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22(R)-hydroxycholesterol and pioglitazone synergistically decrease cholesterol ester via the PPARγ–LXRα–ABCA1 pathway in cholesterosis of the gallbladder

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ABSTRACT

Cholesterosis is a disease of cholesterol metabolism characterized by the presence of excessive lipid droplets in the cytoplasm. These lipid droplets are mainly composed of cholesterol esters derived from free cholesterol. The removal of excess cholesterol from gallbladder epithelial cells (GBECs) is very important for the maintenance of intracellular cholesterol homeostasis and the preservation of gallbladder function. Several lines of evidence have indicated that the activation of either peroxisome proliferator-activated receptor gamma (PPARγ) or liver X receptor α (LXRα) relates to cholesterol efflux. While pioglitazone can regulate the activation of PPARγ, 22(R)-hydroxycholesterol can activate LXRα and is a metabolic intermediate in the biosynthesis of steroid hormones. However, the effect of 22(R)-hydroxycholesterol in combination with pioglitazone on cholesterosis of the gallbladder is unclear. GBECs were treated with pioglitazone, 22(R)-hydroxycholesterol or PPARγ siRNA followed by Western blot analysis for ATP-binding cassette transporter A1 (ABCA1), PPARγ and LXRα. Cholesterol efflux to apoA-I was determined, and Oil Red O staining was performed to monitor variations in lipid levels in treated GBECs. Our data showed that 22(R)-hydroxycholesterol can modestly up-regulate LXRα while simultaneously increasing ABCA1 by 56%. The combination of 22(R)-hydroxycholesterol and pioglitazone resulted in a 3.64-fold increase in ABCA1 expression and a high rate of cholesterol efflux. Oil Red O staining showed an obvious reduction in the lipid droplets associated with cholesterosis in GBECs. In conclusion, the present findings indicate that the anti-lipid deposition action of 22(R)-hydroxycholesterol combined with pioglitazone involves the activation of the PPARγ–LXRα–ABCA1 pathway, increased ABCA1 expression and the efflux of cholesterol from GBECs. Thus, 22(R)-hydroxycholesterol synergistically combined with pioglitazone to produce a remarkable effect on lipid deposition in cholesterosis GBECs.

1. Introduction

While cholecystectomy is the classic method for treating the cholesterol metabolic disease cholesterosis, this procedure can result in numerous postoperative complications that decrease the overall quality of life. The pathological hallmark of cholesterosis is the excessive accumulation of lipid droplets [1]. These lipid droplets are mainly composed of cholesterol esters (CEs) that were derived from excess free cholesterol (FC) [2]. Removal of CEs from gallbladder epithelial cells (GBECs) is very important for the maintenance of intracellular cholesterol homeostasis and the preservation of gallbladder function. FC that is effluxed from cells binds to lipid-poor apolipoprotein A-I (apoA-I) and is then transported to the liver where it is subsequently transformed into bile to maintain whole-body cholesterol homeostasis. This process is referred to as reverse cholesterol transport (RCT) [3].

ATP-binding cassette transporter A1 (ABCA1) can transport FC out of cells, allowing it to be bound by either apoA-I or HDL [4,5]. As a member of the ABC superfamily, ABCA1 is expressed in numerous human organs, including the liver, lungs and small intestine [6]. In GBECs, ABCA1 is localized to the basolateral plasma membrane. ABCA1 expression is regulated by liver X receptor α (LXRα) [7,8], which is itself regulated by peroxisome proliferator-activated receptor gamma (PPARγ) [9]. PPARγ and LXRα are members of the nuclear receptor superfamily and can influence cholesterol transport by inducing the expression of ABCA1 [10].

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PPARγ can be regulated by pioglitazone, which affects blood glucose as well as lipids [11]. LXRα can be activated by 22(R)-hydroxycholesterol, a metabolic intermediate in the biosynthesis of steroid hormones from cholesterol that is involved in the regulation of lipid homeostasis [12]. Several lines of evidence have indicated that ligand-mediated activation of PPARγ and LXRα influences cholesterol efflux [10]. However, the synergistic effect of 22(R)-hydroxycholesterol and pioglitazone on ABCA1 expression and cholesterol efflux in cholesterosis GBECs is unclear.

