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Polyunsaturated fatty acids down-regulate in vitro expression of the key intestinal cholesterol absorption protein NPC1L1: no effect of monounsaturated nor saturated fatty acids

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Abstract

Several transporter proteins regulate intestinal cholesterol absorption. Of these proteins, NPC1L1 is a major contributor to this process. Fatty acids (FAs) modulate cholesterol absorption by a mechanism that remains unknown. We evaluate the effect of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) on the expression of NPC1L1 and others proteins associated with cholesterol absorption (SR-BI, ABCG5, ABCG8, ABCA1, CAV-1, ANX-2) in human enterocytes in vitro. The role of SREBPs, PPARs, LXR and RXR in this process was also investigated. Caco-2/TC-7 enterocytes were incubated for 24 h with a wide range of concentrations of FA–bovine serum albumin (50–300 μM). Gene expression was analyzed by quantitative real-time PCR. The NPC1L1 protein present in enterocyte membranes was analyzed using Western blot. *NPC1L1* mRNA levels were reduced 35–58% by the n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) ($P<.05$). Linoleic acid (n-6), palmitic acid and oleic acid did not affect *NPC1L1* mRNA expression. *ABCA1* mRNA levels were reduced 44–70% by n-6 arachidonic acid and 43–55% by n-3 EPA ($P<.05$). LXR and LXR+RXR agonists decreased *NPC1L1* mRNA expression by 28% and 57%, respectively ($P<.05$). A concentration of 200 μM of EPA and DHA decreased NPC1L1 protein expression in enterocyte membranes by 58% and 59%, respectively. We have demonstrated that the PUFAs n-3 EPA and DHA down-regulate *NPC1L1* mRNA expression. In addition, PUFAs also down-regulate NPC1L1 protein expression in enterocyte membranes. LXR and RXR activation induced a similar repression effect. The lipid-lowering effect of n-3 PUFAs could be mediated in part by their action at the NPC1L1 gene level.
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Keywords: NPC1L1; Fatty acids; Intestinal cholesterol absorption; Enterocytes; Gene expression; Transcription factors

1. Introduction

It has been clearly demonstrated in secondary prevention trials that n-3 polyunsaturated fatty acids (PUFAs) from fish or supplements reduce premature mortality and sudden death in individuals with a history of myocardial infarction [1,2] and coronary artery disease [3]. Moreover, primary prevention studies have shown the benefits of high-dose n-3 PUFAs supplements on metabolic risk factors; for instance, reductions in triacylglycerol (TG) levels have been extensively reported [4–6]. Furthermore, it is known that dietary intake of n-3 PUFAs increases low-density lipoprotein (LDL) particle size [7,8] and exerts lipid-lowering effects; in particular, reductions in plasma cholesterol levels have been described in humans and animals models, although consistent results are lacking [4,9,10]. In contrast, previous research has demonstrated that intake of monounsaturated fatty acids (MUFAs) have an LDL-cholesterol reduction effect without affecting high-density lipoprotein (HDL) cholesterol [5,11], and some groups even report increases in its levels [10,12]. It has also been clearly proven that saturated fatty acids (SFAs) increase either LDL or HDL cholesterol [5,10,13]. Interestingly, n-3 PUFAs have been shown to decrease cholesterol absorption in animals models [14–17]. Although the mechanism involved in the lipid-lowering effect of unsaturated FAs (UFAs) is not fully understood, activation of different transcription factors (PPARs, LXRs, RXR and SREBP 1-c) appears to be involved [18].

Cholesterol derived from cholesterol absorption, along with that synthesized de novo by liver, intestine and other extrahepatic tissues, provides the principal source of blood cholesterol. Intestinal cholesterol absorption is a tightly regulated process. LDL cholesterol levels are positively correlated to cholesterol absorption in humans [19]. Those individuals with high absorption rates are hypercholesterolemic and generally have an increased number of cardiovascular events [20,21]. The efficiency of intestinal cholesterol absorption is most likely determined by the net balance between uptake and efflux of cholesterol molecules across the enterocyte membrane [22]. Moreover, inhibition of cholesterol intestinal absorption leads to lower LDL

