

Taurine Decreases Cellular Cholesterol Level in HepG2 Cells Partly through Upregulating Calcineurin

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Short report

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Abstract

Objective: Taurine exerts cholesterol-lowering effect through inducing CYP7A1 and promoting the biotransformation of cholesterol into bile acids in livers, but its molecular mechanism remains unclear. Taurine also suppresses the expression of MCIP1, a calcineurin inhibitory protein. Here we aimed to explore whether calcineurin involves in the cholesterol-lowering effect and upregulation of CYP7A1 by taurine.

Methods: High cellular cholesterol conditions were obtained by incubating with 0.2mM cholesterol contained DMEM in HepG2 cells. FK506, a calcineurin inhibitor, was used to depress cellular calcineurin. CnAb^{-/-} cells are the HepG2 cells of which calcineurin was knocked down. Taurine was cultured in wild type, high-cholesterol conditions, calcineurin inhibition or deficiency HepG2 cells respectively for 24h or 48h. The levels of intracellular total cholesterol were determined by an enzymatic method and the expressions of CYP7A1, calcineurin, MEK1/2, c-Jun/p-c-Jun and SHP-1 were detected by western blotting.

Results: High cellular cholesterol conditions in HepG2 cells were established and resulted in increased CYP7A1 and calcineurin expression. Taurine exhibited the decreasing-cholesterol effects on HepG2 cells regardless of whether cells with high cholesterol conditions or inhibited / deleted intracellular calcineurin. However, the extent of decreasing cholesterol after calcineurin repression or deficiency was much less than that of controls. Taurine could induce the expression of CYP7A1 but this induction was abolished when the cellular calcineurin was inhibited or deleted. Taurine was able to suppress MEK1/2, p-c-Jun and SHP-1, which are several key molecules in one inhibitory pathway of CYP7A1 transcription, whereas this suppression on MEK1/2 but not p-c-Jun or SHP-1 was reversed after completely knocking down calcineurin.

Conclusions: Calcineurin was found to be required partly in taurine-decreasing cholesterol effect through inhibiting MEK1/2 which resulted in CYP7A1 upregulation.

1 Introduction

Taurine (2-aminoethanesulfonic acid), rich in seafood, is well known to have several beneficial physiological roles, including antioxidation, detoxification, osmoregulation, cell membrane stabilization and hypolipidemia etc. (Lambert et al., 2015; Schaffer et al., 2010). Increasing studies revealed that taurine had cholesterol-lowering effect due to the increased biotransformation of cholesterol into bile acids in livers and the excretion of bile acids in feces (Chen et al., 2005; Mochizuki et al., 1999; S. Murakami et al., 2016). Taurine has been found that it upregulated dramatically the mRNA expression and the activity of Cholesterol 7 α -hydroxylase (CYP7A1), which is the rate-limiting enzyme in the major pathway of cholesterol conversion to bile acids, either in livers of hypercholesterolemia rats and mice or *in vitro* hepatocytes (Chang et al., 2011; Lam et al., 2006; S. Murakami et al., 2016). However, the molecular mechanism of CYP7A1 expression induced by taurine are under extensive investigation.

It has been proved that hepatocyte nuclear factor 4 α (HNF4 α) and liver receptor homolog-1 (LRH-1) are essential factors for CYP7A1 expression (Chiang, 2009). In addition, there still are two pathways to feedback repress CYP7A1 transcription, one is the bile acids receptor–farnesoid X receptor (FXR)-dependent pathway which interacts with HNF4 α and LRH-1 via atypical nuclear receptor small heterodimer partner-1 (SHP-1) induced by FXR, and the other is the FXR-independent pathway which involves many factors such as the mitogen-activated protein kinase/c-Jun N-terminal kinase (MAPK/JNK) and the mitogen-activated protein kinase /extracellular signal-regulated kinase (MAPK/ERK, MEK) signal transduction etc. (Goodwin et al., 2000; Li et al., 2006). Our previous experiments point out that taurine could enhance CYP7A1 expression by inducing HNF4 α and inhibiting MEK1/2 and phosphorylated-c-Jun (p-c-Jun) expressions to promote intracellular cholesterol metabolism in HepG2 cell (Guo et al., 2017).

Calcineurin is a calcium and calmodulin dependent serine/threonine protein phosphatase (also known as calcium-dependent serine-threonine phosphatase), which composes of calcineurin A catalytic subunit (CnA), calcineurin B regulatory subunit (CnB), and calmodulin for calcium binding (Aramburu et al., 2000; Rusnak & Mertz, 2000). Two isoforms of CnA (CnA α and CnA β) and one isoform of CnB (CnB1) are present in somatic cells (Aramburu et al., 2000). Calcineurin, which mainly dephosphorylates its substrates, interacts with numerous protein phosphokinases to control the phosphorylation status of related signaling molecules, such as c-Jun, JNK and MEK et al (Liang et al., 2003). Nuclear factor of activated T cell cytoplasmic (NFATc) is dephosphorylated by calcineurin and translocated into nucleus, which is the essential step to activate the immune system (N. Murakami et al., 2014; Rusnak & Mertz, 2000). Due to this immunomodulatory activity, calcineurin has been developed as the target for a class of immunosuppressive drugs called calcineurin inhibitors (CNI), which include cyclosporine A (CsA) and tacrolimus (FK506). However, dyslipidemia is a frequent finding following CsA and FK506, of which hypercholesterolemia is one of the most common manifestations. The use of immunosuppressive regimens report that the overall incidence of hypercholesterolemia in posttransplant recipients following CsA and FK506 treatment is 40–50%, and that in renal transplantation recipients is even as high as 60–70% (Kockx et al., 2010; Riella et al., 2012).

