

## Pravastatin Modulate Niemann-Pick C1-Like 1 and ATP-Binding Cassette G5 and G8 to Influence Intestinal Cholesterol Absorption

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**ABSTRACT - Purpose.** Niemann-Pick C1-like 1 (NPC1L1), ATP-binding cassette (ABC)G5, and ABCG8 mediate intestinal cholesterol absorption. It is unclear whether pravastatin (PR) or ezetimibe (EZ) affect expression of these transporters. We examined the effects of PR and EZ on NPC1L1, ABCG5, and ABCG8 expression in human hepatoma HepG2 cells and the murine small intestine. We also assessed expression of the transcription factors liver X receptor (LXR) $\alpha$ , LXR $\beta$  and sterol regulatory element-binding protein. **Methods.** Transporter mRNA levels were determined in murine small intestines 6 and 24 h after oral PR and EZ administration by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). In PR- and EZ-treated HepG2 cells, transporter and transcription factor mRNA and protein levels were examined by RT-PCR and western blot, respectively. **Results.** Significant decreases in *NPC1L1*, *ABCG5*, and *ABCG8* mRNA expression were observed in the duodenum, but not jejunum and ileum, of mice 24 h after treatment with PR, but not EZ. In HepG2 cells, PR but not EZ treatment for 24 h also significantly decreased NPC1L1 protein and *ABCG5*, and *ABCG8* mRNA expression, while increasing *LXR $\alpha$*  mRNA levels. **Conclusion.** PR but not EZ treatment reduced duodenal cholesterol transporter expression in mice. PR-induced increases in *LXR $\alpha$*  mRNA levels may be involved in attenuation of NPC1L1 expression, subsequently decreasing intestinal cholesterol absorption.

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### INTRODUCTION

Cholesterol homeostasis in the body is maintained by biosynthesis, intestinal absorption, and biliary excretion. Niemann-Pick C1-like 1 (NPC1L1) is expressed in the epithelial cells of the duodenum, jejunum, and proximal ileum, where it mediates intestinal cholesterol and phytosterol absorption (1-3). Statins, such as pravastatin (PR), suppress 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, and regulate hepatic low-density lipoprotein receptors in animals and humans (4). Ezetimibe (EZ) inhibits intestinal cholesterol absorption via NPC1L1 to reduce plasma cholesterol levels (5-7). Thus, NPC1L1 is a therapeutically important molecular target for dyslipidemia, metabolic syndromes, and obesity. Additionally, ATP-binding cassette (ABC)G5 and ABCG8, which are located on the apical membrane of enterocytes, and are regulated by the liver X receptor (LXR) (8, 9), play important roles in cholesterol homeostasis by mediating its efflux (10, 11). Genetic ablation of *ABCG5* and *ABCG8* in mice resulted in disruption of cholesterol homeostasis (12).

When combined, PR and EZ caused a synergistic reduction in plasma cholesterol levels in patients with dyslipidemia (13). However, it is unclear whether PR and EZ affect the expression of transporters involved in cholesterol absorption, such as NPC1L1, ABCG5, and ABCG8. As plasma cholesterol levels could be affected by the intestinal expression and activity of NPC1L1, ABCG5, and ABCG8, it is important to elucidate the effects of PR and EZ on these proteins.

To investigate the effects of PR and EZ on NPC1L1, ABCG5, and ABCG8, we examined the expression levels of these genes in human hepatoma HepG2 cells, and the murine small intestine, following PR and EZ treatment. HepG2 cells express NPC1L1, ABCG5, and ABCG8 and are used for functional evaluation of these genes (14, 15). We also determined mRNA levels of the transcription factors *LXR $\alpha$* , *LXR $\beta$*  and *sterol regulatory element-binding protein (SREBP)2* in HepG2 cells.

