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# Effects of ezetimibe on Niemann Pick C1 Like 1 expression in human hepatocyte-like C3A cells

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## Abstract

Niemann-Pick C1-Like 1 (NPC1L1) is expressed in human liver, but its mechanism of action and how it is regulated in this tissue is not well-understood. Herein, we decided to examine the effects of the inhibitor of NPC1L1, ezetimibe, in the presence and absence of a low cholesterol medium (MITO+ medium), on the expression of NPC1L1 in the human hepatocyte-like C3A cell line. It was demonstrated that ezetimibe and MITO+ medium were able to independently decrease NPC1L1 protein expression while increased the mRNA expression of this transporter. Treating with ezetimibe and MITO+ medium together additively decreased NPC1L1 protein levels, but their effects on NPC1L1 mRNA expression were counteracting. Cycloheximide studies showed that these treatments enhanced the stability of the NPC1L1 protein in an additive manner, supporting the possibility that the translation efficiency of the NPC1L1 mRNA was decreased under these conditions or that the factors required to degrade the NPC1L1 transporter were inhibited by the presence of cycloheximide. In addition, the data confirmed that growing the cells in flasks coated with poly-D-lysine (should induce polarization) was required for maximum expression levels of NPC1L1 protein but not for localization of this transporter to the apical membrane. The information derived from this research is critical to our understanding of the role of hepatic NPC1L1 in removing cholesterol from the bile and in cholesterol homeostasis in general.

**Keywords:** NPC1L1; Ezetimibe; Cholesterol; Hepatic cells

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## Introduction

Hypercholesterolemia is associated with an enhanced risk of atherosclerosis leading to coronary heart diseases and stroke, two of the principal causes of death and disability in the United States [1-3]. The mortality due to atherosclerotic diseases increases when total serum cholesterol levels rise above 200 mg/dl [1-3]. Plasma cholesterol levels are greatly depending on the rate of cholesterol absorption in the lumen of the small intestine [4-7]. It has been determined that about 50% of the total cholesterol that enters the intestine (2/3 from the bile and 1/3 from the diet) is absorbed [4-7]. In fact, it has been shown that inhibiting intestinal cholesterol absorption using the drug ezetimibe is able to significantly reduce total and Low Density Lipoprotein (LDL) cholesterol levels in the serum of dyslipidemic patients [8-14].

Several studies have shown that the molecular target for ezetimibe is the Niemann-Pick C1-like 1 (NPC1L1) transporter [8-14]. Inactivation or inhibition of intestinal NPC1L1 in mice results in significant decreases in intestinal sterol absorption and serum cholesterol levels, and prevents the development of atherosclerosis [15-19]. Thus far, several patients carrying rare NPC1L1 variants, that decrease intestinal cholesterol absorption, have lower LDL levels than non-carriers, further confirming the association between intestinal cholesterol absorption and plasma

cholesterol levels [19-21]. On the other hand, several single polymorphisms within the NPC1L1 gene have been identified as predictors of cardiovascular risk in coronary patients [22, 23].

NPC1L1 is a membrane bound protein that is primarily expressed in the jejunum of the small intestine where the cholesterol is absorbed [24, 25]. Several studies have established that, in human and species such as monkeys, pigs, dogs, and rats, NPC1L1 is also expressed in the liver [6, 26, 27], the most important organ where most metabolic processes take place. In hepatocytes, NPC1L1 has been implicated in reducing biliary cholesterol levels, and as a result, it enhances cholesterol levels in the blood [6, 27]. Due to its function in liver cells, NPC1L1 appears to be involved in diseases such obesity, diabetes, nonalcoholic fatty liver disease, and gallstone formation [11, 28-32]. It is currently believed that the main site of NPC1L1 expression in liver cells is the canalicular/apical membrane [6, 17, 24].

