

Pioglitazone reduces lipid droplets in cholesterolosis of the gallbladder by increasing ABCA1 and NCEH1 expression

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Abstract As a cholesterol-induced metabolic disease, cholesterolosis of the gallbladder is often resected clinically, which could lead to many complications. The histopathology of cholesterolosis is due to excessive lipid droplet accumulation in epithelial and subcutaneous tissues. The main components of lipid droplets are cholesterol esters (CEs). Removal of CEs from gallbladder epithelial cells (GBECs) is very important for maintaining intracellular cholesterol homeostasis and for treating cholesterol-related diseases. In this study, pioglitazone was used to reduce intracellular CEs. To further elucidate the mechanism, cholesterolosis GBECs were treated with pioglitazone, 22-(R)-hydroxycholesterol (a liver X receptor α (LXR α) agonist), or peroxisome proliferator-activated receptor gamma (PPAR γ) siRNA. Western blotting for PPAR γ , LXR α , ATP-binding cassette transporter A1 (ABCA1), and neutral cholesteryl ester hydrolase 1 (NCEH1) was performed. At length, cholesterol efflux to apoA-I was measured, and oil red O staining was used to

visualize lipid droplet variations in cells. In conclusion, we observed that pioglitazone increased ABCA1 expression in an LXR-dependent manner and NCEH1 expression in an LXR α -independent manner, which mobilized CE hydrolysis and cholesterol efflux to reduce lipid droplet content in cholesterolosis GBECs. Our data provide a plausible alternative to human gallbladder cholesterolosis.

Keywords Cholesterolosis · Pioglitazone · PPAR γ · LXR α · ABCA1 · NCEH1

Introduction

Gallbladder cholesterolosis is becoming more common among Asian populations. Clinically, these cases are often treated with different types of cholecystectomy, which could result in bile duct injury, post cholecystectomy syndrome or other serious complications during and after the operation. As a benign disease, patients with cholesterolosis suffer too much misery, and there should be a better treatment.

Histopathologically, cholesterolosis is characterized by excessive lipid droplet accumulation in epithelial and subcutaneous tissues. The main components of lipid droplets are cholesterol esters (CEs) [1]. Under physiological conditions, intracellular cholesterol levels can be regulated in two ways. On the one hand, they are regulated by cholesterol hydrolysis or esterification to maintain intracellular homeostasis involving two processes working in tandem [2, 3]. Free cholesterol (FC) can be esterified to form CEs by acyl-CoA cholesterol acyltransferase-1 (ACAT 1), or CEs can be hydrolyzed to form FC by neutral cholesteryl ester hydrolase 1 (NCEH 1) [4, 5]. On the other hand, FC can be effluxed out of gallbladder epithelial cells (GBECs) by ATP-binding

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cassette transporter A1 (ABCA1) [6, 7], which localizes to the basolateral plasma membrane [8].

Regulation of ABCA1 or NCEH1 should influence intracellular cholesterol levels. NCEH1 up-regulation could transform CE into FC; however, if the activity of ABCA1 is limited, excess FC that is detained in the cytoplasm must be re-esterified into CE. ABCA1 up-regulation could provide a pathway to efflux FC; however, the lipid droplets in gallbladder cholesterosis are mainly composed of CE that cannot efflux out of cells. Up-regulation of both NCEH1 and ABCA1 should be optimal.

In human macrophages, NCEH1 can be regulated by a PPAR γ agonist. In THP-1-derived macrophages, ABCA1 expression is regulated by liver X receptor α (LXR α) [9, 10], and LXR α expression is regulated by peroxisome proliferator-activated receptor gamma (PPAR γ) [11, 12]. However, in human hepatocytes, PPAR γ ligand treatment inhibited LXR α expression, thereby negatively regulating ABCA1 expression [13]. As members of the nuclear receptor superfamily, PPAR γ and LXR α can be regulated by ligands to influence cholesterol homeostasis [14, 15].

In cholesterosis GBECs, specific pathways that modify NCEH1 and ABCA1 to regulate intracellular cholesterol levels and reduce lipid droplets remain unclear and should be clarified. In this study, we used pioglitazone (a PPAR γ agonist) to decrease lipid droplets in cholesterosis GBECs in an attempt to treat gallbladder cholesterosis [16].

Materials and methods

Materials

Pioglitazone hydrochloride was obtained from Cayman Chemicals (USA). 22-(R)-hydroxycholesterol was purchased from Sigma (USA). ApoA-I, fetal bovine serum (FBS), phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, NBD-cholesterol and Lipofectamine were purchased from Invitrogen/Life Technologies (CA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (USA). Anti- β -actin antibodies were obtained from Santa Cruz Biotechnology (USA).

Patients

The use of human gallbladders in our experiment was approved by the medical ethics committee of Zhongda Hospital, which is affiliated with Southeast University. In the study, we harvested 6 normal gallbladders from patients who had undergone pancreaticoduodenectomies for pancreas head carcinoma, and their gallbladders were normal.

In addition, we harvested 282 cholesterosis gallbladders from patients who had undergone cholecystectomy for gallbladder cholesterosis. In total, 6 normal gallbladders and 6 cholesterosis gallbladders were used to compare the NCEH1, PPAR γ , LXR α , and ABCA1 protein expression. The other specimens were used for the remaining experiments.

Cell culture

Cell culture plates were coated overnight with 0.2 ml of a 1:1 mixture of collagen IV (rat-tail collagen, Sigma, USA) and FBS. Gallbladder mucosa were incubated with 0.25 % collagenase type IV for 20 min. Next, GBECs were collected, washed twice with DMEM, and seeded onto 25 cm culture plates with 10 ml DMEM supplemented with 15 % FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cells were incubated at 37 °C in 5 % CO $_2$, and the medium was changed every 3 days.

Cell viability assay (MTT)

GBECs were treated with 0.01–10 μ M pioglitazone for 3–24 h. Cell viability was then examined by MTT assay.