Taurine promotes the conversion of cholesterol to bile acid [3–5], while bile acid can activate the activity of calcineurin (Muili et al., 2013). Taurine also has been found that it could reduce the expression of MCIP1, a calcineurin inhibitory protein, in C2C12 fibroblast myotubes and mouse fibroblasts (Miyazaki et al., 2013). In *C. elegans* experiments, taurine showed a positive effect on the expression of calcineurin (Ko et al., 2017). Base on the above evidences, we investigated HepG2 cells, which was established to high cholesterol conditions by excess-cholesterol-contained medium and inhibited calcineurin through pharmacologic or genetic approaches, to explore the possible role of calcineurin in taurine decreasing cellular cholesterol process.

2 Materials And Methods

2.1 Reagents

The reagents were purchased from the following sources: taurine, FK506 and all main reagents for the determination of cellular cholesterol level were from Sigma (St. Louis, MO, USA); all the cell culture reagents were from Hyclone (Logan, USA) except fetal bovine serum (FBS) was from Gibco (New York, USA). PowerOpti-ECL Western blotting detection reagent was obtained from GenView (Florida, USA). Antibodies (anti-MEK1/2, #8727; -calcineurin, #2614; -c-Jun and -p-c-Jun #9260; -GAPDH, #2118 and -rabbit immunoglobulin G, #7074s) were obtained from Cell Signaling Technology (USA). Primary anti-CYP7A1 (sc-25536) antibody was from Santa Cruz Biotechnology (USA), anti- β -actin antibody from GenView (USA) and anti-SHP-1 (ab32559) antibody from Abcam (UK). Cell Lysis Buffer, PMSF and BCA Protein Assay Kit were purchased from Beyotime (China). All the other chemicals were purchased from Dingguo Changsheng Biotechnology Co. Ltd (China).

2.2 Cell culture and treatment

HepG2 cell from Cell Resource Center (School of Basic Medicine Peking Union Medical College) and CnA β -gene-deleted HepG2 cell from SyngenTech (Beijing, China) were routinely grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were cultured in 75mm² flasks at 37°C in a humidified atmosphere of 95% air and 5% CO₂ before treatment. For the experiment, the cells were plated out in 6-well plates and incubated in media containing 10 mM taurine for 24 h or 48 h. The cells with 0 mM taurine treatment were taken as the control and were given PBS instead of taurine. Each treatment was carried out at least in triplicate.

2.3 Cellular cholesterol determination

The intracellular cholesterol level was detected as described previously (Guo et al., 2017). Briefly, the cells were treated with 1 mL hexane/isopropanol (2:1, v:v) for 30 min at room temperature and also were cleaned with additional 1 ml hexane/isopropanol. The organic solvents after treating cells twice were blended in a separate test tube. Then the lipid extracted samples were dried and resuspended in 130 μ L isopropanol with 10% TritonX-100. The levels of cellular total cholesterol (TC) and free cholesterol (FC) were measured as following: 50 μ L of the lipid extracted samples was treated with TC or FC working solution (shown in Table 1) for 30 min at 37°C and optical density was determined at 500 nm using a μ Quant microplate spectrophotometers (Bio-Tek instruments INC, USA). The cellular ester cholesterol (EC) was calculated by TC minus FC.

After being treated by organic solvent, the rest of cells were lysed in Cell Lysis Buffer and then protein concentrations were measured by BCA Protein Assay Kit.

Table 1
Composition of working solutions for cholesterol detection

Chemicals	TC (μL)	FC (μL)
dipotassium hydrogen phosphate (0.1M)	192	198
cholesterol oxidase (5U/mL)	6	6
horse radish peroxidase (50U/mL)	6	6
sodium taurocholate hydrate (20 mM)	15	15
TritonX-100(1%)	15	15
4-aminoantipyrine (5.5 mM)	45	45
phenol (280 mM)	15	15
cholesterol esterase (25U/mL)	6	0
Total volume (μL)	300	300

2.4 Immunoblotting assay

HepG2 cells were lysed in ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.4), 0.1M EDTA, 10 mM NaCl, 0.5% Triton X-100 and PMSF. Homogenates were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were used for subsequent analyses. Protein concentration was detected using BCA Protein Assay Kit. Protein lysates with same amount protein were denatured in sample buffer (5% β -mercaptoethanol) for 5 min, then resolved by SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). The primary antibodies were diluted with TBS containing 0.05% Tween (TBS-T) and 5% milk. Membranes were incubated overnight with the primary antibodies at 4°C. Subsequently the membranes were incubated with secondary antibody for 1hr, and immunoreactive bands were visualized with chemiluminescence (ECL) western blotting substrates. Finally, the immunoblots were scanned and quantified using a Gel imaging system (GE healthcare, USA).