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cholesterol levels [22]. Therefore, understanding the mechanisms involved in intestinal cholesterol absorption is highly relevant. Cholesterol absorption is the cooperation result between several intestinal cholesterol proteins including SR-BI, ABCA1, ABCG5/G8 and CAV1/ANX2 complexes [23], and the recently identified NPC1L1, which appears to be crucial for cholesterol absorption [23,24]. NPC1L1 knockout mice have a cholesterol absorption capacity that is decreased by 69% [24]. This reduction in cholesterol absorption was similar to that seen in NPC1L1 wild-type mice treated with ezetimibe, while NPC1L1 null mice were insensitive to ezetimibe treatment [24–26]. These observations suggest that NPC1L1 is the molecular target of ezetimibe, a potent and specific cholesterol absorption inhibitor. In humans, ezetimibe reduces cholesterol absorption by 50% and plasma LDL cholesterol by approximately 22% [27]. These clinical observations have underlined the crucial role of NPC1L1 in cholesterol homeostasis. NPC1L1 is widely expressed in many human tissues, with the highest expression observed in the liver and small intestine [24,28,29], but the subcellular localization of NPC1L1 protein remains controversial [24,29–33]. Little is known about the molecular regulation of intestinal NPC1L1 expression. Few studies have addressed this question. Activation of PPARδ and PPARα has been shown to down-regulate *NPC1L1* gene expression in vitro both in human and mouse models [34,35]. Moreover, two putative sterol response elements (SREs) have been identified in the human NPC1L1 promoter [36,37]. Alrefai et al. [37] have suggested that the increase in *NPC1L1* mRNA levels achieved in response to cellular cholesterol depletion is a SREBP-2-dependent mechanism.

Because cholesterol levels are largely dependent on diet, it seems logical that dietary components could intervene in the regulation of absorption mechanism. Among dietary components, fatty acids are the main candidates to affect this regulation because it has been shown that n-3 PUFAs are cholesterol-lowering agents [4,9,10]. A relatively high concentration of FAs in the form of TGs is transported to the intestinal lumen for absorption and, following TG hydrolization, these FAs are absorbed by enterocytes [38]. Little is known regarding the effects of FAs on the mechanisms of cholesterol absorption in the enterocyte. Moreover, FAs are natural ligands of several transcription factors and have the capacity to modulate gene expression [39,40]. Thus, in the present paper, we hypothesized that FAs could affect gene expression and protein production of molecules directly involved in cholesterol absorption at the intestinal level.

2. Methods and materials

2.1. Caco-2/TC-7 cell culture

The human colon colorectal adenocarcinoma cell line Caco-2/TC-7 was purchased from Celltec (Barcelona, Spain). The cells (Passages 47–49) were routinely grown in 75-cm^2 plastic flasks at a density of 1.25×10^4 cell/cm^2 and cultured in Dulbecco's modified Eagle medium (DMEM) high glucose with L-glutamine supplemented with 20% (v/v) heat-inactivated fetal bovine serum, 100U/ml penicillin and 100 μg/ml streptomicyn (Invitrogen, Paisley, UK) at 37°C in a humidified atmosphere with 5% CO_2. After reaching 80% confluence (Days 4–5 after seeding), cells were trypsinized.

The RNA experiments were performed using cells cultured as described above in six-well culture plates. For the Western blot experiments, cells were grown on 10-cm^2 culture plates. For all experiments, cells were grown for an additional 21 days after reaching confluence (Days 6–7 after seeding). During this period, Caco-2/TC-7 cells differentiate into an enterocyte-like cell with microvilli and basolateral membranes separated by tight junctions [41,42]. Differentiated cells express intestine-specific enzymes and the activities of these enzymes reach approximately half the activities observed in the small intestine. The medium was changed three times per week for all cultures.

2.2. Gene expression experiments

To study the effect of fatty acids on *NPC1L1*, *ABCG5*, *ABCG8*, *SR-BI*, *ABCA1*, *CAV1*, *ANX2*, *SREBP-1* and *SREBP-2* mRNA levels, Caco-2/TC-7 enterocytes were incubated with FA–bovine serum albumin complexes [FA–BSA: palmitic acid (PA, C16:0), oleic acid (OA, C18:1), linoleic acid (LA, C18:3), arachidonic acid (AA, C20:4), eicosapentaenoic acid (EPA, C22:6) and docosahexaenoic acid (DHA, C22:6)] at 50, 100, 200 and 300μM for 24 h in serum-free medium. After the incubation period, total RNA was isolated from the cells using the ABI PRISM 6100 Nucleic Acid PrepStation extraction system (Applied Biosystems, Foster City, USA) following the manufacturer's instructions. The mRNA from the above-mentioned genes was analyzed (see below). Absorbance at 260 nm was used to measure RNA concentration, and an absorbance ratio of 260/280 nm was used to analyze RNA quality. Cells not treated with BSA or FA–BSA complexes (untreated cells) were designated as controls.