Even though the findings mentioned above demonstrate that ezetimibe is able to decrease serum cholesterol levels, the clinical significance of treating for a long-term with ezetimibe was under controversy for a while. Several clinical trials reported that ezetimibe does not clinically improve the incidence of major coronary events, and even made some risk factors, such as artery wall thickness, worse [33-35]. One possible explanation for the disappointing results was that the clinical trials did not last long enough to allow ezetimibe to do its work [33-35]. Another explanation was that, as demonstrated for other drugs, the reduction of cholesterol does not always lead to a decrease in the incidence of atherosclerosis [33-35]. However, the results of the IMProved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) clearly confirmed ezetimibe efficacy, safety, and role in preventing cardiovascular diseases [36, 37]. However, additional information is still missing in order to understand the role of NPC1L1 in the liver. The aim of this study was to use ezetimibe as a tool to examine the regulatory mechanisms of NPC1L1 expression in hepatic cells.

## Materials and Methods

### Materials

The human hepatocyte-like C3A and the hepatocellular carcinoma HepG2 cell lines were purchased from the American Type Culture Collection (Manassas, VA). Ezetimibe was obtained from Merck (Whitehouse Station, NJ). Low glucose (1 g/L) Dulbecco's modified Eagle's medium (LG-DMEM), fetal bovine serum (FBS), RIPA buffer, the BCA protein assay kit, precast 4-20% SDS-PAGE, the SuperSignal West Pico chemiluminescence substrate, cycloheximide, poly-D-lysine, Corning™ MITO+ Serum Extender, the reverse transcriptase system, and SYBR green PCR Master Mix were acquired from Thermo Fisher Scientific (Pittsburg, PA). TRI Reagent was obtained from Molecular Research Center (Cincinnati, OH). The Turbo DNA-free kit was purchased from Ambion (Austin, TX). The specific antibody against NPC1L1 was from Novus Biologicals (Littleton, CO). The actin specific antibody and all the HRP-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cell culture

Cells were maintained between experiments in LG-DMEM medium supplemented with 10% FBS and antibiotics (Regular medium), at 37ºC, under humidified atmosphere and 5% carbon dioxide. For the experiments, cells were treated with either Regular medium or MITO+ medium for 4-6 days, with medium changes every other day. The MITO+ medium was prepared as previously described [38]. For some experiments, cell polarization was induced using poly-D-lysine as previously described [39]. Briefly, coating solution was prepared by dissolving poly-D-lysine at the concentration of 50 µg/mL in serum-free medium. To coat, 5 ml of the poly-D-lysine solution (50 µg/ml) was added to each T75 flask. Flasks were gently rotated to make sure all the coating solution was covering the bottom surface of the flask. Coating was carried out for 1 hour at room temperature. After incubation, the coating solution was removed, and flasks were washed twice in serum-free medium. Cells were plated as usual. Treatment with 6.1 µM of ezetimibe was carried for 4-6 days in the two media. This concentration of ezetimibe is within the range of ezetimibe concentrations used in a previous publication [40]. Furthermore, this dose of ezetimibe was confirmed in a dose-response pilot study examining the viability of the cells in the presence of this drug employing Vybrant MTT Cell Proliferation Assay from Invitrogen. Incubating with 6.1 µM ezetimibe for 6 days caused reductions in the viability of C3A (8.2%; p=0.1734) and HepG2 (7.7%; p=0.0792) cells (data not shown). For the cycloheximide experiments, cells treated with and without ezetimibe in the different media were incubated with 100 µM of cycloheximide

for 5.5 hours. This dose of cycloheximide was sufficient to achieve 95% inhibition of protein synthesis (data not shown).

### Preparation of protein samples

Total cell lysates were prepared using ice-cold RIPA buffer as previously described [41]. Plasma membranes were prepared as a modification of the method developed by Ellis *et al.* [42]. Briefly, cells were washed twice with ice-cold PBS and then scrapped into cold-PBS containing protease inhibitors. Cells were sedimented at 500 x g for 10 min. After centrifugation, the cell pellet was homogenized in a buffer containing 150 mM NaCl, 5 mM DTT, 5 mM EDTA, 25 mM Tris-HCl, pH 7.4, and protease inhibitors. The homogenate was then centrifuged at 500 x g for 5 minutes to remove unbroken cells, nuclei and debris. The supernatant was saved as "total plasma membranes" or processed to separated apical and basolateral membranes. To separate the two types of membranes, calcium chloride was added to a final concentration of 10 mM. After mixing, membrane fractions were incubated for 30 minutes on ice. Centrifugation was carried out at 15,000 x g for 15 minutes. Supernatant was saved as "apical membranes". The pellet was resuspended in homogenization buffer and saved as "basolateral membranes". Concentrations of protein samples were determined using the BCA protein assay. Proteins were stored at -80ºC until needed for Western blotting analysis.