Western blot analyses

GBECs were cultured in DMEM containing 15 % FBS. Cells were treated with pioglitazone (0.01, 0.1, 1, 10 μ M) and/or 10 μ M 22-(R)-hydroxycholesterol. Cells were then washed with PBS, harvested, and lysed in buffer containing protease inhibitor cocktail to obtain protein extracts. After gel electrophoresis, the extracted proteins were transferred onto polyvinylidene difluoride membranes in buffer for 1 h at room temperature. Residual binding sites on the membranes were blocked, and the blots were incubated with the following antibodies in Tris-buffered saline: monoclonal PPAR γ antibodies (1:800 dilution, sc-7273, Santa Cruz Biotechnology, USA), polyclonal LXR α antibodies (1:400 dilution, sc-1202, Santa Cruz Biotechnology, USA), ABCA1-specific antibodies (1:400 dilution, 21676, Signalway Antibody Co, Ltd, USA), NCEH1-specific antibodies (1:400 dilution, 01-P2002, ARP, USA), and monoclonal β -actin-specific antibodies (1:4000 dilution). Membranes were then washed with PBST and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) in PBST for 20 min. Membranes were then washed and incubated with WB detection reagent for 3 min, after which photography was conducted. β -actin immunoreactivity was used as an internal control. The proteins were analyzed and quantified with Image J 1.42.

RNA interference

Cells were seeded on 6 cm culture plates (10^7 per well) containing 5 ml DMEM and 15 % FBS for 3 days. On day 4, 200 pmol PPAR γ siRNA (sc-29455 Santa Cruz Biotechnology, USA) or control siRNA (sc-36869 Santa Cruz Biotechnology, USA) were added to 500 μ l culture media, and 10 μ l Lipofectamine was added to a separate 500 μ l aliquot of culture media. After 5 min, the two 500 μ l media aliquots were mixed gently for 20 min at room temperature to form a siRNA-Lipofectamine mixture. To each culture plate, 1 ml siRNA mixture was added, and the cells were incubated at 37 °C for 48 h. The cells were then lysed and subjected to Western blotting with anti-PPAR γ antibodies to analyze the effect of RNA interference on PPAR γ expression.

Luciferase reporter assays

Briefly, following the protocols provided (E1910, Promega, USA), 10^7 GBECs in 2 ml DMEM were supplemented with 80 μ l Lipofectamine 2,000, 40 μ g luciferase reporter construct or control vector, and 10 μ g CMV- β -galactosidase plasmid as an internal control and incubated at 37 °C for 10 h. Cells were then harvested, washed twice, and re-cultured in DMEM. 24 h later, cell extracts were collected, and lysate luciferase activity was measured using the Dual-Luciferase Reporter Assay System.

Cellular lipid efflux

GBECs were seeded in each well of 96-well culture plates. After incubation with 10 mM NBD-cholesterol for 24 h, the cells were washed twice with PBS and serum-starved overnight. The starved GBECs were cultured in serum-free medium with 0.01, 0.1, 1, or 10 μ M pioglitazone for 24 h or with 1 μ M pioglitazone for 3, 6, 12, and 24 h. Next, the medium in each well was aspirated carefully. The cells were washed twice with PBS and incubated in PBS containing 15 % FBS and 10 μ g/ml apoA-I. Finally, the fluorescently-labeled cholesterol in the medium was collected and measured with a fluorescence microplate reader at 485 nm (excitation) and 535 nm (emission). All of the data were measured six times, and DMSO was added as a negative control.

Cytoplasmic lipid droplet staining

After 3 days of primary cell culture, cholesterosis GBECs that had been seeded in 24-well culture plates were washed twice with PBS. The cells were cultured in a serum-free medium with 1 μ M pioglitazone for 6, 12, 24, and 48 h. Next, the medium was replaced with PBS containing 15 % FBS and 10 μ g/ml apoA-I, and the cells were

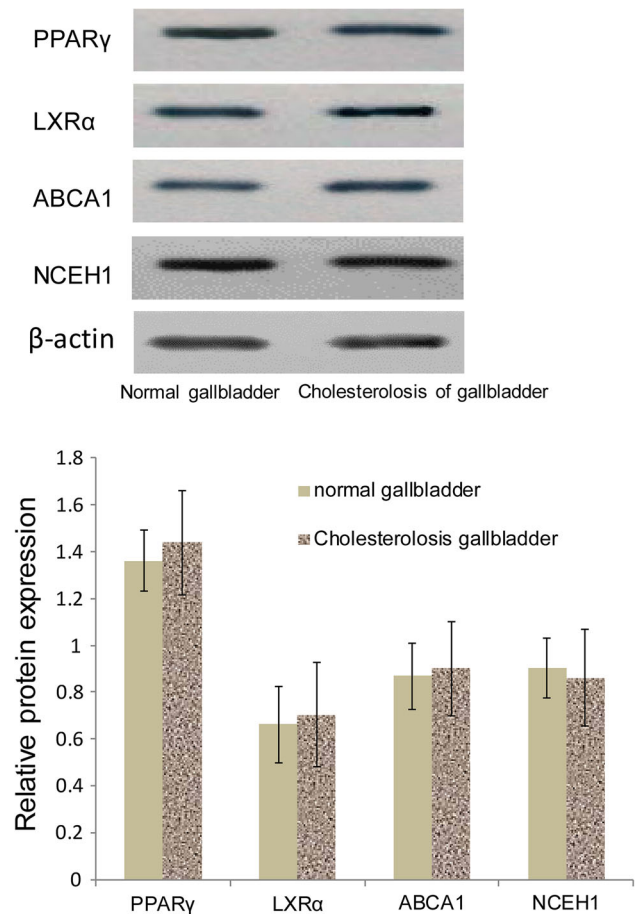


Fig. 1 Comparison of PPAR γ , LXR α , ABCA1 and NCEH1 protein expression in normal and cholesterosis gallbladders. There were no statistically differences between cholesterosis and normal gallbladders for all four proteins. ($P > 0.05$)

incubated for 6, 12, 24, and 48 h. Finally, the cells were collected and fixed in 10 % formaldehyde in PBS. After 25 min, the cells were washed twice again and stained with oil red-O by incubation at 37 °C overnight. After washing three times with 75 % alcohol, the cells were photographed using a microscope that had been fitted with a digital camera. We chose 3 regions in each photo and six cells in each region. The red portion of each cell was quantified by Image J 1.42 software. At each time-point, the mean value and standard deviation was obtained. All of the data were merged into a curve graph.