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## METHODS

### Compounds and reagents

PR (sodium salt) and EZ were purchased from LKT Laboratories (St Paul, MN, USA) and Sequoia Research Products (Pangbourne, UK), respectively. GlutaMAX supplement I and TRIzol were obtained from Life Technologies (Carlsbad, CA, USA). All other chemicals and solvents were of the highest purity commercially available.

### Animals and treatment

Five-week-old male C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Animals were housed in a temperature-controlled room with free access to standard laboratory rodent food (MF diet, Oriental Yeast, Tokyo, Japan) and water. Mice were treated orally once with PR (5 mg/kg or 15 mg/kg) and EZ (5 mg/kg). We chose the dose of PR and EZ for previous pharmacokinetics studies (16-18). The plasma PR concentrations could be comparable to clinical therapeutic concentrations (19). After administering anesthesia by diethyl ether inhalation and sacrifice by cervical dislocation, the duodenum (1–8 cm from the stomach), jejunum (10–18 cm from the stomach), and ileum (8 cm above the cecum) were excised 6 (short-term) and 24 h after PR or EZ injection. Each sample was flash frozen in liquid nitrogen and preserved at  $-80^{\circ}\text{C}$  until RNA extraction. The study protocol was approved by the Committee for the Care and Use of Laboratory Animals of the School of Pharmacy of Kinki University (Osaka, Japan).

### Cell Culture

HepG2 cells were obtained from the RIKEN Cell Bank (Ibaragi, Japan). HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 2 mM GlutaMAX supplement I (at  $37^{\circ}\text{C}$  in the presence of 5%  $\text{CO}_2$  and 95% air). HepG2 cells were seeded at  $2.5 \times 10^4$  cells per well onto a 24-well plate (Sumitomo Bakelite, Tokyo, Japan), and cultured for 1 day. After the medium was replaced with fresh DMEM, HepG2 cells were treated with PR (3, 10, 30, or 300  $\mu\text{M}$ ) and EZ (3, 10, 30, or 300  $\mu\text{M}$ ) for 3, 6, or 24 h.

### Determination of mRNA levels by real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from murine small intestines and HepG2 cells using TRIzol. mRNA expression was measured using RT-PCR, as described previously (20, 21). The oligonucleotide sequences for each mRNA target are shown in Table 1. Data were analyzed using ABI Prism 7000 SDS software (Life Technologies), using the multiplex comparative method.

**Table 1.** Primer sequences used in PCR assays

Gene	Primer sequence (5'-3')
Mouse	
<i>NPC1L1</i>	for; AGGACATCTGCTATGCTCCCCT rev; AGCAGTCAGTGAGGCTGGTGTT
<i>ABCG5</i>	for; CGTGGCGGACCAAATGATT rev; CCACTGGAAATCCCCAAA
<i>ABCG8</i>	for; CCCTCCGATTGCTTCTTTCAG rev; AGCTACCCTATAAAGCCAGCGG
<i>Villin</i>	for; CAGGTTGAGGTGCAGAAT rev; GAGCATCAAACCTCACCTGT
Human	
<i>NPC1L1</i>	for; CCAAGTCGACTGGAAGGACC rev; AGGGCCTCTGCCTCAGAATA
<i>ABCG5</i>	for; GGCAGATGATGCTGGCCTAT rev; CCAGTAGCACACACTGCTGA
<i>ABCG8</i>	for; CACTCGCAGCCCTGTTTCTA rev; GCATCTTCGTAGGACTCGGG
<i>LXR<math>\alpha</math></i>	for; AGACTTTGCCAAAGCAGGG rev; ATGAGCAAGGCAAACCTCGG
<i>LXR<math>\beta</math></i>	for; TGATGTCCCAGGCACTGATG rev; CCTCTTCGGGATCTGGGAT
<i>SREBP2</i>	for; TTGTCCTGAGCGTCTTTGTG rev; AGCAGCTGCAAATCTCCTC
<i>18S</i>	for; CCTGCGGCTTAATTTGACTC rev; AGACAAATCGCTCCACCAAC
<i>rRNA</i>	