### Western Blotting Analysis

Electrophoresis of protein samples on precast 4-20% SDS-PAGEs, electroblotting onto nitrocellulose membranes, Western blotting analysis, and imaging/quantitation of the blots were performed as described in another publication [41]. Primary antibodies used herein were NPC1L1 (diluted 1:500) and actin (diluted 1:250; internal control).

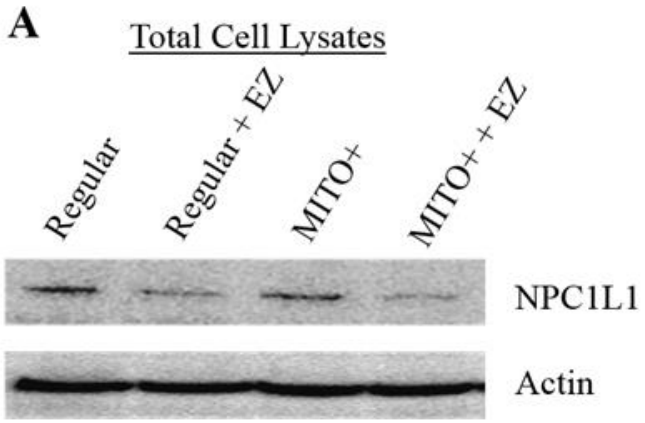
### RNA preparation and quantitative real-time PCR

Total RNA was prepared using TRI Reagent [43]. After DNase I treatment and reverse transcription, real-time PCR analysis was performed using 100 ng of the produced ssDNA, the SYBR green PCR Master Mix, and the AB real-time PCR system as previously described [41]. Human NPC1L1 and 18s rRNAa specific primers were obtained from SA Biosciences (Frederick, MD), which amplified fragments of 166 and 100 bp, respectively. The parameters for the PCR reactions were: denaturation at 95ºC for 10 minutes, followed by 45 cycles of denaturation at 95ºC for 72ºC for 30 seconds. Quantitation was done using the Comparative CT method [44].

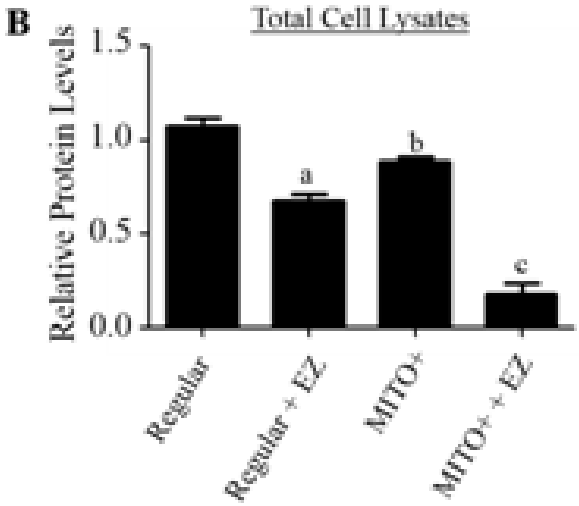
## Results

We started by determining whether ezetimibe affects the protein expression of the hepatic NPC1L1. For this, human hepatocyte-like C3A cells grown in either Regular or MITO+ medium were treated with 6.1 μM of ezetimibe for 6 days. Media supplemented with and without ezetimibe was replaced every two days. Cells were harvested on the 6$^{th}$ day after the initial treatment and used for lysate preparation. Western blotting analysis for NPC1L1 was then carried out. Ezetimibe reduced the expression of the NPC1L1 protein by 37.4 ($p=0.0002$) and 80% ($p<0.0001$) in both Regular and MITO+ media, respectively, as compared to the Regular medium without ezetimibe (Figure 1A & 1B). Incubating with MITO+ medium alone caused a 17.5% ($p<0.0001$) reduction in NPC1L1 protein expression levels (Figure 1A & 1B).