To study the effect of PPARγ, PPARδ, LXR and RXR agonist on *NPC1L1* gene expression, Caco-2/TC-7 enterocytes were incubated with 2μM PPARγ agonist (BRL), 100 nM PPARδ agonist (GW501516), 2 μM LXR agonist (TO901317) and LXR+RXR agonist (TO901317+9cRA) at concentrations of 2 and 10 μM for 24 h in serum-free medium. After 24 h incubation, cells were used for total RNA extraction, and *NPC1L1* gene expression was evaluated. Cells incubated with vehicle alone (DMSO) were designated as controls. All the experiments were performed in triplicate and the experiments were repeated three times.

2.3. Preparation of FA sodium salt and FA–BSA complex

Preparation was made according to the method of Wu et al. [43] with some modifications. Briefly, 10 mg of FAs (Sigma, St. Quentin Fallavier, France) was mixed with 0.5 ml EtOH and 5 M NaOH in a volume adjusted so that the molar ratio of FAs to NaOH was 1:1. The mixture was dried under gas nitrogen until FA sodium salt was obtained and then dissolved in 2 ml of sterile water (stock solutions of FA). To avoid FA oxidation, 1 μM butylated hydroxytoluene was added to the stock solutions of FAs. Stock solutions of FAs complexed to fatty acid free BSA (Sigma, St. Quentin Fallavier, France) were made by combining FAs and 5 mM BSA in a volume adjusted so that the FA-to-BSA molar ratio was 3:1. The FA–BSA solutions were sterile-filtered and used fresh.

2.4. Analysis of mRNA by real-time quantitative PCR

1 μg of RNA was reverse transcribed to cDNA using Random Hexamers and SuperScript II (Invitrogen, Paisley, UK), following the manufacturer's protocol and using a PE Biosystems 2400 thermocycler (Applied Biosystems, Foster City, USA). TaqMan primers and probes for *NPC1L1*, *ABCG5*, *ABCG8*, *SR-BI*, *ABCA1*, *CAV1*, *ANX2*, *SREBP-1*, *SREBP-2*, *GAPDH* and *18S* were obtained from predesigned and validated "Assays-on-Demand" products (Applied Biosystems, Foster City, USA) and were used for real-time PCR amplifications. The respective mRNA expression for each sample was calculated using the recommended $2^{-\Delta\Delta Ct}$ method [ABI Prism 7700 Sequence Detection System; user bulletin no. 2. Revision A. Foster City (USA): Applied Biosystems, 1997]. The control group (untreated cells) was used for calibration in this experiment. *GAPDH* and *18S* were used as housekeeping genes to normalize the results of the genes of interest.

2.5. Determination of NPC1L1 protein in whole cell lysates and enterocyte membranes

Membrane protein extracts from cells were generated as described in the Mem-Per Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce, Rockford, USA) and whole cell lysates were obtained by repeated freezing and thawing in homogenization buffer containing PBS pH 7.4 and complete miniprotease inhibitors (Roche, Manheim, Germany) after incubation for 24 h with EPA and DHA at a concentration of 200 μM. Samples were kept at −80°C until the assay was performed. Total protein was measured using the Bradford method (Bio-Rad Laboratories, CA, USA). Electrophoresis and blotting were performed using the NUPAGE Large Protein Analysis System (Invitrogen). The blotting membrane was blocked with 2% ECL Advance Blocking Reagent (Amersham Biosciences, Bucks, UK) and then incubated with an anti-NPC1L1 antibody (1:2500, NB 400-127, Novus Biologicals, Littleton, USA). Antigen–antibody complexes were detected by incubating the membrane with IgG-HRP (1:20000, P0448, Dako, Glostrup, Denmark). Signals were detected with ECL Advanced Reagent Mixture (Amersham Biosciences, Bucks, UK), and the bands were visualized on film by autoradiography. Bands were captured using a Versadoc 4000 imaging system and analyzed using the Quantity One software (Bio-Rad Laboratories). As a control for equal protein loading, the blotted proteins were stained with Ponceau S and band quantification was normalized to actin. The protein sizes were confirmed by comparison with molecular weight standards (Invitrogen Ltd, Paisley, UK). All the experiments were performed in triplicate, and the experiments were repeated three times.

2.6. Determination of fatty acid oxidation and cytotoxicity experiments

Fatty acid oxidation of EPA and DHA stock solutions at concentrations of 100 and 200 μM were measured with thiobarbituric acid reagent using malondialdehyde (MDA) as a standard using the usual methods [44]. As a positive control, oxidized solutions of FAs were generated as described by Mishra et al. [45]. Results are expressed as μM MDA/μM FA.