Statistical analysis

All of the outcomes represented six separate experiments and were expressed as the mean \pm SD. The data were analyzed with SPSS 20.0 statistical software. Student's *t* test or one-way ANOVA were used to analyze the differences between groups. Values of $P < 0.05$ were considered to be statistically significant.

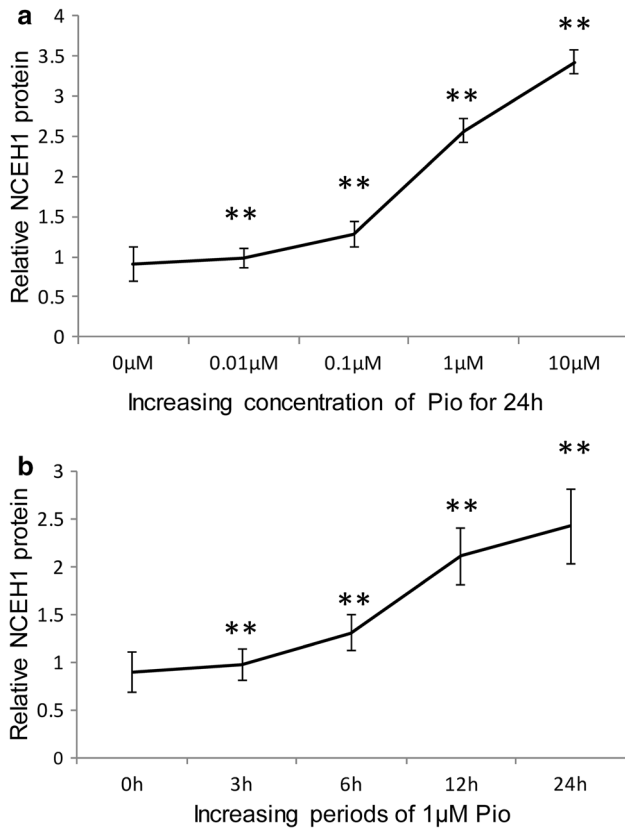


Fig. 2 Pioglitazone (Pio) induced NCEH1 protein expression. GBECs were treated with **a** increasing pioglitazone concentrations for 24 h or **b** 1 μM Pio for 0–24 h. Pio dose-dependently enhanced NCEH1 expression, which reached $342 \pm 14.79\%$ at 10 μM for 24 h and time-dependently increased NCEH1 expression to $242 \pm 39.19\%$ at 1 μM for 24 h. All of the data are shown as the mean \pm SD. (** $P < 0.01$)

Results

Pioglitazone did not affect cell viability

GBECs were treated with increasing concentrations of pioglitazone ranging from 0.01 to 10 μM for 3–24 h. Cell viability was not remarkably decreased. This result indicated that pioglitazone at this dose did not inhibit cell viability.

Comparing PPAR γ , LXR α , ABCA1, and NCEH1 expression

Though the expression of PPAR γ , LXR α , and ABCA1 in cholesterosis of the gallbladder were slightly higher than in normal GBECs, and the mean NCEH1 expression in cholesterosis of the gallbladder was slightly lower than in normal gallbladder. In this experiment, we determined that there was no statistically difference between cholesterosis gallbladders and normal gallbladders for all four proteins. ($P > 0.05$; Fig. 1) [17]

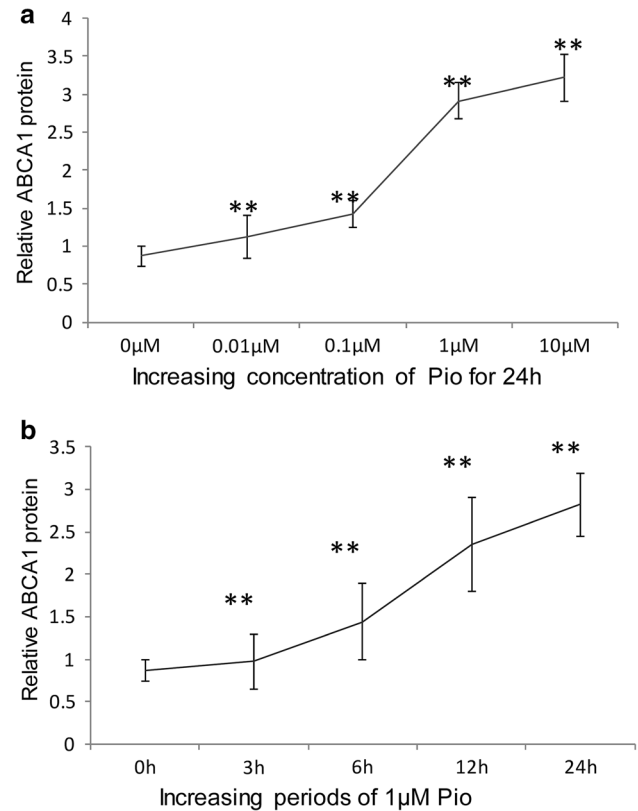


Fig. 3 Pioglitazone (Pio) induced ABCA1 protein expression. GBECs were treated with **a** increasing concentrations of pioglitazone for 24 h or **b** 1 μM Pio for 0–24 h. Pio dose-dependently activated ABCA1 expression, which reached $322 \pm 31.2\%$ at 10 μM for 24 h and time-dependently increased ABCA1 expression to $281 \pm 66.5\%$ at 1 μM for 24 h. All of the data are shown as the mean \pm SD. (** $P < 0.01$)