**Figure 1**: Effects of ezetimibe (EZ) on protein expression of NPC1L1 in human hepatocyte-like C3A cells. Cells were grown in the indicated media (Regular or MITO+) and treated with and without 6.1 μM EZ for 6 days. (A) Total cell lysates were prepared and analyzed by Western blotting. Typical blots for NPC1L1 and actin are shown.



(B) Blots were quantitated using the Kodak imaging software, and the NPC1L1 signal was corrected using the internal control actin. Data (mean ± SEM) are from n=5 for each condition. "a" refers to p=0.0002, when comparing Regular medium ± EZ. "b" refers to p=0.0056, when comparing Regular and MITO+ media in the absence of EZ. "c" refers to p<0.0001, when comparing MITO+ medium ± ezetimibe.




The next experiment was performed to find out whether similar results could be obtained using plasma membrane fractions. Total membrane fractions were prepared from cells treated as described above and analyzed by Western blotting analysis. Once again, the presence of ezetimibe and/or MITO+ medium caused a reduction in the expression levels of NPC1L1 protein (Fig. 1C). Ezetimibe reduced NPC1L1 protein levels by 30 and 88% in both Regular and MITO+ media, respectively. The MITO+ medium alone caused a 41% reduction in NPC1L1 protein expression levels (Fig. 1C). These experiments demonstrated that the effects of ezetimibe and MITO+ medium on NPC1L1 protein expression were additive. It is important to point out that although they were less pronounced, similar results were seen in HepG2 cells (Fig. 2), the cells from which C3A are derived [45].

(C) Total plasma membranes were prepared and analyzed by Western blotting. Typical Western blots for NPC1L1 and actin are shown. The data under the blot are represented as relative NPC1L1 protein levels (mean ± SEM) where the normalized value for the sample grown in the presence of Regular medium was set to 1.0. This experiment was repeated four times.