In this study, we investigated the possible synergistic effects of 22(R)-hydroxycholesterol and pioglitazone on ABCA1 through the PPARγ–LXRα–ABCA1 pathway. Our *ex vivo* experiments, in which cholesterosis GBECs were treated with pioglitazone and/or 22(R)-hydroxycholesterol, showed that the synergistic effect was sufficient to decrease the deposition of CE. These observations suggest that cholesterosis may be effectively treated by this drug regimen rather than by cholecystectomy.

2. Materials and methods

2.1. Materials

Pioglitazone hydrochloride was obtained from Cayman Chemicals (USA). 22(R)-hydroxycholesterol and piperine were 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). β-Actin was obtained from Santa Cruz Biotechnology (USA).

2.2. Cell culture

Human cholesterosis gallbladders were isolated during cholecystectomies. Gallbladders obtained from pancreaticoduodenectomies were designated as normal. 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 mucous membranes were incubated with 0.25% collagenase type IV for 20 min. GBECs were then collected, washed twice with DMEM and seeded in 25 cm culture plates containing 10 ml DMEM supplemented with 15% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C with 5% CO_2, and the medium was changed every 3 days.

2.3. Cell viability assay (MTT)

GBECs were treated with 10 μM 22(R)-hydroxycholesterol and/or 1 μM pioglitazone for 24 h. Cell viability was determined using the MTT assay.

2.4. Western blot analyses

GBECs were cultured in DMEM containing 15% FBS and treated with 10 μM 22(R)-hydroxycholesterol and/or 1 μM pioglitazone. The cells were then washed with PBS, harvested, and lysed in buffer containing a protease inhibitor cocktail. Cellular protein was extracted and subjected to gel electrophoresis, after which the proteins were transferred from the gel onto a polyvinylidene difluoride membrane in buffer for 1 h at room temperature. Residual binding sites were blocked and the blots were incubated with the following antibodies in tris-buffered saline: monoclonal PPARγ antibody (1:800 dilution, sc-7273, Santa Cruz Biotechnology, USA), LXRα-specific antibody (1:400 dilution, sc-1202, Santa Cruz Biotechnology, USA), ABCA1-specific antibody (1:400 dilution, 21676, Signalway antibody Co., Ltd., USA) and monoclonal β-actin antibody (1:4000 dilution). The membranes were then washed with PBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) in PBST for 20 min. The membrane was then washed and incubated with WB detection kit for 3 min, followed by visualization by autoradiography. β-Actin was used as an internal control. Proteins were quantified using an image analysis software program.

2.5. RNA interference

Cells was seeded into 6 cm culture plates (10^7 cells per well) containing 5 ml DMEM and 15% FBS and incubated for three days. On day 4, 200 pmol of PPARγ siRNA (sc-29455 Santa Cruz Biotechnology, USA) or control siRNA (sc-36869 Santa Cruz Biotechnology, USA) was diluted in 500 μl of culture media, while 10 μl of Lipofectamine was added to a separate aliquot of 500 μl of culture media, according to the manufacturer's instructions. After 5 min, both dilutions were mixed gently for 20 min at room temperature to form a siRNA mixture. For each culture plate, 1 ml of siRNA mixture was added, and the cells were incubated at 37 °C for 48 h. The cells were then lysed and subjected to Western blot analysis with anti-PPARγ antibody to analyze the effect of RNA interference on PPARγ expression.

2.6. Luciferase reporter assays

Briefly, in accordance with established protocols (E1910, Promega, USA), 10^7 GBECs in 2 ml of DMEM were incubated with a mixture of 80 μl of Lipofectamine 2000, 40 μg of luciferase reporter construct or control vector, and 10 μg of CMV-β-galactosidase plasmid as an internal control at 37 °C for 10 h. The cells were then harvested, washed twice and re-cultured in DMEM. Cell extracts were collected after 24 h, and luciferase activity was determined using the Dual-Luciferase reporter assay system.