2.5 Statistical analysis

All the data are expressed as the mean \pm standard deviation, and statistical analysis was performed by one-way ANOVA. The differences are considered to be statistically significant at $p < 0.05$.

3 Results

3.1 Calcineurin Correlated with Intracellular Cholesterol Levels

To clarify the hepatic molecular mechanism of cholesterol-lowering effect by taurine, high-cholesterol hepatocyte models were built by being treated HepG2 cells with exogenous high cholesterol (0.02 mM) to simulate hypercholesterolemia. As Fig. 1.A shown, the intracellular cholesterol levels of HepG2 cells

exhibited significant increases by 0.02 mM cholesterol treatment for either 24 h or 48 h, compared with the control cells. Meanwhile, we also observed a large induction of the calcineurin expression in high-cholesterol HepG2 cells for both 24 h and 48 h incubation (Fig. 1.B). To further confirm the role of calcineurin in hepatocytic cholesterol mechanism, FK506 was administrated in HepG2 cells, which is an inhibitor of the calcineurin activity. The increases of the total cellular cholesterol concentration were seen at 5.0 $\mu\text{g}/\text{mL}$ FK506 or 10.0 $\mu\text{g}/\text{mL}$ FK506 for either 24 h (Fig. 1.C) or 48 h (Fig. 1.D). And when the CnA β of HepG2 cells was knocked out, increased cellular cholesterol could be clearly observed compared as to wild type control cells (Fig. 1.E). These results showed that calcineurin may be an important molecular in the development of liver high cholesterol conditions.

3.2 Calcineurin Involved in Taurine Lowering Cholesterol Process

To explore whether calcineurin plays crucial roles in the decrease of cellular cholesterol by taurine, we confirmed the cholesterol lowering effect of taurine in our current cells. Consistent with the data from mice or rats (Chen et al., 2005; Mochizuki et al., 1999; S. Murakami et al., 2016), taurine could decrease cellular cholesterol levels at 10 mM concentration for 24 h incubation in either normal HepG2 or high-cholesterol HepG2 (Fig. 2A). After inhibiting calcineurin by FK506, the decreased cholesterol effect of taurine before-mentioned was still seen, but the extent of cholesterol decreases in FK506 treated cells tended to be smaller than that of in control cells (12.5% versus 16.6%) (Fig. 2B). When CnA β was completely deleted, taurine still exhibited cellular cholesterol-decreasing effect, but the extent of decrease in CnA $\beta^{-/-}$ cells was less significant as compared to that of in control cells (13.2% versus 25.3%) (Fig. 2C and Fig. 2D). Moreover, for taurine, the reduction of total cholesterol in wild type cells was mainly due to the reduction of free cholesterol whereas in CnA $\beta^{-/-}$ cells it was due to the decrease in cholesterol ester (Fig. 2D). Our results showed that calcineurin participates in the cholesterol-lowering effect of taurine, at least by partially.

3.3 Calcineurin Promoted Taurine to induce CYP7A1 Expression

We next validated the role of calcineurin in taurine cholesterol-decreasing effect through CYP7A1. As Fig. 3A/3C/3D showed, taurine induced significantly the expression of CYP7A1 in the HepG2 cells. In high-cholesterol HepG2 cells, the treatment with 0.02 mM cholesterol for 24 h stimulated CYP7A1 protein expression compared to the untreated cells, and taurine could induce even more CYP7A1 expression than that of high cholesterol alone treatment (Fig. 3A). However, this CYP7A1 induction effect of taurine disappeared when FK506 was administrated (Fig. 3C) or CnA β was deleted (Fig. 3D) in HepG2 cells. Taurine also led to a significant increased expression of calcineurin in HepG2 cells and failed to induce its further increases in high-cholesterol HepG2 cells, which had resulted in increased expression of calcineurin (Fig. 3B). These data indicate that calcineurin may be a crucial molecule in the upregulating CYP7A1 expression of taurine.

3.4 The effect of calcineurin on several key signaling molecules regulated by taurine in CYP7A1 expression

To confirm the above findings, several key molecules involved in the FXR-independent pathway of CYP7A1 inhibitory regulation were measured. When CnA β of HepG2 cells was knocked out, the protein expressions of MEK1/2 and p-c-jun were induced, and the expressions of c-Jun and SHP-1 were not affected compared as to that of the wild type cells (Fig. 4). We further found that taurine significantly decreased the expression of MEK1/2, p-c-jun and SHP-1 in HepG2 cells, this MEK1/2 downregulation could be rescued by deleting calcineurin (Fig. 4A), while the downregulation of p-c-jun and SHP-1 were not affected by deleting calcineurin (Fig. 4B/4C). These data suggest that calcineurin plays a key role in the inhibitory effect on MEK1/2 expression by taurine.