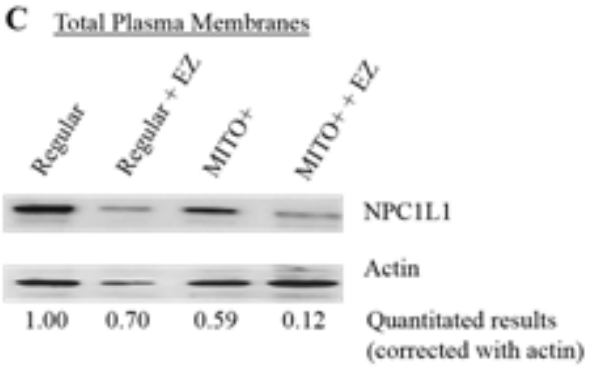
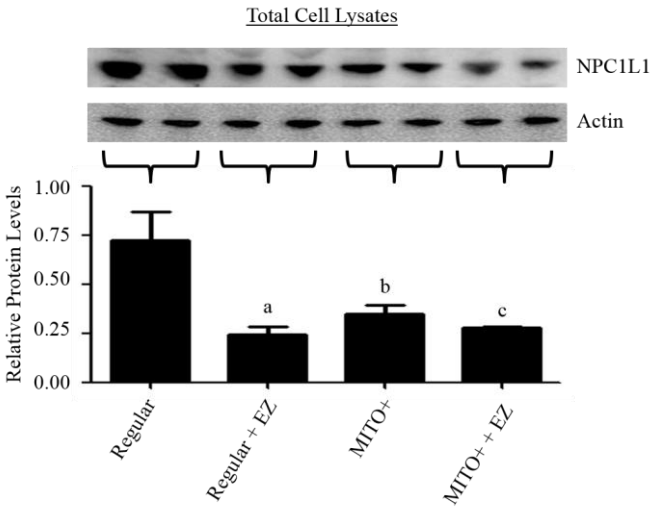
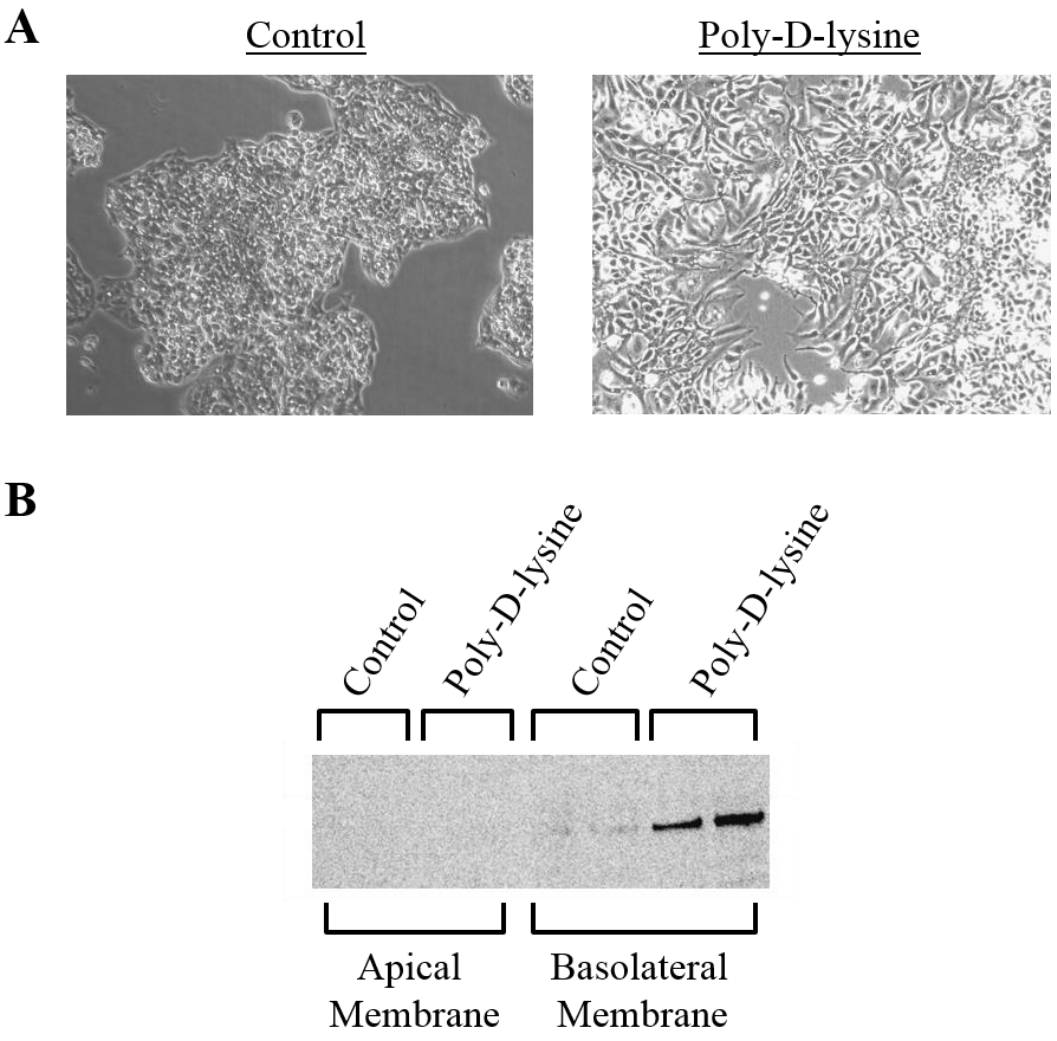



**Figure 2:** Effects of ezetimibe (EZ) on protein expression of NPC1L1 in human hepatocellular carcinoma HepG2 cells. Cells were treated as indicated in Fig. 1. Total cell lysates were prepared and analyzed by Western blotting. Typical blots for NPC1L1 and actin are shown. Blots were quantitated and the data (mean ± SEM) from n=4 per each condition are shown. "a" refers to p=0.0348, when comparing Regular medium ± EZ. "b" refers to p=0.0718 (not significant), when comparing Regular and MITO+ media in the absence of EZ. "c" refers to p=0.0185, when comparing MITO+ medium ± ezetimibe.



Since it is believed that the main site of NPC1L1 expression in hepatocytes is the canalicular membrane [6, 17, 24], membrane fractions were prepared from non-polarized (control) and poly-D-lysine-induced polarized cells grown in Regular medium. It is important to mention that we did not use any method to confirm the polarity of the cells. However, we observed that the cells spread more evenly in the presence of poly-D-lysine than without (Figure 3A). NPC1L1 protein was detected in the basolateral membranes, but not in the canalicular (apical) membranes (Figure 3B). Similar results were obtained in the presence of MITO+ medium (data not shown). These results indicated that growing the cells in poly-D-lysing (polarization) alone did not affect the plasma membrane localization of NPC1L1. Interestingly, Figure 3B also demonstrates that poly-D-lysine coating enhanced the expression levels of the NPC1L1 protein.