2.7. Cellular lipid efflux

Normal GBECs were seeded into 96-well culture plates. After incubation with 10 μM NBD-cholesterol for 24 h, the cells were washed twice with the PBS and serum starved overnight. The starved GBECs were cultured in serum-free medium containing 10 μM 22(R)-hydroxycholesterol and/or 1 μM pioglitazone for 12 or 24 h. The media in all wells were then carefully aspirated, and the cells were washed twice with PBS and incubated in PBS containing 15% FBS and 10 μg/ml apoA-I. Finally, fluorescence-labeled cholesterol in the media was collected and measured with a fluorescence microplate reader at wavelengths of 485 and 535 nm (Ex/Em). All data were measured 6 times with DMSO acting as a control.

2.8. Staining of cytoplasmic lipid droplets

After 3 days in culture, cholesterosis GBECs were seeded into 24-well culture plates and washed twice with PBS. The cells were then cultured in a serum-free medium with 10 μM 22(R)-hydroxycholesterol and/or 1 μM pioglitazone for 6 h, 12 h, 24 h or 48 h at which point the media were changed, and the cells were treated with PBS containing 15% FBS and 10 μg/ml apoA-I. The cells were subsequently collected and fixed in 10% formaldehyde in PBS. After 30 min, the cells were washed twice and stained with Oil Red O at 37 °C incubator overnight. After washing 3 times with 75% alcohol, the cells were imaged using a microscope fitted with a digital camera.

2.9. Statistical analysis

All reported outcomes represent at least three separate experiments and are expressed as the means ± SD. Data were analyzed with the Stat View Version 9.2 software package. Differences between the groups were calculated using Student's t test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Pioglitazone and 22(R)-hydroxycholesterol did not affect cell viability

After treatment of GBECs with 1 μM pioglitazone and/or 10 μM 22(R)-hydroxycholesterol for 24 h, cell viability was not remarkably decreased, indicating that pioglitazone and 22(R)-hydroxycholesterol do not inhibit cell viability at this dose.

3.2. The expression of PPARγ, LXRα and ABCA1 in GBECs

The expression of PPARγ, LXRα and ABCA1 in GBECs was not significantly different between normal (Fig. 1A) and cholesterosis (Fig. 1B) gallbladders. However, the protein levels of PPARγ, LXRα and ABCA1 increased dramatically 24 h after treatment with 1 μM pioglitazone (Fig. 1C). After treatment with 10 μM 22(R)-hydroxycholesterol (Fig. 1D), LXRα protein levels increased slightly while ABCA1 protein levels increased by 56%.

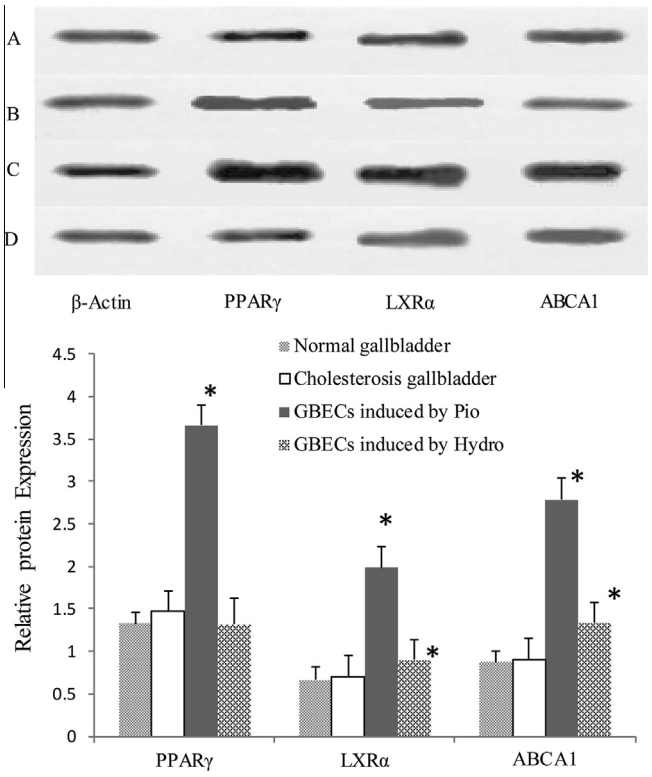

Fig. 1. Protein expression of PPARγ, LXRα and ABCA1 in normal gallbladder (A), cholesterosis gallbladder (B), GBECs treated with 1 μM pioglitazone (Pio) for 24 h (C), GBECs treated with 10 μM 22(R)-hydroxycholesterol (Hydro) for 24 h (D). There is no significant difference between normal gallbladder and cholesterosis gallbladder. PPARγ, LXRα and ABCA1 protein levels increased remarkably in response to pioglitazone. Treatment with 10 μM 22(R)-hydroxycholesterol for 24 h mildly increased the levels of LXRα protein, but increased ABCA1 levels by 56%. $^{*}P < 0.05$ for student's t-test.