The cytotoxic effects of the studied FAs were determined by lactate dehydrogenase (LDH) release in the culture medium and morphologic observation. The amount of LDH released was measured in an automatic analyzer (Cobas-Mira; Roche, Switzerland) using an enzymatic method (Boehringer Mannheim, Ingelheim, Germany). LDH release was expressed in units of LDH per liter (U/L). Viability of cells was estimated as well by observation of morphological changes using phase-contrast microscopy.

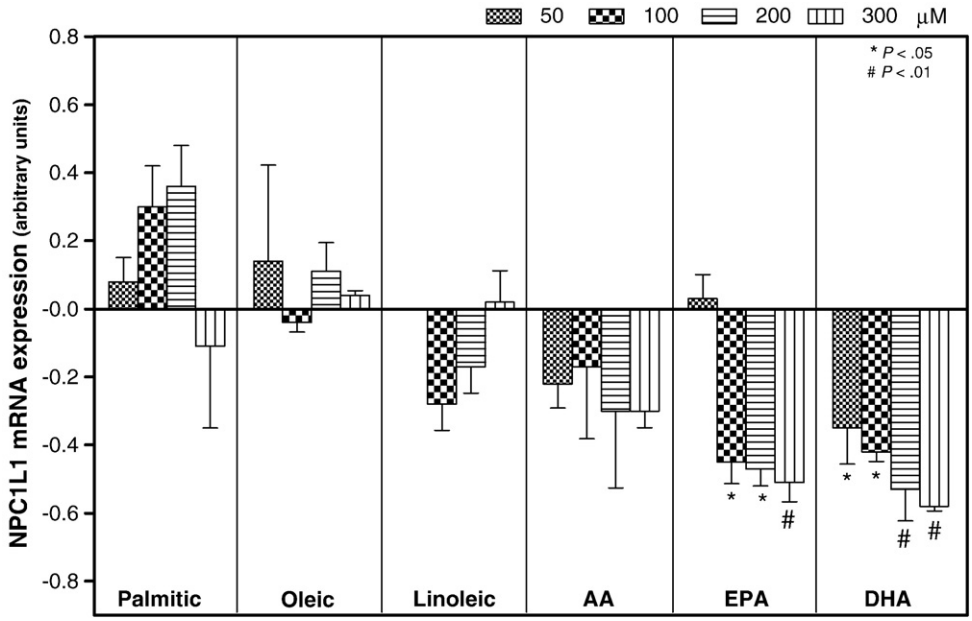

Fig. 1. Dose–response relationship for FAs on *NPC1L1* mRNA expression. Enterocytes Caco-2/TC-7 cells were incubated with increasing noncytotoxic concentrations of each FA, as described in the Methods and Materials section. Expression values were normalized to housekeeping genes, and expression in untreated cells was set to 0. *$P<.05$, #$P<.01$ compared to untreated cells (controls).

2.7. Statistical analysis

2.7.1. Cytotoxicity

Statistical analyses of LDH measurements were performed using one-way analysis of variance (ANOVA) followed by the Bonferroni posttest for multiple comparisons. Differences were considered significant at $P<.05$. All analyses were performed using GraphPad Prism 5.0 software Inc.

2.7.2. mRNA levels

The results are presented as mean ± S.E.M. The statistical significance between the groups was assessed by one-way ANOVA and was calculated using the Bonferroni multiple comparison test. Statistical significance was set at a level of $P<.05$. The statistical package for the Social Sciences software version 14.0 was used for this statistical analysis.

3. Results

3.1. Effect of fatty acids on NPC1L1 mRNA expression

Treatment of Caco-2/TC-7 enterocytes with PA (SFA), OA (n-9 MUFA) and LA (n-6 PUFA) fatty acids did not affect *NPC1L1* mRNA