*Determination of protein levels by western blot*

Membrane proteins were extracted from HepG2 cells using the Mem-PER Eukaryotic Membrane Protein Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were measured using a BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 7.5% e-Pagell (Atto, Tokyo, Japan) and 5 µg of membrane protein per well. Resolved proteins were transferred onto Hybond-P polyvinylidene difluoride membranes (GE Healthcare, Milwaukee, WI, USA). Immunoreactive NPC1L1 proteins were detected using polyclonal NPC1L1 antibodies (NB400-128, Novus Biologicals, Littleton, CO, USA), monoclonal β-actin antibodies (Acris Antibodies, Herford, Germany), and an ECL Prime Western Blotting Detection system (GE Healthcare).

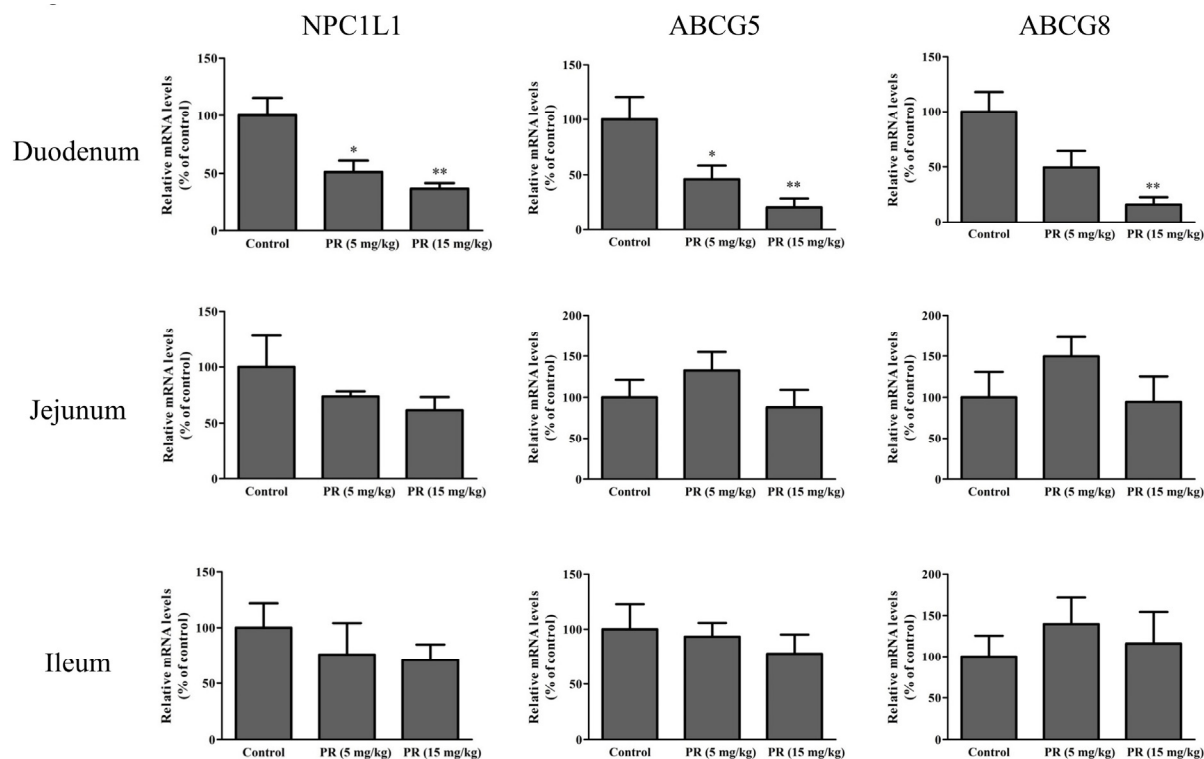
**STATISTICAL ANALYSES**

Significant differences between mean values were determined by analysis of variance followed by the Bonferroni correction. Significance levels were