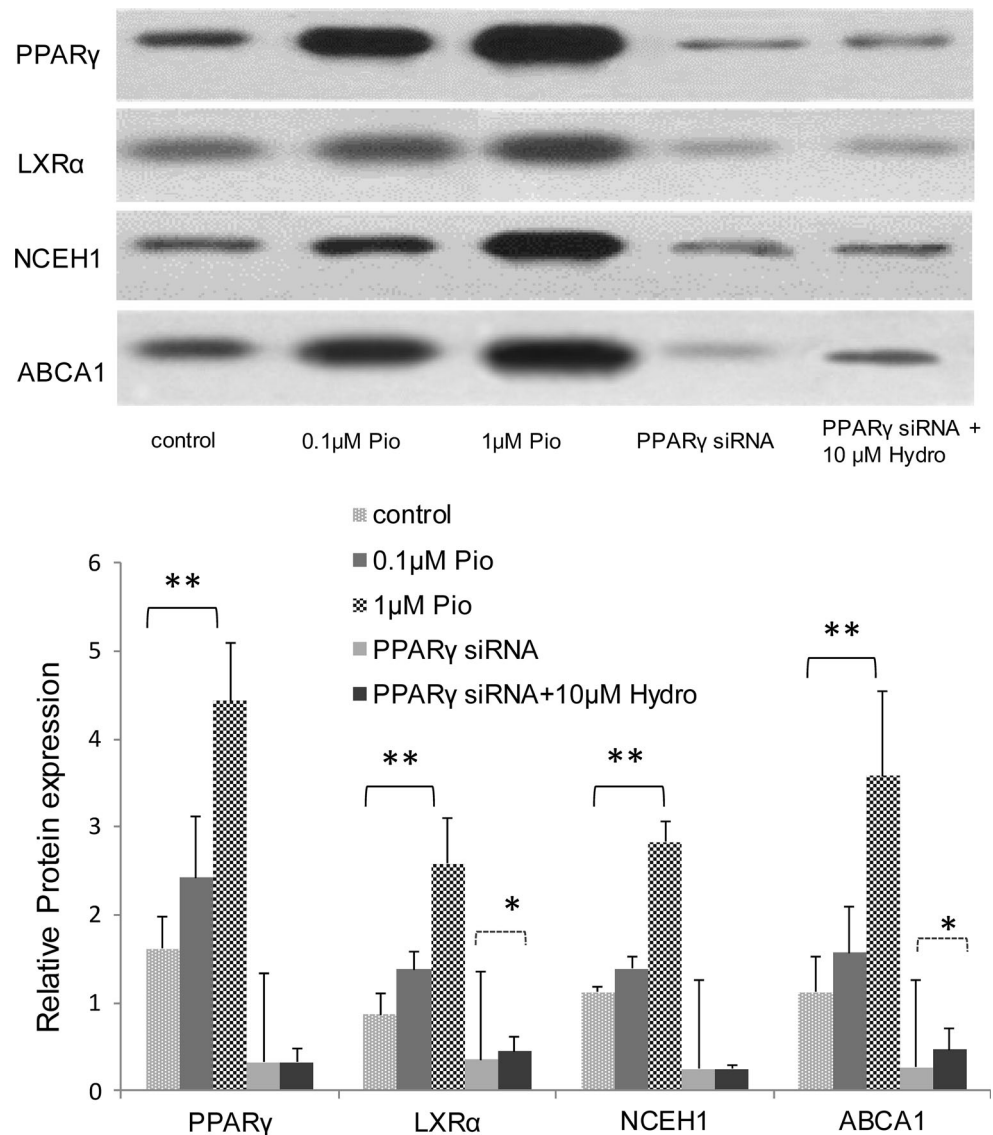
Pioglitazone markedly increased NCEH1 expression

Cholesterosis GBECs were treated with 0, 0.01, 0.1, 1, or 10 μM pioglitazone for 24 h, and a dose-dependent effect of the drug on NCEH1 expression was observed. NCEH1 transcription increased to 90.22 ± 21.53 , 98.18 ± 11.47 , 127 ± 16.04 , 257 ± 14.33 , and $342 \pm 14.79\%$, respectively, ($P < 0.01$; Fig. 2a). After treatment with 1 μM pioglitazone for 0, 3, 6, 12, and 24 h, NCEH1 protein levels were gradually up-regulated to 90.22 ± 21.53 , 97.87 ± 16.92 , 131 ± 19.18 , 210 ± 29.12 , and $242 \pm 39.19\%$, respectively, ($P < 0.01$; Fig. 2b). In all of the experiments, 10 μM pioglitazone for 24 h resulted in the highest level of NCEH1 protein, and 1 μM pioglitazone resulted in 75.13 % of the highest protein expression.

Pioglitazone markedly induced ABCA1

Cholesterosis GBECs were treated with 0, 0.01, 0.1, 1, or 10 μM pioglitazone for 24 h, and ABCA1 expression

Fig. 4 Pioglitazone (Pio) up-regulated NCEH1 and ABCA1 protein expression. GBECs were treated with 0.1 μ M pioglitazone, 1 μ M pioglitazone, PPAR siRNA, and PPAR siRNA + 10 μ M 22-(R)-hydroxycholesterol (Hydro). PPAR γ , LXR α , NCEH1 and ABCA1 protein expression were increased by 0.1 and 1 μ M pioglitazone treatment. All of the proteins were decreased markedly by PPAR γ siRNA, and treatment with 10 μ M 22-(R)-hydroxycholesterol (an LXR α agonist) up-regulated LXR α and ABCA1 but not PPAR γ or NCEH1 expression. (* $P < 0.05$; ** $P < 0.01$)



increased to $86.95 \pm 12.7 \%$, $112 \pm 27.8 \%$, $142 \pm 18.2 \%$, $291 \pm 24.6 \%$, and $322 \pm 31.2 \%$, respectively, ($P < 0.01$; Fig. 3a). Furthermore, after 1 μ M pioglitazone treatment for 0, 3, 6, 12, and 24 h, ABCA1 expression was induced to 86.95 ± 12.7 , 97 ± 32.6 , 144 ± 45.1 , 235 ± 55.2 , and $281 \pm 66.5 \%$, respectively, ($P < 0.01$; Fig. 3b). In all of the experiments, 10 μ M pioglitazone for 24 h resulted in the highest levels of ABCA1 protein, and 1 μ M pioglitazone for 24 h resulted in 93.63 % of the highest protein expression.