**Figure 3:** Effects of coating plates with poly-D-lysine (should induce polarization) on NPC1L1 protein expression and localization. C3A cells were set in un-coated (control) and poly-D-lysine coated flasks and incubated for 6 days in Regular medium. (A) Pictures were taken after incubating cells for 4 days. (B) Preparation of apical and basolateral plasma membranes was done after 6 days in the media. Western blotting analysis for NPC1L1was then done, and a typical blot is shown. This experiment was repeated four times.



At this point, we decided to determine whether ezetimibe could also affect the mRNA expression of the hepatic NPC1L1. As depicted for C3A cells (Figure 4A), in the Regular medium, ezetimibe enhanced the expression of NPC1L1 mRNA by 1.87-fold (p=0.0411). Incubating C3A cells in the presence of the MITO+ medium also enhanced NPC1L1 mRNA expression by 5.51-fold (p=0.0049; Figure 4A). Although this enhancement in NPC1L1 mRNA with MITO+ medium correlates with the finding that the NPC1L1 gene is controlled by sterol regulatory element binding protein 2 (SREBP-2) [46, 47], these findings do not agree with the protein results shown above. Treating C3A cells with ezetimibe in the presence of MITO+ medium caused a reduction (64%; p=0.0247) in the levels of NPC1L1 mRNA when compared to MITO+ medium alone, but caused an enhancement (2-fold; p=0.1268; not significant) relative to the levels in Regular medium (Figure 4A). Once again, similar results were obtained in the presence of HepG2 cells (Figure 4B). Thus, it appears that there is dissociation between hepatic NPC1L1 mRNA and protein responses to the different treatments.

In an effort to explain this dissociation between NPC1L1 mRNA and protein responses, we performed cycloheximide (CH) experiments. For this, some cells treated with ezetimibe in the presence of the two media, also received 100 µM of CH on the 6$^{th}$ day. To determine the half-life of NPC1L1 in cells treated with Regular medium, incubations with CH were carried out for 0, 2.5, 5, and 24 hours. After plotting the remaining levels of NPC1L1 protein for every time point (compared to "0"), it was determined that the half-life of this transported in cells treated with Regular medium was 4.5 hours (Figure 5A). Then, cells treated with the different conditions for 6 days and then with and without CH for 5.5 hours were used in the preparation of total plasma membrane fractions. Unexpectedly, treating with ezetimibe and MITO+ medium independently stabilized the NPC1L1 protein by 2.45- (p=0.1083) and 2.64-fold (p=0.0095), respectively, as compared to the Regular medium. Maximum

