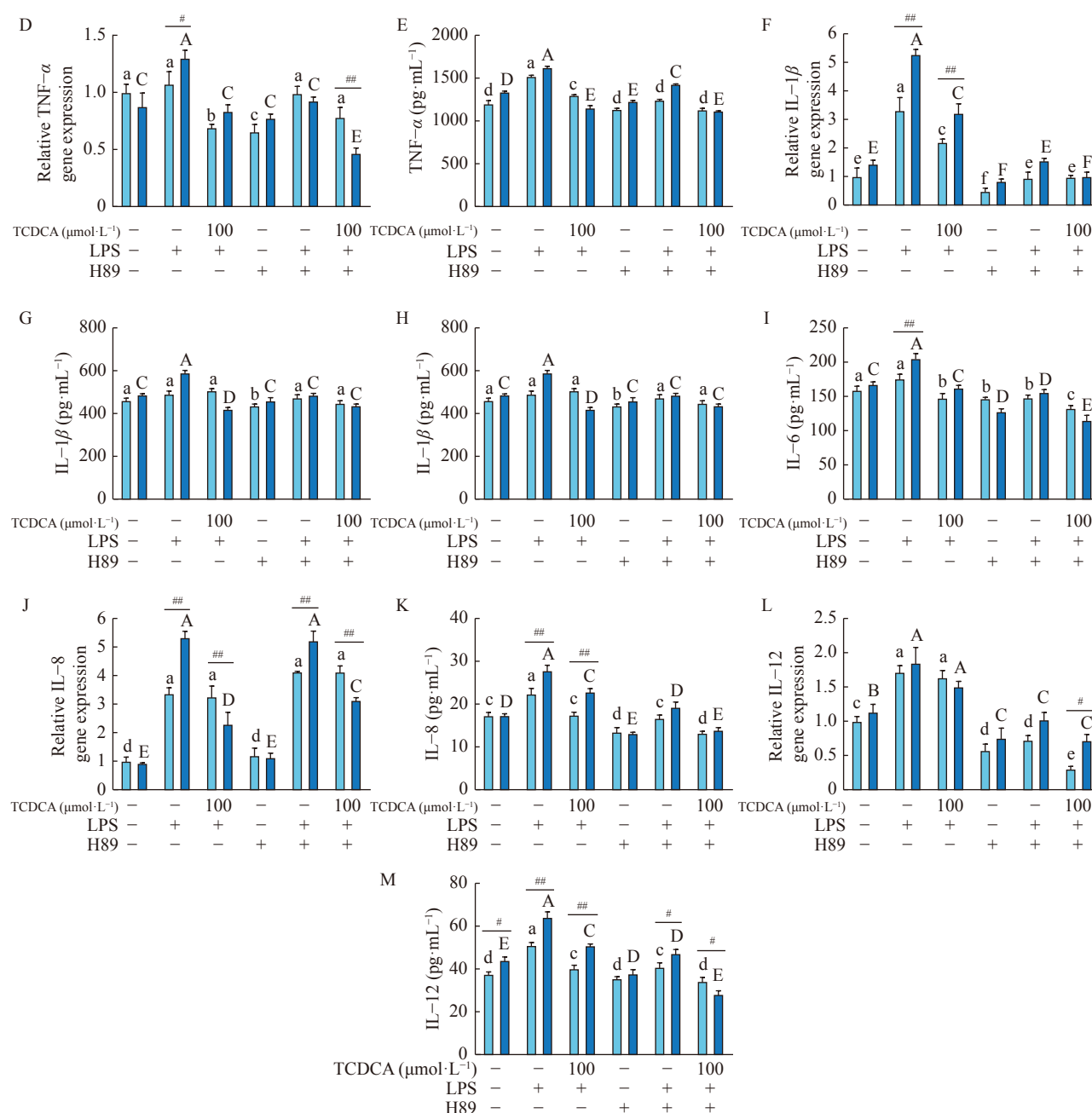


Fig. 6 Effects of TCDCA on NF- κ B expression and cytokines production by TGR5. All experiments carried on the NR8383 cells with either vector or TGR5 overexpression. TLCA was as a positive control. (A) I κ B α gene expression was detected by qPCR in NR8383 cells. (B, C) I κ B α phosphorylation level was detected by western blot. (D) TNF- α gene expression was detected by qPCR in NR8383 cells. (E) TNF- α content was detected by ELISA. (F) IL-1 β gene expression was detected by qPCR in NR8383 cells. (G) IL-1 β content was detected by ELISA. (H) IL-6 gene expression was detected by qPCR in NR8383 cells. (I) IL-6 content was detected by ELISA. (J) IL-8 gene expression was detected by qPCR in NR8383 cells. (K) IL-8 content was detected by ELISA. (L) IL-12 gene expression was detected by qPCR in NR8383 cells. (M) IL-12 content was detected by ELISA. Representative of three independent experiments with similar results are shown. Data are presented as mean \pm SD. The adjacent lowercase letters indicate significant differences in NR8383 cells ($P < 0.05$), the interphase lowercase letters indicate that differences are extremely significant in NR8383 cells ($P < 0.01$); The adjacent uppercase letters indicate that differences are extremely significant in NR8383-TGR5 cells ($P < 0.05$), the interphase uppercase letters indicate that differences are extremely significant in NR8383-TGR5 cells ($P < 0.01$); # indicates that significant differences compared with the adjacent group ($P < 0.05$), ## indicates that differences is extremely significant compared with the adjacent group ($P < 0.01$)

cAMP response element binding; AC: adenylate cyclase;
TNF- α : tumour necrosis factor- α ; IL-1 β : interleukin-1 β ; NF-

κ B: nuclear factor kappa light chain enhancer of activated B cells; BAs: Bile acids; CA: cholic acid; CDCA: chenodeoxy-

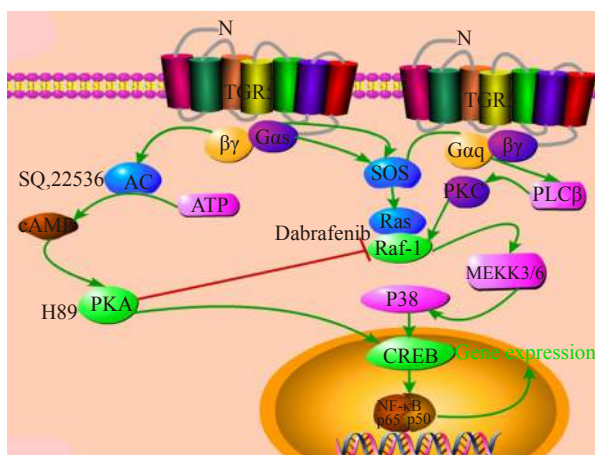


Fig. 7 Molecular mechanism of anti-inflammatory signal transported pathway of TCDCA induced by TGR5

cholic acid; PKC: protein kinase C; JNK: c-Jun N-terminal kinase; TCA: taurocholic acid; GPCR: G-protein-coupled receptor; LPS: lipopolysaccharides; FBS: fetal bovine serum; qPCR: Quantitative real-time PCR; SD: standard deviation; Ik-κB: inhibitor of κB; FXR: farnesoid-X receptor; GRs: glucocorticoid receptor; TLRs: Toll like receptors.

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