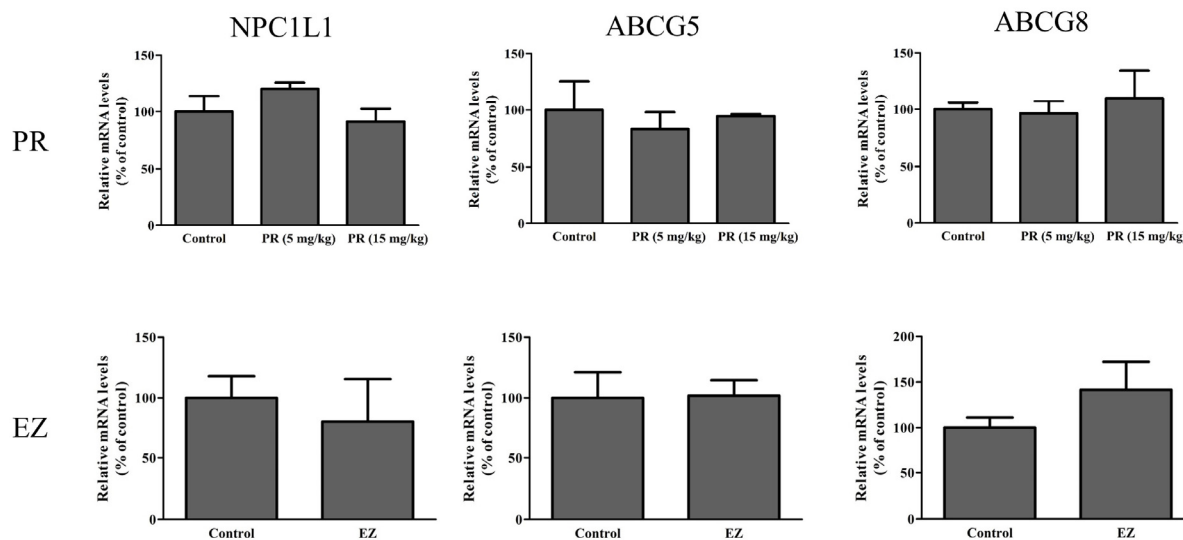
determined at  $p < 0.05$ .

**RESULTS**

Relative *NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels in the duodenum, jejunum, and ileum of mice orally administered PR (5 mg/kg and 15 mg/kg) at 24 h are shown in Fig. 1. Following PR treatment, *NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels in the duodenum were significantly lower, with dramatic decreases at higher PR doses (15 mg/kg). However, in the jejunum and ileum, few changes in *NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels were observed following PR treatment. To clarify whether expression of these transporters were affected by 6 hr (short-term) after PR administration, *NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels in the duodenum were also determined 6 h post-treatment; however, no expression changes were observed at this time point (Fig. 2). *NPC1L1*, *ABCG5*, and *ABCG8* mRNA expression in the duodenum of mice treated orally with EZ (5 mg/kg) at 24 h are shown in Fig. 2; mRNA levels of these genes were not altered by EZ treatments.



**Figure 1.** Changes in relative *NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels in the duodenum, jejunum, and ileum of mice 24 h after oral PR administration (5 mg/kg and 15 mg/kg). Data are expressed as means ± standard deviation (SD; n = 4). Significant differences between control and PR-treated mice are shown (\* $p < 0.05$  and \*\* $p < 0.01$ ).



**Figure 2.** Changes in relative *NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels in the duodenum of mice 6 h after oral PR administration (5 mg/kg and 15 mg/kg) or 24 h after oral EZ administration (5 mg/kg). Results are expressed as means  $\pm$  SD (n = 4).