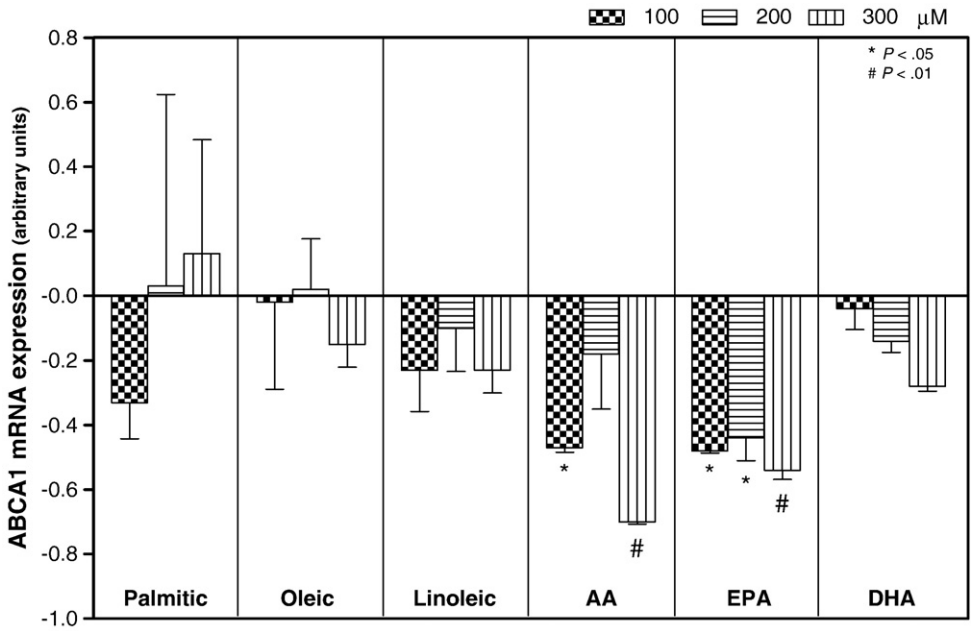

Fig. 2. Dose–response relationship for FAs on *ABCA1* mRNA expression. Enterocytes Caco-2/TC-7 cells were incubated with increasing noncytotoxic concentrations of each FA, as described in the Methods and Materials section. Expression values were normalized to housekeeping genes, and expression in untreated cells was set to 0. *$P<.05$, #$P<.01$ compared to untreated cells (controls).