Pioglitazone activated NCEH1 expression in an LXR α -independent manner

After treatment with 10 μ M pioglitazone for 24 h, NCEH1 protein expression increased remarkably. Upon incubation with PPAR γ siRNA, there was a 77.85 % reduction in NCEH1 protein expression. To further determine whether

LXR α agonists directly regulated NCEH1 expression, GBECs transfected with PPAR γ siRNA were incubated with 10 μ M 22-(R)-hydroxycholesterol (an LXR α agonist) for 24 h. As demonstrated in Fig. 4, there was no effect of the LXR α agonist on NCEH1 and PPAR γ expression.

Pioglitazone induced ABCA1 expression in an LXR α -dependent manner

After treatment with 10 μ M pioglitazone for 24 h, PPAR γ , LXR α , and ABCA1 expression increased noticeably. This result suggested that LXR α and ABCA1 transcription was likely mediated by increased PPAR γ expression. To test this hypothesis, GBECs were incubated with PPAR γ siRNA, which reduced PPAR γ expression to 20.11 %. In accordance with this change in PPAR γ expression, LXR α levels decreased to 40.49 % and ABCA1 levels declined to

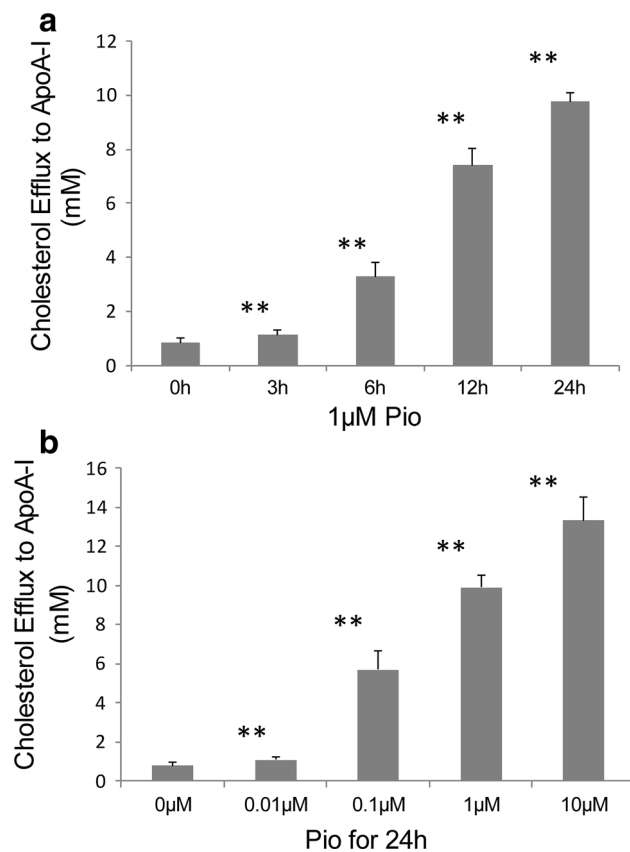


Fig. 5 Pioglitazone (Pio) promoted cholesterol efflux into apoA-I. GBECs were incubated with 10 mM NBD-cholesterol for 24 h and were then induced by 10 μg/ml apoA-I+ **a** 1 μM Pio for 0, 3, 6, 12 and 24 h or **b** 0, 0.01, 0.1, 1 and 10 μM Pio for 24 h (DMSO was used as a negative control). Media cholesterol concentrations were measured. All of data are shown as the mean ± SD. (** $P < 0.01$)

23.62 %. To further determine the relationship among PPAR γ , LXR α , and ABCA1, studies were conducted using 10 μM 22-(R)-hydroxycholesterol. LXR α protein expression increased to 128.28 % and ABCA1 expression increased to 176.98 %; however, PPAR γ expression was not noticeably affected by 22-(R)-hydroxycholesterol treatment (Fig. 4).

Pioglitazone-induced cholesterol efflux

To reduce the interference of intracellular lipid droplets to cholesterol efflux, NBD-cholesterol was used. After stimulation with 1 μM pioglitazone for 0, 3, 6, 12, and 24 h, NBD-cholesterol concentration in the supernatant was 0.79 ± 0.19 , 1.13 ± 0.16 , 3.27 ± 0.52 , 7.39 ± 0.63 , and 9.75 ± 0.36 mmol/L, respectively, (Fig. 5a). Conversely, after treatment with 0, 0.01, 0.1, 1, and 10 μM pioglitazone for 24 h, NBD-cholesterol concentrations were 0.79 ± 0.19 , 1.07 ± 0.16 , 5.71 ± 0.98 , 9.89 ± 0.65 , and 13.31 ± 1.23 mmol/L, respectively, (Fig. 5b). Therefore, pioglitazone

time- and dose-dependently promoted apoA-I-mediated cholesterol efflux from human cholesterosis GBECs.

Photomicrographs demonstrated reduced lipid droplets in cholesterosis GBECs

To determine the influence of PPAR γ activation on cholesterosis of the gallbladder, GBECs were cultured for 3 days and then incubated with 1 μM pioglitazone for 0, 6, 12, 24, and 48 h, and the cells were stained by oil red-O. Representative images of intracellular lipid droplets are shown in Fig. 6. Untreated control cells were dark red at 200 \times (Fig. 6a) and 400 \times (Fig. 6b) magnification and were filled with lipid droplets. After time, at 200 \times magnification, the cell color gradually faded and became pink in the 48 h group (Fig. 6i). At 400 \times magnification, red color decreased gradually and began to appear as needle-like features (Fig. 6f). Finally, the needle-like region also gradually reduced or disappeared (Fig. 6j). Using Image J 1.42, lipid droplets were counted, and a curve demonstrated that pioglitazone might effectively decrease lipid accumulation in cholesterosis GBECs ($P < 0.01$; Fig. 6k).

Discussion

Supersaturated cholesterol in bile can be absorbed through both passive and active mechanisms by GBECs. If cholesterol levels exceed cellular transport capacity, excess cholesterol may be esterified and deposited in cells, which is known as cholesterosis. Altering cholesterol metabolism and transport in GBECs may potentially prevent or treat cholesterosis [18]. For the first time, our study demonstrated that pioglitazone mitigated cholesterosis by two pathways. As demonstrated in Fig. 7, pioglitazone up-regulated NCEH1 expression, resulting in CEs hydrolysis into cytoplasmic FC. Pioglitazone also up-regulated ABCA1 via an LXR α -dependent pathway to efflux hydrolyzed FC into apoA-I. Ultimately, pioglitazone decreased cholesterol deposits in cholesterosis GBECs.