We also examined effects of PR and EZ on *NPC1L1*, *ABCG5*, and *ABCG8* expression *in vitro* using HepG2 cells. Dose- and time-dependent effects of these compounds on *NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels are shown in Fig. 3a and 3b. *NPC1L1*, *ABCG5*, and *ABCG8* mRNA expression were dose-dependently suppressed by PR treatments (Fig. 3a). At 30 and 300  $\mu$ M PR, *NPC1L1* and *ABCG8* mRNA levels were significantly decreased in HepG2 cells. When we analyzed the time course of *NPC1L1* mRNA levels following PR treatment, *NPC1L1* expression was significantly reduced after 24 h (Fig. 3b). We also assessed the effects of PR on *NPC1L1* protein levels (Fig. 3c). *NPC1L1* mRNA and protein levels showed corresponding significant decreases following PR administration (at 30 and 300  $\mu$ M). Similar *NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels to baseline controls were observed at each EZ-treatment concentration in HepG2 cells (Fig. 4).

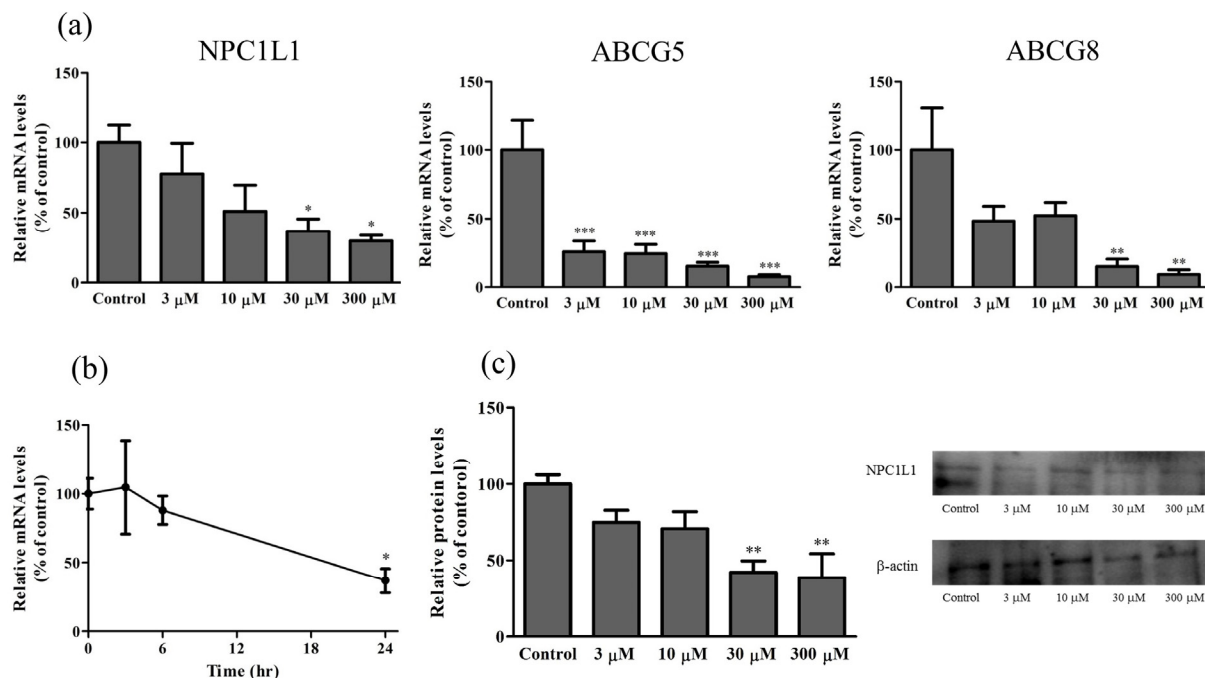
To investigate the underlying mechanisms of PR-induced changes in *NPC1L1*, *ABCG5*, and *ABCG8* levels, we examined mRNA expression of the transcription factors *LXR $\alpha$* , *LXR $\beta$*  and *SREBP2* (Fig. 5). Significant increases in *LXR $\alpha$*  mRNA levels were observed following treatment with 3–300  $\mu$ M PR. Higher *LXR $\beta$*  mRNA levels were also observed following administration of 30  $\mu$ M PR; however, few changes in *SREBP2* expression were observed.