4 Discussion

In this study, we found that cellular high cholesterol condition in HepG2 cells resulted in increased expression of CYP7A1 and calcineurin, and calcineurin could affect MEK1/2 and p-c-Jun pathway which repress the transcription of CYP7A1, suggesting calcineurin involves in cellular cholesterol balances through CYP7A1 regulation. Then we showed that taurine could decrease the intracellular cholesterol level, but the extent of cholesterol decreasing was much smaller in calcineurin inhibition or calcineurin deficiency cells than in untreated controls, indicating calcineurin is required partly for taurine decreasing cholesterol process. Furthermore, we clarified that calcineurin is a key molecule to reduce cellular cholesterol by upregulating CYP7A1 through targeting to MEK1/2 but not p-c-Jun or SHP-1.

Our data demonstrated that the expression of CYP7A1 and calcineurin were followed by cellular high cholesterol condition, which induced by treatment with high cholesterol medium, and inhibiting or deleting calcineurin led to elevate the cellular cholesterol level and the expression of CYP7A1. Hypercholesterolemia, one of the characteristics of metabolic syndrome, is well known for contributing to the formation of atheromatous plaques in arteries (Bhatnagar et al., 2008; Mannu et al., 2013). Calcineurin inhibitors such as CsA and FK506 are the clinically used drugs that cause hypercholesterolemia (Kockx et al., 2010). It is also reported that mice lacking calcineurin A β are hyperlipidemic, including high level of cholesterol, triglyceride and free fatty acid (Suk et al., 2013). Our results agree with these previously reported studies. CYP7A1, as a rate limiting enzyme in the process of transforming cholesterol into bile acid, plays an important role in regulating cholesterol balance and is often as a target to improve hypercholesterolemia. Our current *in vitro* data showed that CYP7A1 expression were dramatically increased in CnA β ^{-/-} cells and FK506- cells, suggesting that the elevated cholesterol due to calcineurin deficiency may also induce CYP7A1 expression to deal with this cholesterol stress compensatively. However, few *in vivo* experiments indicated that CsA administration for 3 weeks resulted in the reduction of hepatic CYP7A1 expression and marked down-regulation of skeletal muscle and adipose tissue lipoprotein lipase abundance in rats (Vaziri et al., 2000). These puzzling data might be explained by the difference in compensatory mechanisms between systemic regulations *in vivo* and

direct hepatocytic effects *in vitro*. Further, we also noticed that calcineurin deficiency was coupled to the increase of MEK1/2 and p-c-jun but not SHP-1, which are several key molecules in FXR-independent inhibitory pathway of CYP7A1 transcription. Together, calcineurin may play a critical part in cellular cholesterol homeostasis via regulation of CYP7A1 expression.

Our study revealed that taurine was able to reduce the intracellular cholesterol level not only in wild type cells and high-cholesterol cells but also in calcineurin inhibited or deleted cells, whereas the extent of reducing cholesterol in the latter two conditions was less than that of untreated or wild type cells. This indicates that calcineurin participates partly in cholesterol-decreasing effect of taurine. Many feeding studies with guinea pigs, rats, mice and hamsters demonstrated that taurine affected cholesterol metabolism, and showed cholesterol-lowering effect in hypercholesterolemia induced by high cholesterol diet, but it also showed cholesterol-raising effect in hypercholesterolemia induced by administration of polychlorinated biphenyl (PCB, the synthetic organic compound widely distributed in the environment) and phenobarbital (PB, one of the widely used sleeping drugs) (Chen et al., 2012). Here, calcineurin is found to play an important role in the cholesterol-lowering effect of taurine *in vitro*, which may help understand its cellular mechanism.

Taurine also could induce notably the expression of CYP7A1 in both wild type and high-cholesterol HepG2 cells, but this induction was abolished by inhibition or deletion of calcineurin. Furthermore, taurine suppressed MEK1/2, p-c-Jun and SHP-1 in wild type HepG2 cells, whereas this suppression on MEK1/2 but not p-c-Jun or SHP-1 was reversed after completely knocking down calcineurin. As for the expression of calcineurin, taurine caused an increase in wild type HepG2 cells but failed to more increases in high-cholesterol cell condition, and this failure probably because calcineurin had already been induced to a very high level. Ko et al addressed that taurine exerts a positive effect on the expression of calcineurins in *C. elegans* (Ko et al., 2017). Miyazaki et al demonstrated that the expression of calcineurin inhibitory protein, MCIP1, was specifically inhibited at the transcriptional level by taurine (Miyazaki et al., 2013). There are evidences that taurine can not only increase the concentration of Ca^{2+} in cells, but also reduce the phosphorylation level of related signal molecules (Bkaily et al., 1998; Wei et al., 2012). CYP7A1 expression is regulated by several signal regulating pathway, including one enhancing pathway and two feedback repressing pathways (Chiang, 2013; Goodwin et al., 2000; Li et al., 2006). Our previous study showed that taurine can promote intracellular cholesterol metabolism in HepG2 cell by promoting CYP7A1 expression via inducing HNF4 α in the enhancing pathway and inhibiting MEK1/2 and phosphorylated-c-Jun expressions in the feedback repressing pathways (Guo et al., 2017). Taken together, we clarify that calcineurin is required partly in taurine's cholesterol-decreasing effect through inhibiting MEK1/2 which resulted in CYP7A1 upregulation (schematic in Fig. 5).