In this study combined with our previous studies, PPAR γ , LXR α , NCEH1, and ABCA1 expression in normal and cholesterosis gallbladders was similar. All of these data provide the basis for regulation of intracellular cholesterol homeostasis. In this study, GBECs treated with 0.01–10 μM pioglitazone were viable. Clinically, the maximum plasma concentration should be 3.6 μM [19]. Therefore, 1 μM pioglitazone was selected as the optimal drug concentration.

Endoplasmic reticulum-localized NCEH1 promoted CE hydrolysis to FC, but the relationship between PPAR γ and NCEH1 was not distinct. NCEH1 expression may be increased [19], decreased [20], or unchanged [21] by

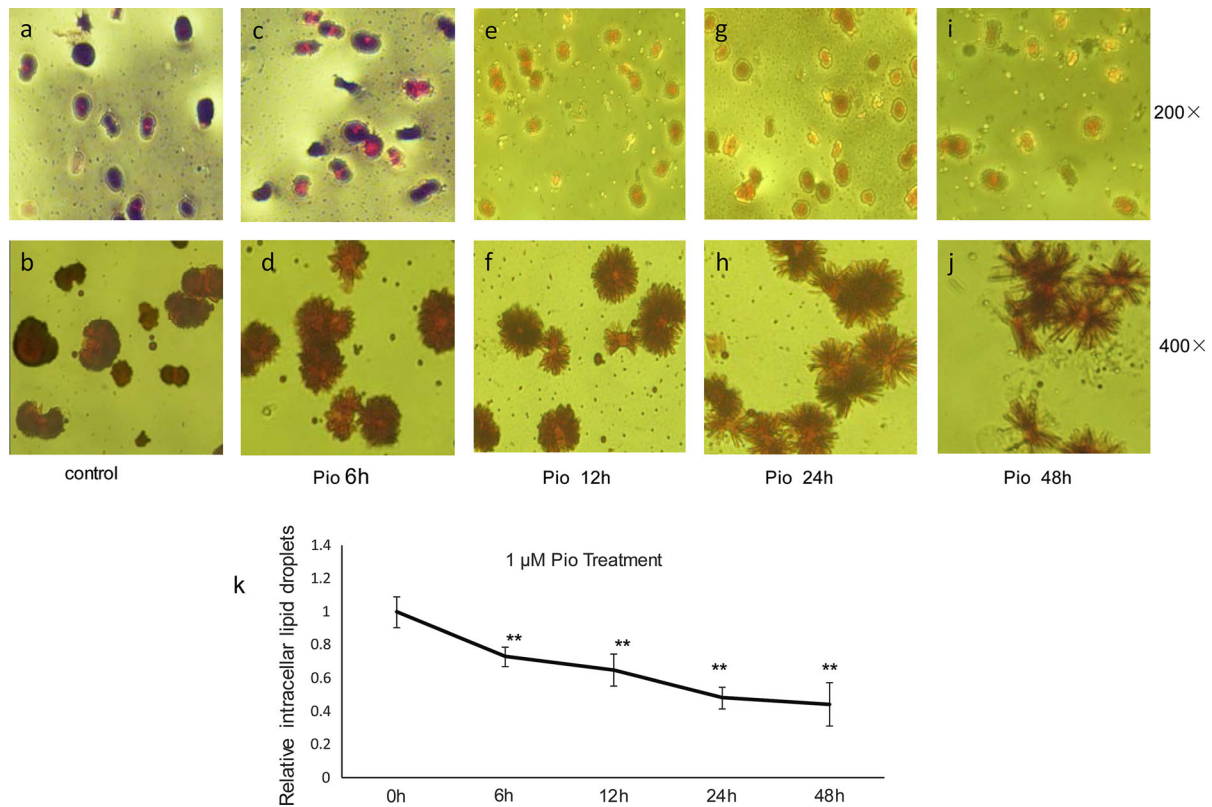
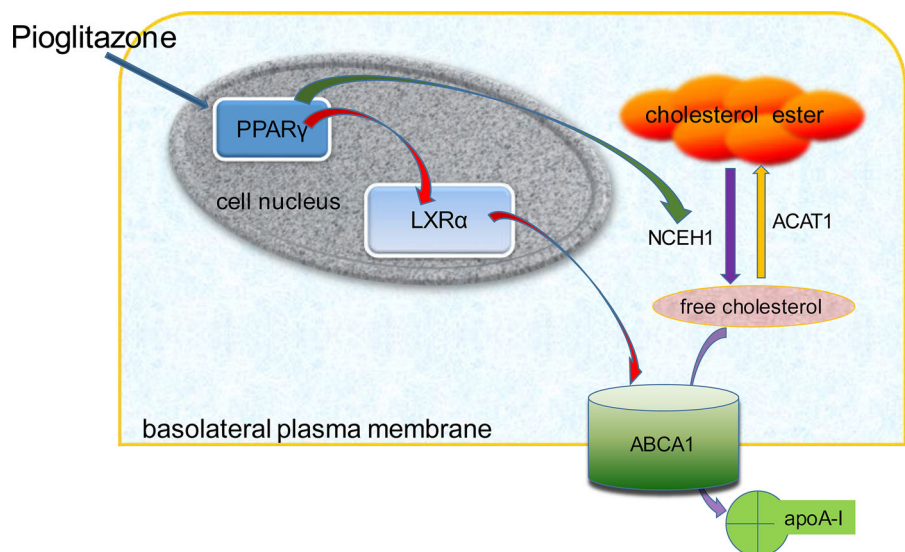


Fig. 6 Pioglitazone (Pio) effluxed lipid droplets from cholesterolosis GBECs. Primary cells from cholesterolosis gallbladders were incubated with 1 μ M Pio + 10 μ g apoA-I for 0, 6, 12, 24 and 48 h. Representative images of intracellular lipid droplets are demonstrated in Fig. 6. Lipid droplets were counted using Image J 1.42, and a curve

was made. A time-dependent effect on the reduction of intracellular lipid droplets was observed. (** $P < 0.01$; Fig. 6k) **a** control, $\times 200$; **c** 6 h, $\times 200$; **e** 12 h, $\times 200$; **g** 24 h, $\times 200$; **i** 48 h, $\times 200$; **b** control, $\times 400$; **d** 6 h, $\times 400$; **f** 12 h, $\times 400$; **h** 24 h, $\times 400$; **j** 48 h, $\times 400$