## DISCUSSION

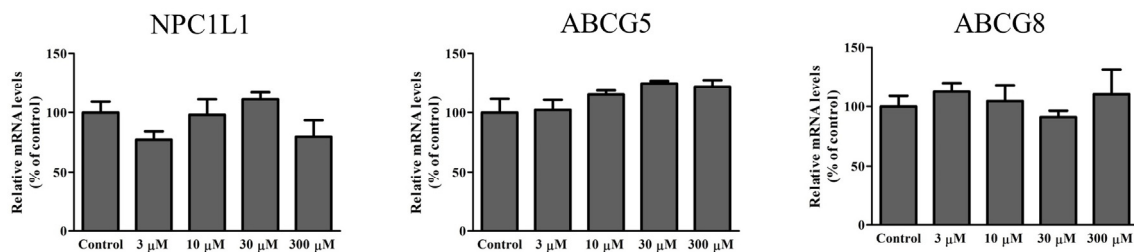
Intestinal cholesterol absorption via *NPC1L1* and its *de novo* synthesis are important factors for cholesterol homeostasis. Because PR and EZ affect expression of the intestinal cholesterol-absorbing proteins *NPC1L1*, *ABCG5*, and *ABCG8*, the present study demonstrates that these compounds are potentially therapeutic drugs for dyslipidemia.

*NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels were significantly decreased in the duodenum, but not jejunum and ileum, of mice 24 h after PR treatment (Fig. 1), indicating that PR regionally affected the expression of these genes in the small intestine of mice. *NPC1L1* is highly expressed in the duodenum and jejunum, but not other tissues, in mice (1, 3, 22). PR reaches a maximum plasma concentration within 2 h of oral administration, and is eliminated with a relatively short half-life (1–3 h; 23, 24). PR was primarily absorbed from the duodenum (25). Thus, the region-specific effects of PR may have been the result of higher duodenal concentrations (compared with those in the jejunum and ileum) after oral administration.

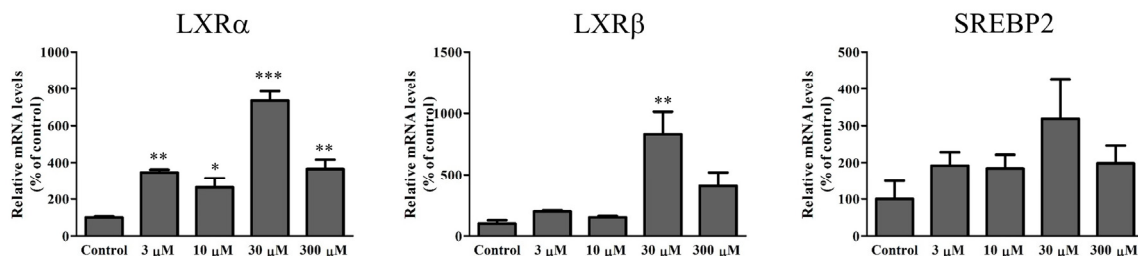
Although PR and EZ both pharmacologically act to reduce plasma cholesterol levels (26), *NPC1L1*, *ABCG5*, and *ABCG8* mRNA expression did not change following EZ treatment (Figs. 2 and 4).



**Figure 3** The effects of PR on *NPC1L1*, *ABCG5*, and *ABCG8* expression in HepG2 cells. (a) Relative *NPC1L1*, *ABCG5*, and *ABCG8* mRNA levels 24 h after PR (3, 10, 30, and 300 μM) treatment. (b) Time course of relative *NPC1L1* mRNA expression changes after PR (30 μM) treatment. (c) *NPC1L1* protein expression after PR (3, 10, 30, and 300 μM) treatment. Results are expressed as means ± SD (n = 4). Significant differences between (a) 0 h (control) and each time, (b), and (c) no treatment (control) and PR-treated HepG2 cells are shown (\* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ ).