5 Conclusion

In conclusion, the present study shows that calcineurin may play a critical part in cellular cholesterol homeostasis via regulation of CYP7A1 expression. Moreover, calcineurin was firstly found to be required

partly in taurine's cholesterol-lowering effect through inhibiting MEK1/2 which resulted in CYP7A1 upregulation.

Declarations

Acknowledgments

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Author Contributions

JXG carried out most of the experiments, interpreted the data and drafted the manuscript. YG and YXZh participated in the detection of protein expression. JZh and YZHZh participated in the interpretation of the data and statistical analysis. WCh participated in experimental design and coordination, corrected the manuscript and supervised the study. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interests.

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Figures

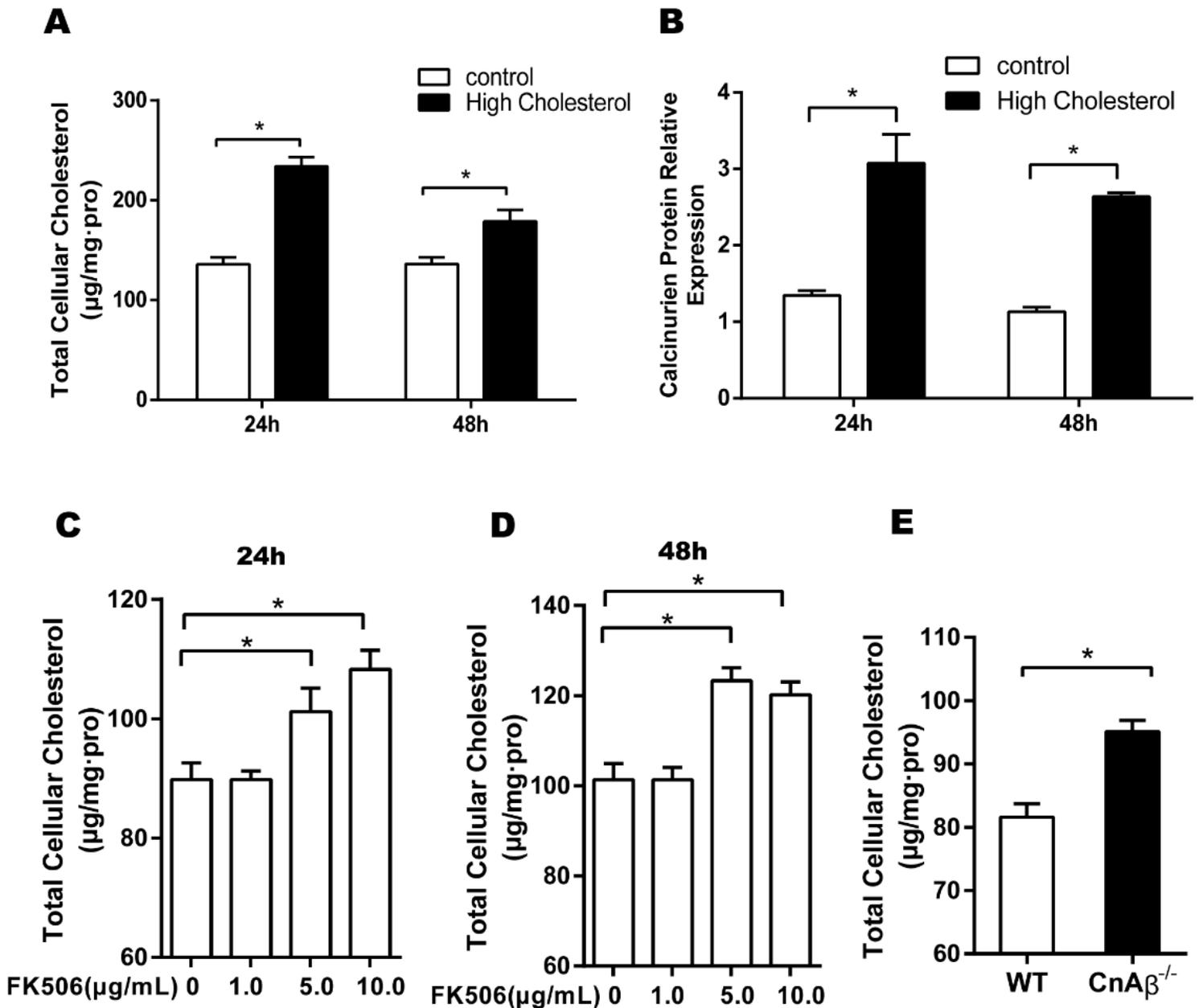


Figure 1

The effect of calcineurin on total intracellular cholesterol levels. The cellular cholesterol levels (A) increased significantly while the expression of calcineurin (B) was induced due to high cholesterol treatment in HepG2 cells. The cellular cholesterol content was promoted apparently after either inhibiting calcineurin activity by FK506 for 24h (C) or 48h incubation (D) or deleting CnAβ (E) in HepG2 cells. * Represents the significant difference ($p < 0.05$) between two groups.