3.3. A PPARγ–LXRα–ABCA1 pathway participated in the regulation of ABCA1 in GBECs

To further confirm the role of the PPARγ–LXRα–ABCA1 pathway, we made use of PPARγ siRNA. The introduction of PPARγ siRNA to GBECs decreased the expression of PPARγ, LXRα and ABCA1, while pioglitazone effectively increased the expression of PPARγ, LXRα and ABCA1. Additionally, treatment of GBECs with either 22(R)-hydroxycholesterol or piperine (a LXRα antagonist) increased or decreased the expression of LXRα and ABCA1, respectively, without affecting PPARγ expression. This indicated that ABCA1 is regulated downstream of PPARγ and LXRα. As shown in Fig. 2C, combined treatment with 22(R)-hydroxycholesterol and pioglitazone effectively increased the levels of ABCA1. Either the combination of pioglitazone and piperine or PPARγ siRNA and 22(R)-hydroxycholesterol resulted in lower levels of ABCA1 protein than pioglitazone alone.

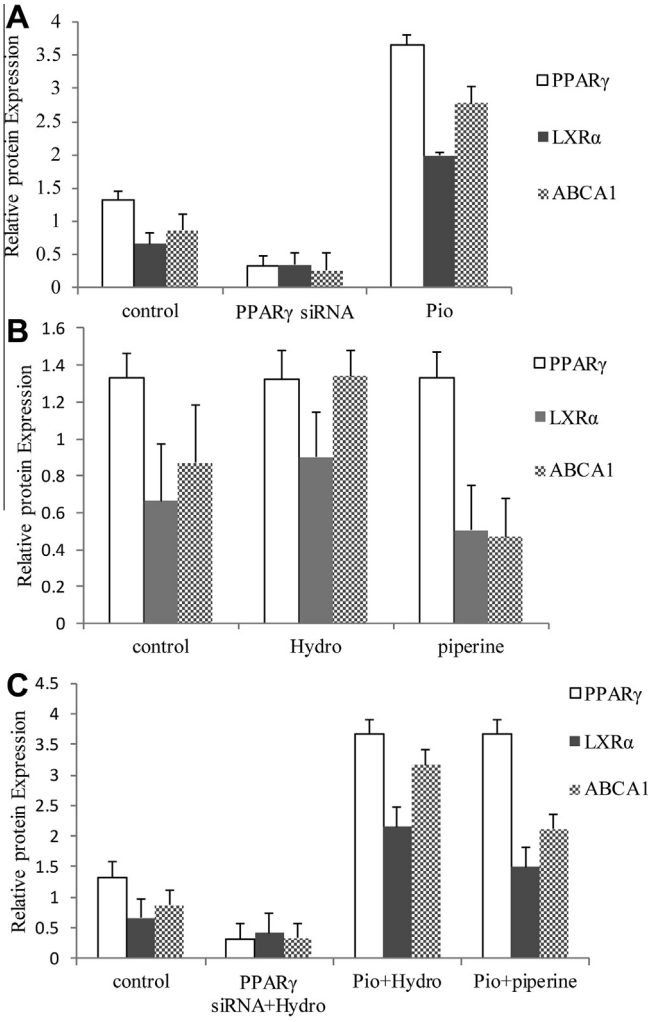

Fig. 2. The PPARγ–LXRα–ABCA1 pathway participated in the regulation of ABCA1 in GBECs. The introduction of PPARγ siRNA decreased the expression of PPARγ, LXRα and ABCA1. Treatment with 1 μM pioglitazone (Pio) effectively increased the expression of PPARγ, LXRα and ABCA1. Treatment of GBECs with 10 μM 22(R)-hydroxycholesterol (Hydro) or 10 μM piperine increased or decreased the expression of LXRα and ABCA1 without affecting PPARγ. Treatment with either 1 μM pioglitazone in combination with 10 μM piperine or PPARγ siRNA in combination with 10 μM 22(R)-hydroxycholesterol resulted in increased ABCA1 protein, although this was lower than that induced by 1 μM pioglitazone alone. Treatment with 10 μM 22(R)-hydroxycholesterol combined with 1 μM pioglitazone resulted in the highest level of ABCA1 protein expression. $^{*}P < 0.05$ for student's t-test.