**Figure 4** Effects of EZ on relative *NPC1L1*, *ABCG5*, and *ABCG8* mRNA expression 24 h after EZ (3, 10, 30, and 300 μM) treatment in HepG2 cells. Results are expressed as means ± SD (n = 4).



**Figure 5** Effects of PR on relative *LXRα*, *LXRβ* and *SREBP2* mRNA expression 24 h after PR (3, 10, 30, and 300 μM) treatment in HepG2 cells. Results are expressed as means ± SD (n = 4). Significant differences between control and PR-treated HepG2 cells are shown (\* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ ).

Unfortunately, the precise mechanism underlying the differential effects of PR and EZ on the expression of these genes is unclear. The primary sites of PR and EZ action are the small intestine and liver, respectively, which could partially account for the observed differential effects on *NPC1L1*, *ABCG5*, and *ABCG8* expression. Further studies are needed to clarify whether higher doses or repeated administration of EZ affect the expression of these genes.

In our *in vitro* experiments using HepG2 cells, the examined PR and EZ concentrations did not promote lactate dehydrogenase leakage (data not shown), suggesting that cytotoxicities induced by PR and EZ were minimal. In accordance with the results of our *in vivo* experiments, PR, but not EZ, treatment suppressed *NPC1L1*, *ABCG5*, and *ABCG8* mRNA expression in HepG2 cells. *LXR $\alpha$*  mRNA was also significantly increased in HepG2 cells treated with PR (Fig. 5). However, the effects of PR on *LXR* protein levels and activity remain unclear. *LXR $\alpha$*  and *LXR $\beta$*  belong to the nuclear receptor family, and are crucial inhibitory *NPC1L1* regulators (27-29). Thus, the present results suggest that PR-induced *LXR $\alpha$*  upregulation may attenuate duodenal *NPC1L1* expression.

It has been reported that cholesterol intake upregulates *ABCG5* and *ABCG8* expression in the small intestine (30). Moreover, cholesterol-free diet has been found to suppress *ABCG5* and *ABCG8* gene expression in the murine small intestine (31). Thus, because PR inhibits cholesterol synthesis in the small intestine and liver, PR may also attenuate *ABCG5* and *ABCG8* expression by such a mechanism.

We determined the effects of single PR and EZ administrations on *NPC1L1*, *ABCG5*, and *ABCG8* the mRNA levels in the small intestine. However, in clinical settings, PR and EZ used long term. Previous reports showed that 6 weeks of statin therapy was associated with an increase in intestinal cholesterol absorption (32). Moreover, atorvastatin decreased cholesterol synthesis, while increasing intestinal *NPC1L1* expression in dyslipidemic men (33), suggesting that reducing cholesterol levels increases the expression of this gene. We have previously shown that *NPC1L1* expression was significantly decreased in the small intestine of mice fed a high-cholesterol diet for 7 days (34). These long-term results differed from those obtained using a single PR administration in this study. Thus, *NPC1L1* feedback regulation in long-term statin treatment should be evaluated separately from that of single

administrations. Further studies are needed to clarify the effects of repeated PR and EZ administration.

The effects of other nutrients on *NPC1L1* expression have also been reported. For example, D-glucose directly modulated *NPC1L1* expression via transcriptional mechanisms and phosphatase-dependent pathways (35). Additionally, dietary-calcium-induced plasma total cholesterol reductions were accompanied by transcriptional *NPC1L1* downregulation, along with *ABCG5* and *ABCG8* upregulation (36). Whether other drugs or nutrients affect intestinal *NPC1L1*, *ABCG5*, and *ABCG8* expression or cholesterol absorption remains to be investigated.

## CONCLUSION

In conclusion, we demonstrated that treatment with PR, but not EZ, decreased *NPC1L1* expression in the duodenum, tentatively as the result of *LXR* upregulation. Moreover, our results show that a single administration of PR could affect intestinal cholesterol absorption.

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