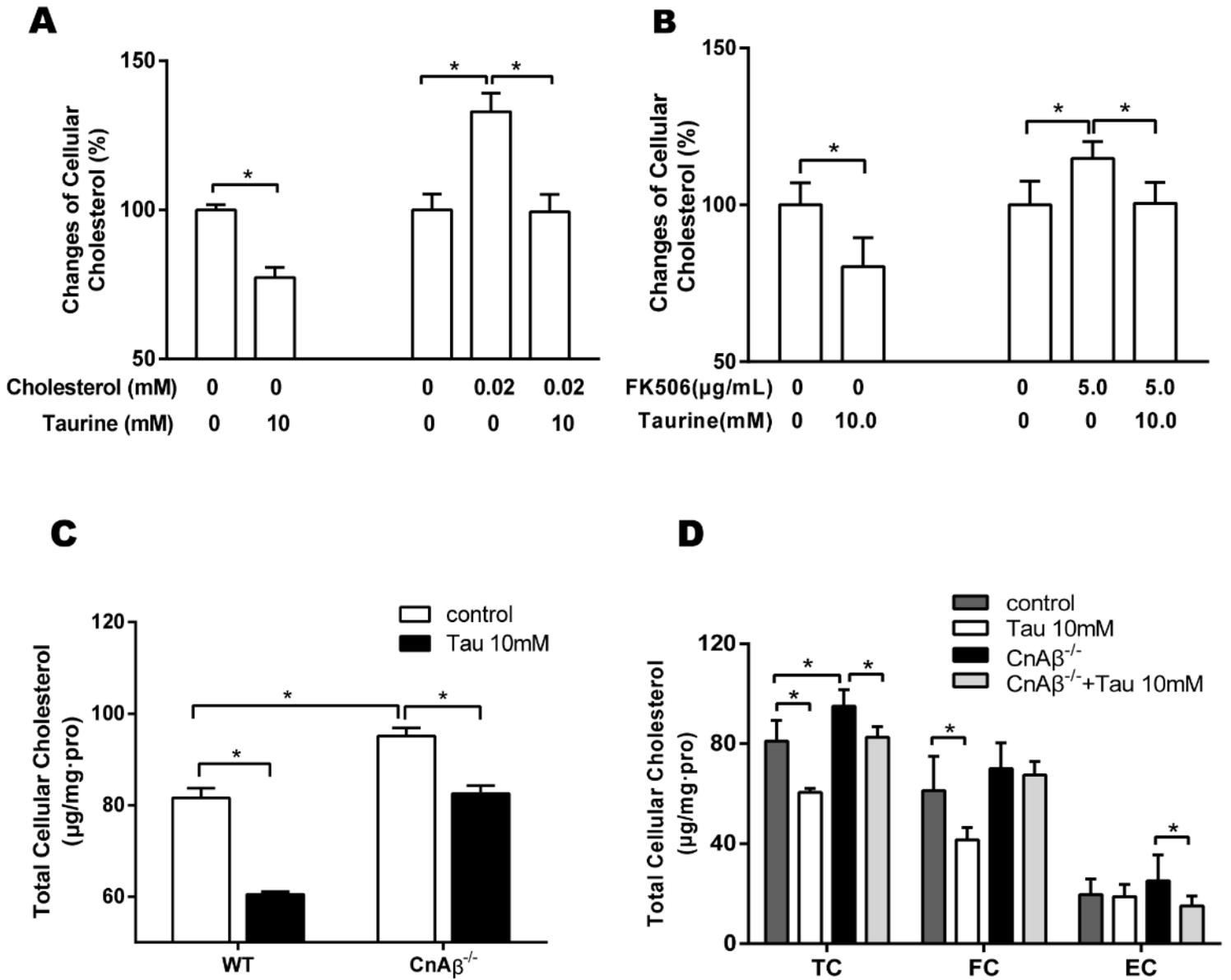


Figure 2

The role of calcineurin on the cellular cholesterol-lowering effect by taurine in HepG2 cells. Comparative results on the cellular cholesterol levels by taurine are shown here in HepG2 cells respectively following being treated with cholesterol (A), inhibiting CnA activity by FK506 (B) and deleting CnAβ (C and D). * Represents the significant difference ($p < 0.05$) between two groups.