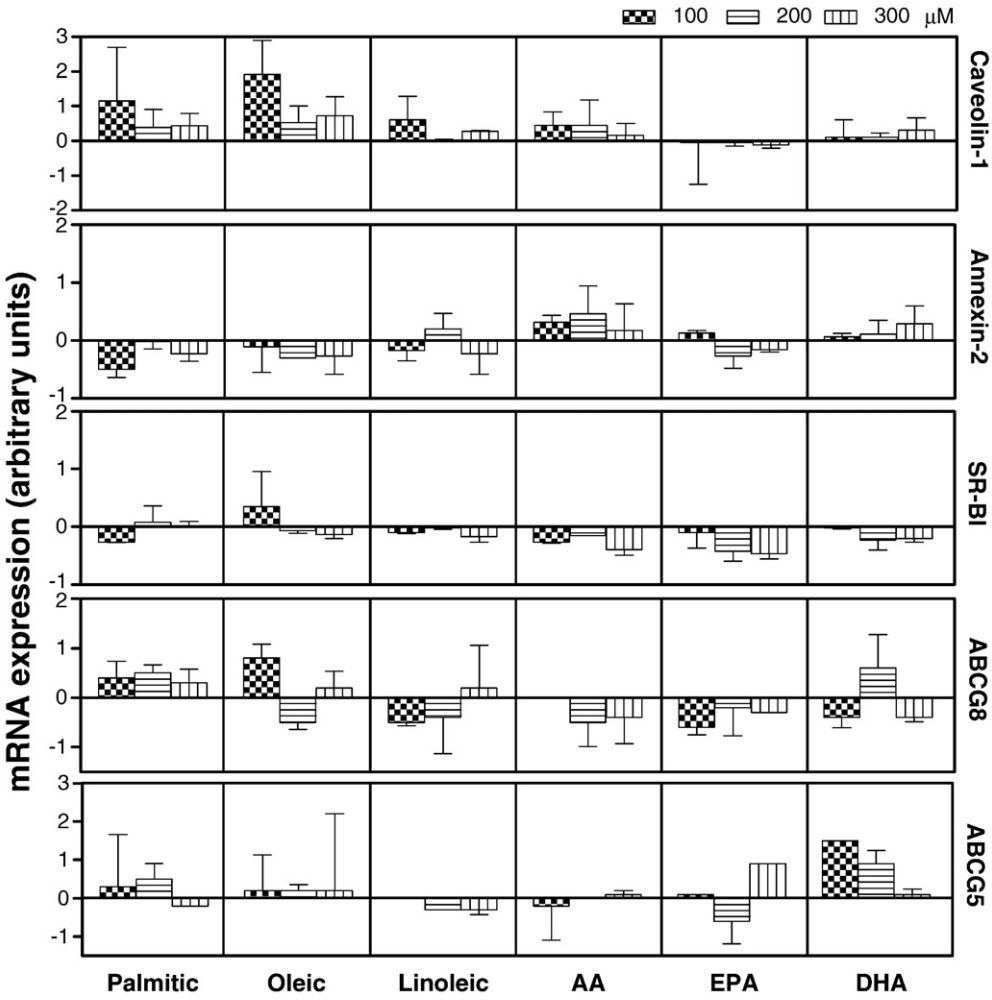

Fig. 3. Dose–response relationship for FAs on *CAV-1*, *ANX-2*, *SR-BI*, *ABCG8* and *ABCG5* mRNA expression. Enterocytes Caco-2/TC-7 cells were incubated with increasing noncytotoxic concentrations of each FA, as described in the Methods and Materials section. Expression levels were normalized to housekeeping genes, and expression in untreated cells were set to 0.

expression (Fig. 1). When we performed dose–response experiments with AA (n-6 PUFA), we saw a trend toward decreased *NPC1L1* mRNA levels with a maximum inhibition effect at doses of 200 and 300 μM. On the other hand, PUFAs n-3 (EPA and DHA) significantly decreased *NPC1L1* mRNA levels with variable intensity (from 20% to 60%). Thus, when we treated Caco-2/TC-7 cells with EPA and DHA, we saw a dose-dependent effect on the decrease in *NPC1L1* mRNA levels. These levels were already reduced by 45% ($P<.05$) and 35% ($P<.05$) at 100 μM EPA and 50 μM DHA, respectively. The maximum inhibition observed with EPA and DHA occurred at 300 μM. At these concentrations, *NPC1L1*

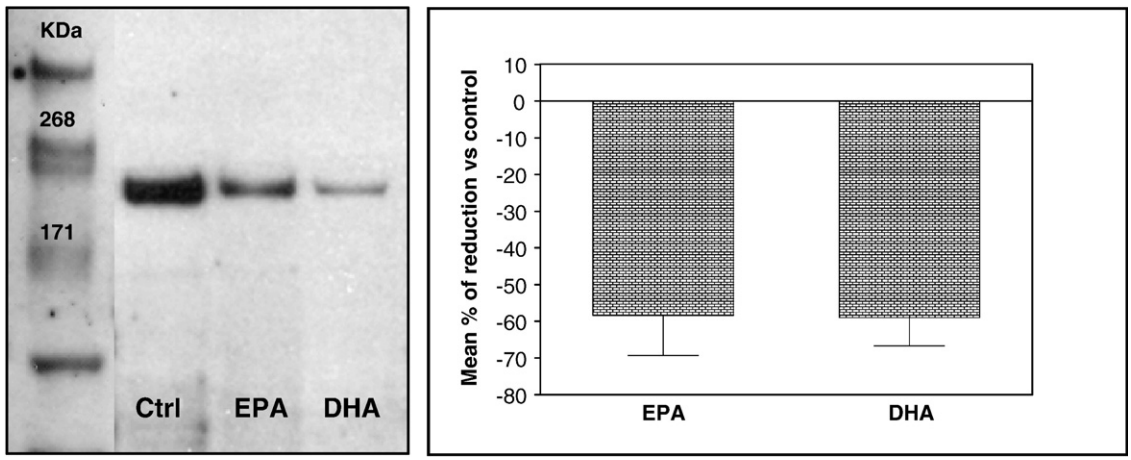

Fig. 4. NPC1L1 protein expression in membrane protein extracts of Caco-2/TC-7 cells incubated with 200 μM of EPA and DHA for 24 h.

mRNA levels were decreased 51% ($P<.01$) and 58% ($P<.01$), respectively. DHA, the strongest repressor, induced a significant decrease in *NPC1L1* mRNA levels at all concentrations tested (Fig. 1).

3.2. Effect of fatty acids on ABCA1 mRNA expression

n-6 and n-3 PUFAs decreased *ABCA1* mRNA levels in Caco-2/TC-7 cells (Fig. 2). The effects of AA and EPA were more marked and reached statistical significance. As such, AA decreased mRNA levels from 47% ($P<.05$) at 100 μM to 70% ($P<.01$) at 300 μM. EPA significantly decreased mRNA levels at all three concentrations tested. A 48% ($P<.05$) reduction at 100 μM and a 54% ($P<.01$) reduction at 300 μM was observed. Although a decrease in *ABCA1* mRNA levels with DHA and LA treatments was detected, the results did not reach statistical significance. PA and OA did not affect *ABCA1* expression (Fig. 2).