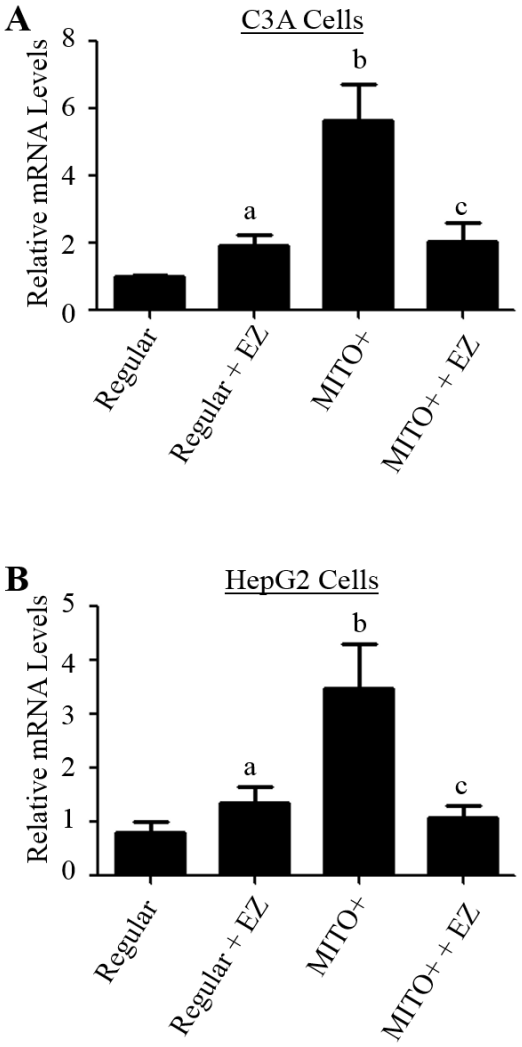



**Figure 4:** Effects of ezetimibe (EZ) on NPC1L1 mRNA expression in C3A and HepG2 cells. Treatments with EZ in the different media were carried out as described in Figure 1. RNA preparation, real-time PCR, and quantitation of the results were done. The data are represented as relative mRNA levels (mean ± SEM) for n=4 in the case of the C3A (A) and for n=3 in the case of the HepG2 (B) cells. (A) "a" refers to p=0.0411, when comparing Regular medium ± EZ. "b" refers to p=0.0049, when comparing Regular and MITO+ media in the absence of EZ. "c" refers to p=0.0247, when comparing MITO+ medium ± ezetimibe. (B) "a" refers to p=0.2134 (not significant), when comparing Regular medium ± EZ. "b" refers to p=0.0353, when comparing Regular and MITO+ media in the absence of EZ. "c" refers to p=0.0495, when comparing MITO+ medium ± EZ.

fold-increase, 12.81 (p=0.004), in the stability of the NPC1L1 protein, was obtained in the presence of both MITO+ medium and ezetimibe (Figure 5B). These findings suggested that MITO+ medium and ezetimibe may reduce the levels of NPC1L1 protein by decreasing the translational efficiency of the NPC1L1 mRNA. However, it might be possible that the protein(s) required degrading NPC1L1 in response to MITO+ medium and ezetimibe may be reduced by the CH treatment giving the impression that there is no degradation of the NPC1L1 protein.

## Discussion

The data reported herein showed that in the presence of Regular medium, ezetimibe decreased the levels of NPC1L1 protein while enhanced mRNA expression for this transporter. In order to explain these results, we were expecting to see an enhancement in the degradation rate of the NPC1L1 protein in hepatic cells, and accordingly, the transcription of this gene would have to be increased resulting in higher mRNA levels in order to compensate for the degraded NPC1L1 protein. Contrary to our expectations, the results established that ezetimibe stabilized the NPC1L1 protein. This finding suggests that ezetimibe reduced the expression of the NPC1L1 protein most likely by decreasing the translational efficiency of the NPC1L1 mRNA or that the protein(s) required to degrade NPC1L1 under these conditions are not synthesized due to the presence of CH. Additional studies are required to confirm these possibilities.