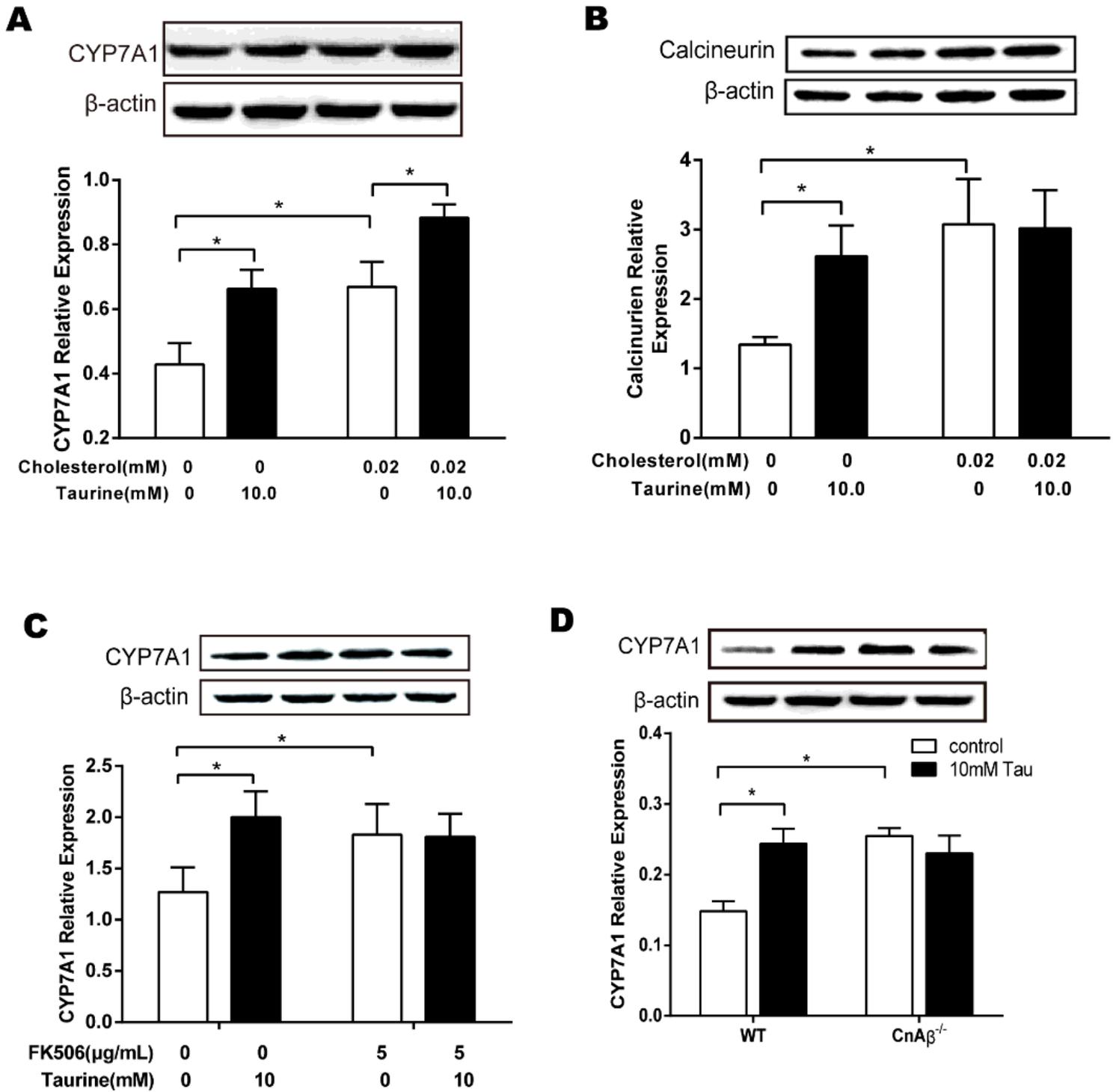


Figure 3

The effect of calcineurin on CYP7A1 expression induced by taurine. The protein expression of CYP7A1 (A) and calcineurin (B) were induced by taurine in HepG2 cells treated with high cholesterol. While the inducing effect of taurine on CYP7A1 expression disappeared after inhibiting CnA activity by FK506 (C) or deleting CnAβ (D). * Represents the significant difference ($p < 0.05$) between two groups.