Fig. 7 Pioglitazone (Pio) could reduce lipid droplets in cholesterolosis GBECs by two pathways. First, Pio up-regulated NCEH1 to hydrolyze cholesterol esters (CEs) into free cholesterol (FC); second, Pio up-regulated ABCA1 in an LXR α -dependent pathway, which then effluxed FC to apoA-I. Ultimately, Pio decreased lipid droplets in cholesterolosis GBECs



PPAR γ agonists. To analyze the underlying relationship between NCEH1 and PPAR γ in GBECs, several tests were performed. In our study, PPAR γ siRNA decreased NCEH1 protein expression by 77.85 %, and pioglitazone

dose- and time-dependently activated NCEH1. Meanwhile, 1 μ M pioglitazone treatment for 24 h resulted in a 2.57-fold increase of NCEH1. To further study the correlation between LXR α and NCEH1 expression, 22-(R)-

hydroxycholesterol was applied to GBECs that had been transfected with PPAR γ siRNA. In those cells, no difference in NCEH1 expression was observed. Thus, LXR α may not regulate NCEH1 expression. Therefore, pioglitazone activates NCEH1 via an LXR α -independent pathway.

ABCA1 is located in the basolateral plasma membrane and is the most predominant transporter for eliminating intracellular cholesterol in GBECs [6]. Previous reports demonstrated that the ABCA1 gene is a target of LXR α [22, 23], and that the LXR α gene is a target of PPAR γ . PPAR γ can regulate ABCA1 protein [24], but there does not appear to be a functional PPAR γ binding site in the ABCA1 promoter, which suggested that PPAR γ -induced ABCA1 expression is likely to be secondary to LXR α regulation. Nevertheless, in intestinal mucosa epithelial cells and human macrophages, PPAR γ can positively regulate LXR α expression; in contrast, PPAR γ negatively regulated LXR α expression in human hepatocytes. Thus, this pathway and its corresponding modulator acted in a tissue-specific manner [25–27]. In our study, PPAR γ siRNA decreased ABCA1 protein expression, and pioglitazone treatment markedly enhanced ABCA1 protein expression in a dose- and time-dependent manner. To further study the correlation between LXR α and ABCA1, 22-(R)-hydroxycholesterol was applied, and LXR α and ABCA1 protein expression increased accordingly. Therefore, a PPAR γ -LXR α -ABCA1 pathway exists, and pioglitazone up-regulated ABCA1 via an LXR-dependent pathway in human GBECs.

Thus, pioglitazone may be useful to promote lipid droplets efflux. To test this hypothesis, we measured cholesterol flow in cell supernatants and stained lipid droplets with oil red-O. Indeed, cholesterol content increased and cytoplasmic lipid droplet content was remarkably reduced after pioglitazone treatment. As demonstrated in Fig. 6, pioglitazone treatment resulted in a marked mobilization to mitigate cholesterol. Furthermore, the observable effects of pioglitazone on cholesterol deposits can be interpreted as a combination of NCEH1 and ABCA1 activities, leading to a much steeper increase in lipid droplet efflux.

The limited availability of animal models to study cholesterosis in vivo was overcome using primary cell cultures from cholesterosis gallbladders. Additionally, pioglitazone administration only represents an acute effect on CE accumulation in GBECs. Many genes have effects not only on cholesterol intake and discharge but also on lipid metabolism. GBECs incubated with siRNA did not attain 100 % inhibition of PPAR γ expression, which might be other mechanism that is involved, although it is unlikely to be a major contribution to NCEH1 and ABCA1 regulation. These unsolved questions would be answered by further experimentation. In any case, this study demonstrated that

pioglitazone caused a net removal of lipid droplets from cholesterosis GBECs. Thus, regulating cholesterol metabolism and transportation may be a new therapeutic method.

In conclusion, this study demonstrated that pioglitazone treatment enhanced cholesterol efflux from cholesterosis GBECs by increasing ABCA1 expression in an LXR-dependent manner and NCEH1 expression in an LXR α -independent manner. Pioglitazone treatment also reduced lipid droplet number in cholesterosis GBECs. Our data provide a plausible alternative to cholecystectomy for the treatment of human gallbladder cholesterosis.