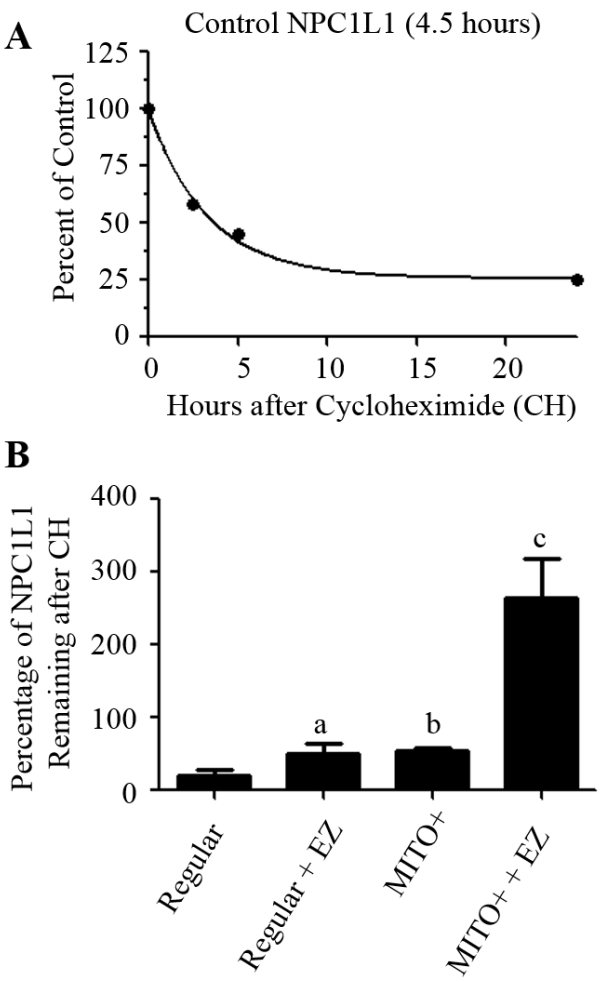



**Figure 5:** Effects of ezetimibe (EZ) and MITO+ medium on the half-life of the NPC1L1 protein in C3A cells. (A) Cells grown in Regular medium for 6 days were treated with 100 µM of cycloheximide (CH) for 0, 2.5, 5, and 24 hours. Protein preparation, Western blotting analysis, and quantitation of the blots were carried out as described above. The data are presented as percentage of NPC1L1 protein remaining after CH (mean ± SEM) relative to the "0" time point. (B) Cells grown in the two media ± EZ for 6 days were treated with CH for 5.5 hours. The data are presented as percentage of NPC1L1 protein remaining after cycloheximide (mean ± SEM) relative to the same treatment without CH. "a" refers to p=0.1083 (not significant), when comparing Regular medium ± EZ. "b" refers to p=0.0095, when comparing Regular and MITO+ media in the absence of EZ. "c" refers to p=0.004, when comparing MITO+ medium ± EZ. These experiments were repeated four times.

Incubating in MITO+ medium, which is characterized by having low levels of cholesterol [38], also decreased NPC1L1 protein expression while increased the transporter mRNA levels and protein stability. The results in the presence of the MITO+ medium were expected considering the role of NPC1L1 in transporting cholesterol back from the bile into the hepatocytes [48]. Since the MITO+ medium does not provide cholesterol to the cell, intracellular cholesterol levels eventually decrease which result in activation of nuclear SREBP-2. This transcription factor is then translocated into the nuclei where transcription of genes involved in cholesterol homeostasis are activated. Among these genes activated by SREBP-2 are the LDL receptor (internalizes LDL-cholesterol from the serum), HMG-CoA reductase (enhances synthesis of cholesterol), and NPC1L1 (internalizes cholesterol from the bile). Increased transcription of the NPC1L1 gene would explain the increase in NPC1L1 mRNA levels seen in our experiments. In fact, transcriptional activation of NPC1L1 by SREBP-2 has been previously demonstrated [49].

Incubating with ezetimibe in the presence of Regular medium may have also caused a reduction in intracellular cholesterol levels that resulted in an up-regulation of the NPC1L1 mRNA, once again, possible mediated by an enhancement in SREBP-2 levels. In agreement with this, an increase in SREBP-2 mRNA and protein expression in response to ezetimibe has been observed in enterocytes [50]. Thus, it is possible that a similar control of NPC1L1 transcription by SREBP-2 due to ezetimibe treatment is occurring in our studies. An intriguing observation from our studies was that in the presence of MITO+ medium, the effects of ezetimibe on NPC1L1 protein expression and protein stability were not only similar but also additive to the effects of the MITO+ medium alone. However, in the case of the mRNA expression, ezetimibe treatment prevented the MITO+ medium-dependent enhancement in NPC1L1 mRNA levels suggesting that when added together, these treatments activate opposite regulatory mechanisms of the transcription of the NPC1L1 gene. Further studies need to be carried out to identify the factors involved in the transcriptional regulation of the NPC1L1 gene in response to the different treatments.

Herein, we were also able to demonstrate that, in our experimental system, NPC1L1 was located at the basolateral membrane and not at the apical membrane as previously reported [6, 17, 24]. These results were unaffected by whether the cells were grown in plates coated with poly-D-lysine (should induce polarization) or not, suggesting that additional factors may be involved in triggering the translocation of NPC1L1 to the apical membrane of hepatocytes. Additional studies are required to identify the factors involved in the transcriptional and post-transcriptional regulation of the NPC1L1 gene by ezetimibe and MITO+ medium. This information is critical in order to identify regulators of NPC1L1, especially in the liver.

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