3.3. Effect of fatty acids on ABCG5, ABCG8, SR-BI, CAV1 and ANX2 mRNA levels

In Fig. 3, we show that treatment of Caco-2/TC-7 enterocytes with the same FAs and using the same experimental conditions as those used for the NPC1L1 and ABCA1 experiments did not produce any significant effect on the mRNA levels of *ABCG5*, *ABCG8*, *SR-BI*, *CAV1* and *ANX2*.

3.4. Effect of fatty acids on NPC1L1 protein expression

Consistent with the decrease in *NPC1L1* mRNA expression, when Caco-2/TC-7 cells were incubated with 200 μM of EPA or DHA for 24 h, there was a decrease in NPC1L1 protein expression, both in the whole cell lysate and enterocyte membrane (Fig. 4). After band quantification and normalization, we saw a 58% and 59% reduction in EPA and DHA-treated samples, respectively.

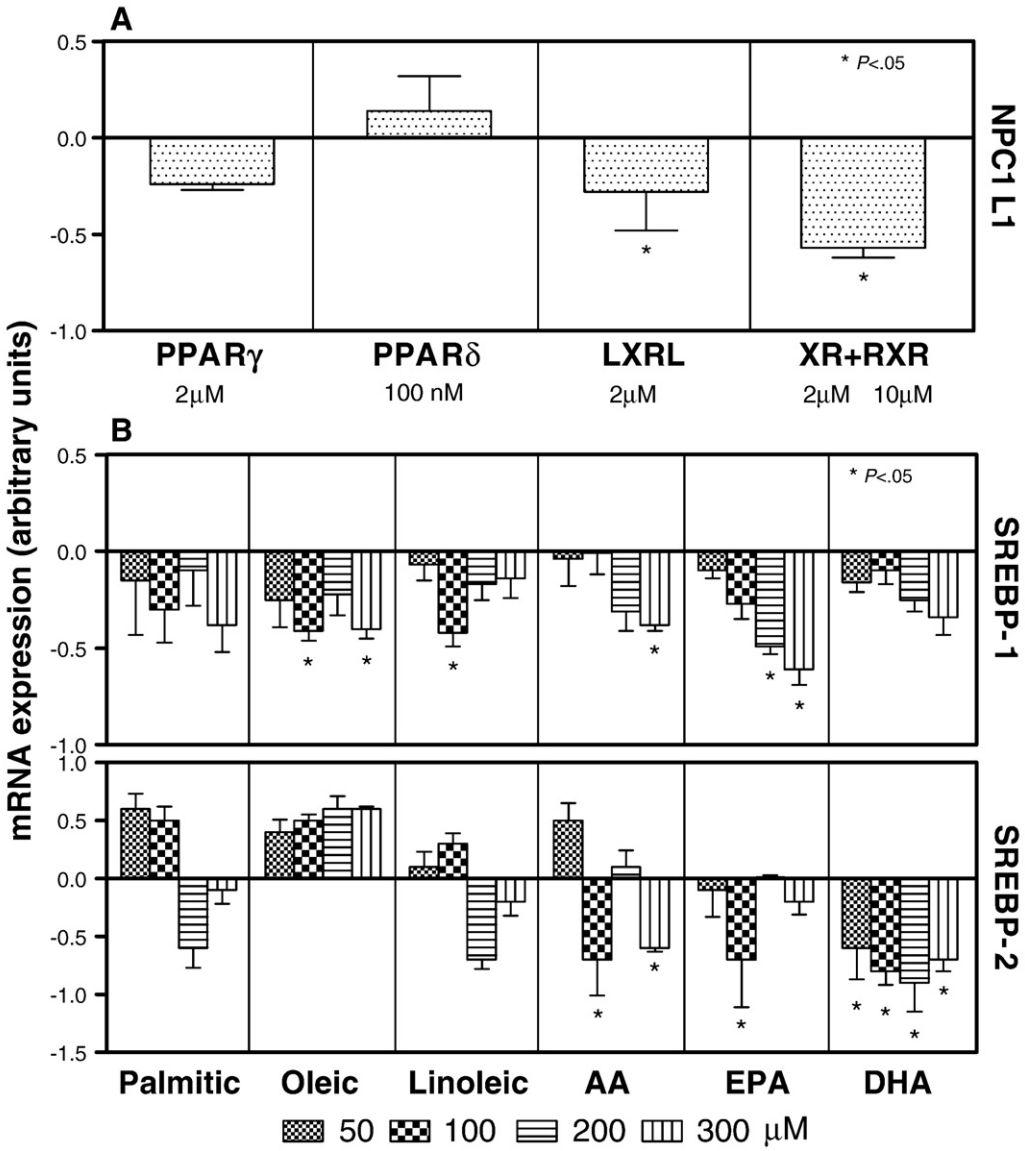

Fig. 5. Transcription factors involved in fatty acid regulation. (A) Effect of nuclear receptors PPARγ, PPARδ, LXR and RXR on *NPC1L1* mRNA expression. Enterocytes Caco-2/TC-7 cells were incubated as described previously. Expression values were normalized to housekeeping genes and expression in untreated cells was set to 0. *$P<.05$ compared with untreated cells (control). (B) Dose–response relationship for FAs on SREBP-1, SREBP-2 mRNA expression. Enterocytes Caco-2/TC-7 cells were incubated with increasing noncytotoxic concentrations of each FA, as described in the Methods and Materials section. Expression values were normalized to housekeeping genes, and expression in untreated cells was set to 0. *$P<.05$ compared to untreated cells (controls).

3.5. Transcription factors involved in fatty acid down-regulation of NPC1L1 mRNA levels

We evaluated whether the transcription factors that have been shown to regulate *NPC1L1* expression [34,35,37] are involved in our experiments. After treatment of Caco-2/TC-7 enterocytes with PPARγ, PPARδ, LXR, and LXR+RXR agonists, we saw that neither 2 μM BRL (a PPARγ agonist) nor 100 nM GW501516 (a PPARδ agonist) affected *NPC1L1* mRNA levels (Fig. 5A). However, 2 μM TO901317 (a LXR agonist) reduced *NPC1L1* mRNA levels by 28% ($P<.05$) and, when 2 μM TO901317 was added in combination with 10 μM 9cRA (an RXR agonist), the reduction effect on *NPC1L1* mRNA levels increased to 57% ($P<.05$) (Fig. 5A). Moreover, when we treated Caco-2/TC-7 enterocytes with the same FAs and used the same experimental conditions as those used for NPC1L1 studies, we observed a general inhibitory effect of FAs on the mRNA levels of SREBP-1 and SREBP-2. FA inhibition was more marked for SREBP-1, as all FAs tested decreased mRNAs levels, although with different degrees of significance (Fig. 5B). In contrast, mRNA levels for SREBP-2 decreased only with AA, EPA and DHA treatment (Fig. 5B).