3.4. Effect of pioglitazone and/or 22(R)-hydroxycholesterol on ABCA1 in GBECs

In our experiments, the level of ABCA1 protein induced by pioglitazone, 22(R)-hydroxycholesterol, or pioglitazone with 22(R)-hydroxycholesterol was consistently lower at 12 h than at 24 h. ABCA1 expression increased by 56% after treatment with 10 μM 22(R)-hydroxycholesterol for 24 h, while 24 h treatment with 1 μM pioglitazone also caused ABCA1 levels to rise. Finally, incubation with the combination of 10 μM 22(R)-hydroxycholesterol and 1 μM pioglitazone for 24 h increased the level of ABCA1 3.64-fold (Fig. 3A).

3.5. Cholesterol efflux assay

All reagents tested promoted apoA-1 mediated cholesterol efflux from human GBECs in a time-dependent and dose-dependent manner (Fig. 3B). However, treatment with 10 μM 22(R)-hydroxycholesterol did not induce a significant cholesterol efflux at 24 h, with the final concentration of cholesterol in the supernatant increasing by only 13.5% compared with the control group. In contrast, 1 μM pioglitazone significantly improved the cholesterol efflux, and combined treatment with both compounds for 24 h resulted in a final cholesterol concentration of 11.01 mmol/L, which was 10.62-fold greater than that of the 22(R)-hydroxycholesterol only group.

3.6. Visualization of lipid droplets in GBECs

To determine the effect on visible lipid droplets, representative images of intracellular lipid droplets were generated, as shown in Fig. 4. In untreated control GBECs, the cells were observed to be full of lipid droplets, resulting dark pink staining at 200× (Fig. 4A) and 400× (Fig. 4B) magnifications. In the pioglitazone-treated group, the staining intensity decreased significantly, with pink cells still visible at 200× (Fig. 4E). The pigmentation volume was also significantly reduced, resulting in a petal-like shape at 400× magnification (Fig. 4F). In the pioglitazone and 22(R)-hydroxycholesterol combined treatment group, the overall color was more pale (Fig. 4G), and the number of petals was decreased (Fig. 4H). However, the effect of 22(R)-hydroxycholesterol on ABCA1 was slow to develop, with no observable effect until 48 h after initial treatment, at which point cellular color was observed to fade slightly in the 200× (Fig. 4C) and 400× magnification fields (Fig. 4D). These results indicated that 22(R)-hydroxycholesterol and pioglitazone synergistically decrease lipid droplet levels in cholesterosis GBECs.

4. Discussion

Cholesterosis of the gallbladder results from the accumulation of excess cholesterol in the cytoplasm. Therefore, developing methods to promote cholesterol efflux is vital for the treatment of cholesterosis. ABCA1 may function to clear excessive amounts of absorbed cholesterol, thereby altering cholesterol concentrations in GBECs [13,14]. Subsequently, apoA-I binds to the exported cholesterol and transports it back to liver, completing the process of RCT. Modulating the level of ABCA1 in GBECs could potentially promote the export of cellular cholesterol and affect the development of cholesterol-related disease. PPARγ or LXRα, induced by cognate ligands, can up-regulate ABCA1 expression and increase the efflux of excess cholesterol. However, it is unclear whether combinatorial up-regulation of PPARγ and LXRα together would