References

1. Strömsten A, Von Bahr S, Bringman S, Saeki M, Sahlin S, Björkhem I, Einarsson C (2004) Studies on the mechanism of accumulation of cholesterol in the gallbladder mucosa. Evidence that sterol 27-hydroxylase is not a pathogenetic factor. *J Hepatol* 40:8–13
2. Yu XH, Fu YC, Zhang DW, Yin K, Tang CK (2013) Foam cells in atherosclerosis. *Clin Chim Acta* 424:245–252
3. Ouimet M, Marcel YL (2012) Regulation of lipid droplet cholesterol efflux from macrophage foam cells. *Arterioscler Thromb Vasc Biol* 32:575–581
4. Igarashi M, Osuga J, Uozaki H, Sekiya M, Nagashima S, Takahashi M, Takase S, Takanashi M, Li Y, Ohta K, Kumagai M, Nishi M, Hosokawa M, Fledelius C, Jacobsen P, Yagyu H, Fukayama M, Nagai R, Kadowaki T, Ohashi K, Ishibashi S (2010) The critical role of neutral cholesterol ester hydrolase 1 in cholesterol removal from human macrophages. *Circ Res* 107:1387–1395
5. Igarashi M, Osuga J, Isshiki M, Sekiya M, Okazaki H, Takase S, Takanashi M, Ohta K, Kumagai M, Nishi M, Fujita T, Nagai R, Kadowaki T, Ishibashi S (2010) Targeting of neutral cholesterol ester hydrolase to the endoplasmic reticulum via its N-terminal sequence. *J Lipid Res* 51:274–285
6. Reboul E, Dyka FM, Quazi F, Molday RS (2013) Cholesterol transport via ABCA1: new insights from solid-phase binding assay. *Biochimie* 95:957–961
7. Ruiz JL, Fernandes LR, Levy D, Bydlowski SP (2013) Interrelationship between ATP-binding cassette transporters and oxysterols. *Biochem Pharmacol* 86:80–88
8. Yoon JH, Choi HS, Jun DW, Yoo KS, Lee J, Yang SY, Kover R (2013) ATP-binding cassette sterol transporters are differentially expressed in normal and diseased human gallbladder. *Dig Dis Sci* 58:431–439
9. Yan JQ, Tan CZ, Wu JH, Zhang DC, Chen JL, Zeng BY, Jiang YP, Nie J, Liu W, Liu Q, Dai H (2013) Neopterin negatively regulates expression of ABCA1 and ABCG1 by the LXR α signaling pathway in THP-1 macrophage-derived foam cells. *Mol Cell Biochem* 379:123–131
10. Lee J, Hong EM, Koh DH, Choi MH, Jang HJ, Kae SH, Choi HS (2010) HMG-CoA reductase inhibitors (statins) activate expression of PPAR α /PPAR γ and ABCA1 in cultured gallbladder epithelial cells. *Dig Dis Sci* 55:292–299
11. Lee SM, Moon J, Cho Y, Chung JH, Shin MJ (2013) Quercetin up-regulates expressions of peroxisome proliferator-activated receptor γ , liver X receptor α , and ATP binding cassette transporter A1 genes and increases cholesterol efflux in human macrophage cell line. *Nutr Res* 33:136–143

12. Xu X, Li Q, Pang L, Huang G, Huang J, Shi M, Sun X, Wang Y (2013) Arctigenin promotes cholesterol efflux from THP-1 macrophages through PPAR- γ /LXR- α signaling pathway. *Biochem Biophys Res Commun* 441:321–326
13. Goldwasser J, Cohen PY, Yang E, Balaguer P, Yarmush ML, Nahmias Y (2010) Transcriptional regulation of human and rat hepatic lipid metabolism by the grapefruit flavonoid naringenin: role of PPAR α , PPAR γ and LXR α . *PLoS One* 5:e12399
14. Ishibashi M, Filomenko R, Rébé C, Chevriaux A, Varin A, Derangère V, Bessède G, Gambert P, Lagrost L, Masson D (2013) Knock-down of the oxysterol receptor LXR α impairs cholesterol efflux in human primary macrophages: lack of compensation by LXR β activation. *Biochem Pharmacol* 86:122–129
15. Li L, Jiang J, Wang L, Zhong T, Chen B, Zhan S, Zhang H, Du L (2013) Expression patterns of peroxisome proliferator-activated receptor gamma 1 versus gamma 2, and their association with intramuscular fat in goat tissues. *Gene* 528:195–200
16. Okunuki Y, Usui Y, Nakagawa H, Tajima K, Matsuda R, Ueda S, Hattori T, Kezuka T, Goto H (2013) Peroxisome proliferator-activated receptor- γ agonist pioglitazone suppresses experimental autoimmune uveitis. *Exp Eye Res* 116:291–297
17. Wang JM, Wang D, Tan YY, Zhao G, Ji ZL (2014) 22(R)-hydroxycholesterol and pioglitazone synergistically decrease cholesterol ester via the PPAR γ -LXR α -ABCA1 pathway in cholesterosis of the gallbladder. *Biochem Biophys Res Commun* 447:152–157
18. Zhao B, Song J, St Clair RW, Ghosh S (2007) Stable overexpression of human macrophage cholesteryl ester hydrolase results in enhanced free cholesterol efflux from human THP1 macrophages. *Am J Physiol Cell Physiol* 292:C405–C412
19. Hirakata M, Tozawa R, Imura Y, Sugiyama Y (2004) Comparison of the effects of pioglitazone and rosiglitazone on macrophage foam cell formation. *Biochem Biophys Res Commun* 323:782–788
20. Ghosh S, Natarajan R (2001) Cloning of the human cholesteryl ester hydrolase promoter: identification of functional peroxisomal proliferator-activated receptor responsive elements. *Biochem Biophys Res Commun* 284:1065–1070
21. Cabrero A, Cubero M, Llaverías G, Jové M, Planavila A, Alegret M, Sánchez R, Laguna JC, Carrera MV (2003) Differential effects of peroxisome proliferator-activated receptor activators on the mRNA levels of genes involved in lipid metabolism in primary human monocyte-derived macrophages. *Metabolism* 52:652–657
22. Watanabe K, Sakurai K, Tsuchiya Y, Yamazoe Y, Yoshinari K (2013) Dual roles of nuclear receptor liver X receptor α (LXR α) in the CYP3A4 expression in human hepatocytes as a positive and negative regulator. *Biochem Pharmacol* 86:428–436
23. Ohara K, Wakabayashi H, Taniguchi Y, Shindo K, Yajima H, Yoshida A (2013) Quercetin-3-O-glucuronide induces ABCA1 expression by LXR α activation in murine macrophages. *Biochem Biophys Res Commun* 441:929–934
24. Huang CX, Zhang YL (2013) The target of regulating the ATP-binding cassette A1 protein (ABCA1): promoting ABCA1-mediated cholesterol efflux in different cells. *Curr Pharm Biotechnol* 14:623–631
25. Jun HJ, Hoang MH, Yeo SK, Jia Y, Lee SJ (2013) Induction of ABCA1 and ABCG1 expression by the liver X receptor modulator cineole in macrophages. *Bioorg Med Chem Lett* 23:579–583
26. Kannisto K, Gåfvels M, Jiang ZY, Slätis K, Hu X, Jorns C, Steffensen KR, Eggertsen G (2014) LXR driven induction of HDL-cholesterol is independent of intestinal cholesterol absorption and ABCA1 protein expression. *Lipids* 49:71–83
27. Ignatova ID, Angdisen J, Moran E, Schulman IG (2013) Differential regulation of gene expression by LXRs in response to macrophage cholesterol loading. *Mol Endocrinol* 27:1036–1047