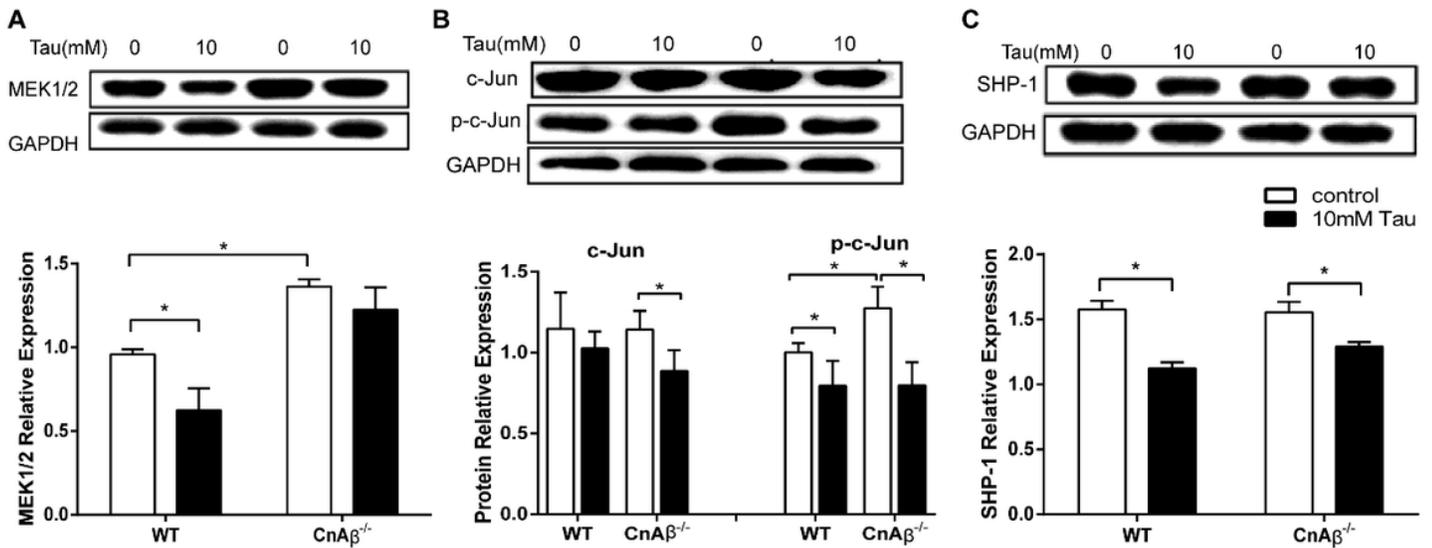


Figure 4

The effect of calcineurin on several key signaling molecules regulated by taurine in CYP7A1 expression. The comparative effects by taurine were shown on the protein expression of MEK1/2 (A), c-jun/p-c-jun (B) and SHR-1 (C) in WT and CnA β ^{-/-} HepG2 cells. * Represents the significant difference (p<0.05) between two groups.

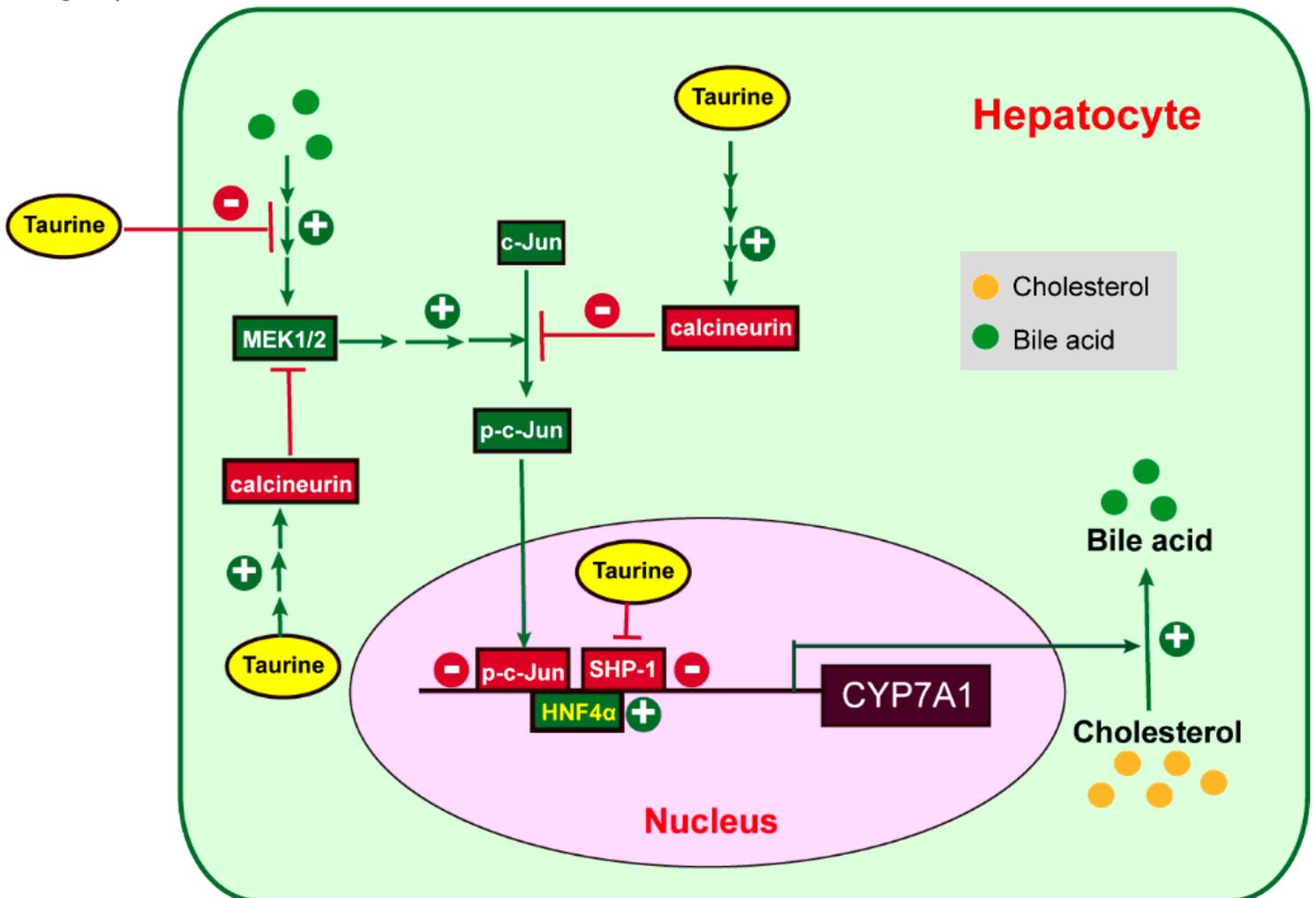


Figure 5

The possible role of calcineurin in cholesterol-lowering effect of taurine.