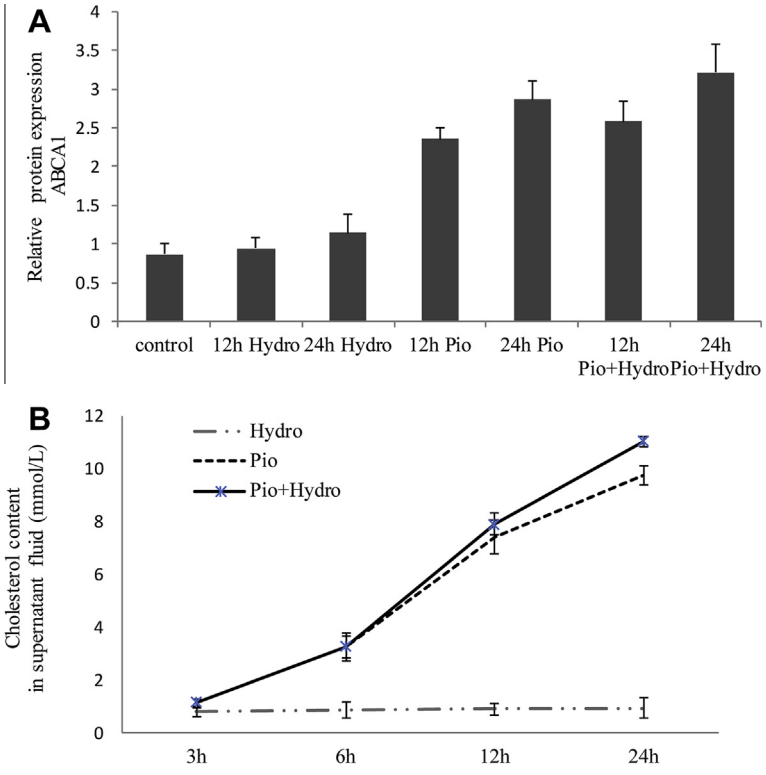

Fig. 3. 22(R)-hydroxycholesterol combined with pioglitazone induced ABCA1 protein expression and cholesterol efflux. (A) ABCA1 expression induced by 1 μM pioglitazone (Pio), 10 μM 22(R)-hydroxycholesterol (Hydro) or 1 μM pioglitazone + 10 μM 22(R)-hydroxycholesterol for 12 h or 24 h. Quantification of the data indicates that ABCA1 levels at 12 h are lower than at 24 h. Treatment with 1 μM pioglitazone and 10 μM 22(R)-hydroxycholesterol for 24 h increased ABCA1 expression approximately 3.64-fold. (B) The concentration of cholesterol in the supernatant was measured. Treatment with 10 μM 22(R)-hydroxycholesterol did not induce a satisfactory cholesterol efflux. Compared to 22(R)-hydroxycholesterol, treatment with 1 μM pioglitazone can significantly improve the cholesterol efflux. However, 24 h treatment with a combination of 22(R)-hydroxycholesterol and pioglitazone resulted in the highest level of cholesterol in the supernatant. $^{*}P < 0.05$ for student's *t*-test.

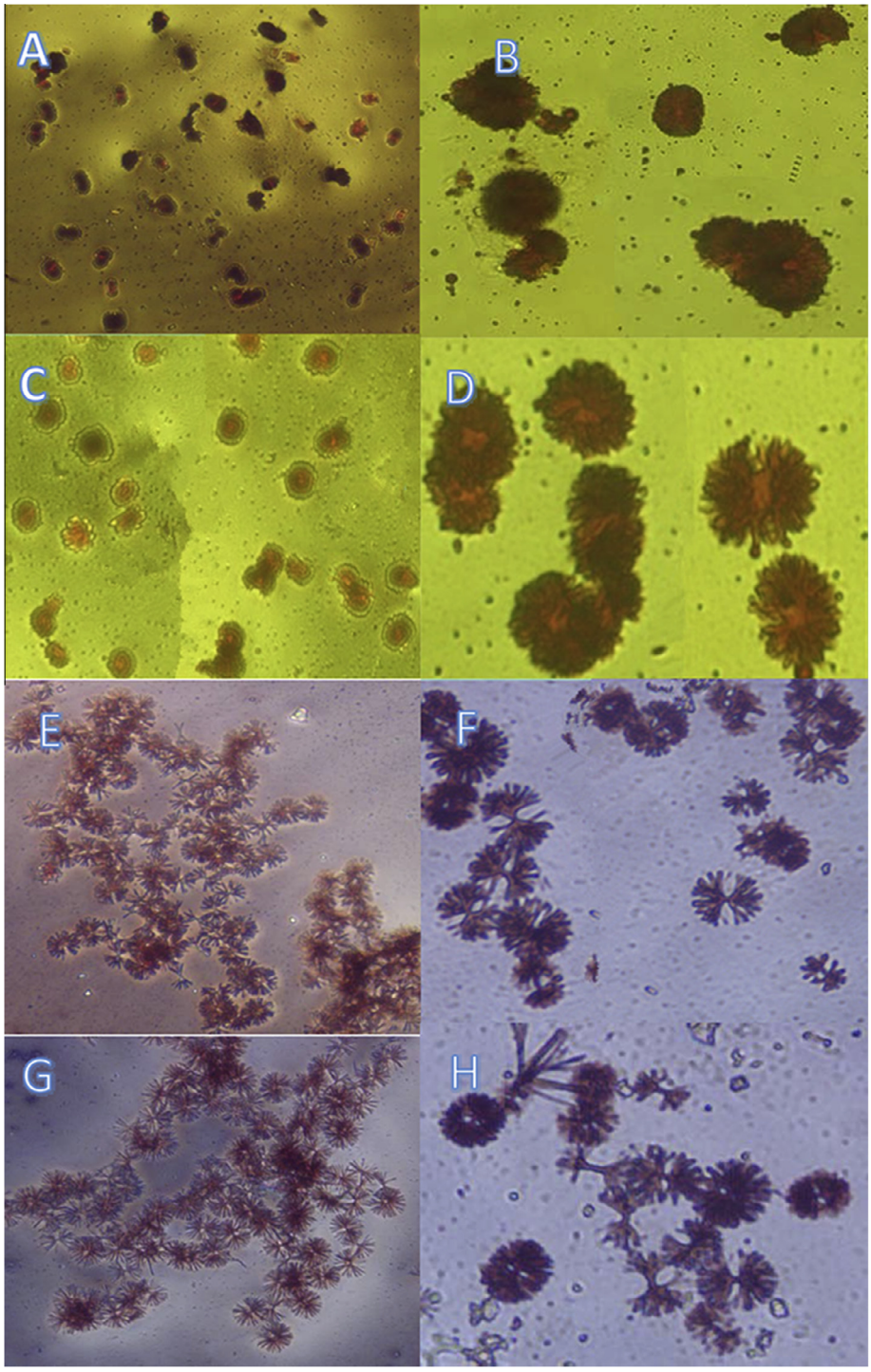

Fig. 4. Representative images of intracellular lipid droplets. Untreated control cells, 200× (A) and 400× (B). Treatment with 1 μM pioglitazone for 24 h (E and F) or 1 μM pioglitazone + 10 μM 22(R)-hydroxycholesterol for 24 h (G and H) significantly reduced the lipid droplets and decreased red coloration. The effect of 10 μM 22(R)-hydroxycholesterol on ABCA1 is slow, with the observable affect not becoming apparent until 48 h (C and D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have a greater effect on ABCA1 expression and the cholesterol efflux in GBECs. In the present study, we demonstrate that pioglitazone and 22(R)-hydroxycholesterol can synergistically up-regulate ABCA1 via the PPARγ–LXRα–ABCA1 pathway, resulting in increased cholesterol efflux in GBECs. Ultimately, 22(R)-hydroxycholesterol and pioglitazone were shown to synergistically reduce lipid droplet levels in cholesterosis GBECs.

It has been previously demonstrated that 1 μM pioglitazone and 10 μM 22(R)-hydroxycholesterol are optimal doses [15]. In our assay, GBECs were treated with 1 μM pioglitazone and/or 10 μM 22(R)-hydroxycholesterol for 24 h. This treatment did not remarkably decrease cell viability, and we therefore used pioglitazone and 22(R)-hydroxycholesterol at these doses for our subsequent experiments. Yoon et al.'s study showed that the expression of ABCA1 in GBECs derived from cholesterol-related diseased gallbladders was slightly higher than in normal gallbladders [16]. In our study, the expression of ABCA1 in cholesterosis gallbladders was similar to that in normal gallbladders. As a physiological activator,

22(R)-hydroxycholesterol increased the levels of LXRα and ABCA1. At physiological concentrations, 22(R)-hydroxycholesterol promotes the transcription of LXRα and is an important factor for the maintenance of cholesterol balance [17]. The concentration of cholesterol in bile varies widely and is often beyond the control of any physiological factor. In cholesterosis, as in other cholesterol-related diseases of the gallbladder, the physiological activity of 22(R)-hydroxycholesterol alone is not sufficient to completely restore cholesterol homeostasis. It is therefore necessary to develop more effective interventions.

An increase or decrease in PPARγ levels resulted in higher or lower expression of LXRα and ABCA1, respectively. These increased or decreased levels of LXRα resulted in similar changes in the expression of ABCA1, but had no effect on PPARγ. We confirmed the existence of the PPARγ–LXRα–ABCA1 pathway in GBECs, and our data indicates that this PPARγ–LXRα–ABCA1 pathway functions in the regulation of ABCA1. Our data showed that pioglitazone alone had a greater ability to increase ABCA1 levels than any other factor (Fig. 2). This may suggest that PPARγ plays a major role in the regulation of the pathway and that LXRα is a minor factor. Previous studies have reported that 22(R)-hydroxycholesterol had an additive effect on ABCA1 expression [15], which is consistent with our results. However, the relationship between PPARγ and LXRα is complex. In THP-1-derived macrophages, PPARγ upregulates LXRα, resulting in increased ABCA1 expression [18,19]. In human hepatocytes, PPARγ inhibits LXRα expression and negatively regulates the expression of ABCA1 [20]. We suggest a tissue-specific relationship between these factors. In GBECs, PPARγ and LXRα functioned together to up-regulate ABCA1. In our study, pioglitazone treatment significantly increased the expression of PPARγ, LXRα and ABCA1. We speculate that, *in vivo*, 22(R)-hydroxycholesterol combined with pioglitazone could act to regulate the PPARγ–LXRα–ABCA1 pathway to synergistically decrease CEs in cholesterosis patients.

In theory, co-regulation of PPARγ and LXRα should increase ABCA1 to a greater extent than either one individually. In our data, the highest induction of ABCA1 protein was achieved with 22(R)-hydroxycholesterol in combination with pioglitazone. In accordance with the protein levels, 22(R)-hydroxycholesterol treatment induced the lowest cholesterol efflux and 22(R)-hydroxycholesterol in combination with pioglitazone induced the highest. To test this notion further, we examined lipid droplets in cholesterosis GBECs treated with 22(R)-hydroxycholesterol and/or pioglitazone. We observed that lipid droplets were reduced much more quickly in the 22(R)-hydroxycholesterol + pioglitazone treatment group than in 22(R)-hydroxycholesterol group. We suggest that 22(R)-hydroxycholesterol and pioglitazone synergistically decrease the levels of cholesterol ester in cholesterosis GBECs.

In a previous study, lipid droplets in human monocytes/macrophages were observed as bright punctate spots [21]. In GBECs, we observed that the stained lipid droplets formed spindle-shaped petals. As the cholesterol levels increased, the gaps between the petals reduced gradually until the petals fused together, a process that was effectively reversed by lipid efflux. When cholesterosis GBECs were treated with pioglitazone or pioglitazone + 22(R)-hydroxycholesterol, we observed that the rich red staining color became weaker, a gap began to appear, and lipid accumulation reduced gradually.

Our study was limited in regards to animal models and *in vivo* data, but focused instead on primary cells from cholesterosis gallbladders, partly compensating for this issue. 22(R)-hydroxycholesterol is only one type physiological factor, and it may be that other physiological factors have a stronger ability to regulate LXRα. However, the potency of any physiological factor is limited and may require the intervention of an exogenous factor.

In conclusion, the present findings clearly indicate that the anti-lipid deposition activity of 10 μM 22(R)-hydroxycholesterol in combination with 1 μM pioglitazone in GBECs involves the activation of the PPARγ–LXRα–ABCA1 pathway, the up-regulation of ABCA1, cholesterol efflux and the abatement of lipid droplets. Additionally, the synergistic effect of 22(R)-hydroxycholesterol and pioglitazone on lipid deposition in GBECs suggests that cholecystectomy maybe not the only treatment option for cholesterosis and that a drug regimen may be a more appropriate treatment.

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