3.6. Oxidation and cytotoxicity analysis of fatty acid experiments

To confirm that the fatty acid solutions used in the experiments were not oxidized, we measured the MDA concentration in these solutions. When fresh FAs solutions were compared to oxidized solutions, the MDA concentration was significantly lower in the fresh fatty acid solutions (data not shown).

In order to use noncytotoxic concentrations of FAs in our experiments, we examined fatty acid cytotoxicity. Our results show that PA, OA, LA, AA, EPA and DHA were not toxic for Caco-2/TC-7 cells at any of the tested concentrations (50–300 μM) after 24 h (data not shown). Twenty-four hours of incubation in serum-free medium did not affect viability of the cells. The noncytotoxicity of FAs was also confirmed at the same concentrations by direct observation of morphological changes under phase-contrast microscopy (data not shown). Gene expression experiments were carried out at a range of noncytotoxic concentrations of FA.

4. Discussion

Diet is the main source of the different types of FAs, with daily intake being on the order of several grams [46]. These FAs, which are transported in the form of TGs, are absorbed by the enterocyte after TGs hydrolysis [38], but little is known regarding the effects of the different types of FAs on the enterocyte. In the current study, we evaluated the effect of different FAs on the expression of genes involved in cholesterol absorption in enterocytes. The main observation of this work is that the n-3 PUFAs, EPA and DHA, down-regulate NPC1L1 gene expression in human enterocytes. Furthermore, we have demonstrated that this gene down-regulation is associated with a decrease in NPC1L1 protein levels. It is particularly interesting that the protein reduction is observed in the enterocyte membrane where NPC1L1 is functional. SFA, MUFA and n-6 PUFAs did not affect *NPC1L1* gene expression.

Circulating plasma cholesterol concentrations depend on the cholesterol synthesized by the liver and also on the cholesterol synthesized and absorbed by the intestine. In fact, the cholesterol absorption rate is directly related to cholesterol plasma levels. Our results show a differential FA modulation of the expression of NPC1L1, which is the key protein involved in cholesterol absorption. Thus, plasma cholesterol levels may be modulated by FAs through their action on cholesterol absorption due to the effect on NPC1L1 expression. Though we cannot rule out an additional effect of BSA on enterocyte gene expression. The reduction in cholesterol levels described in different studies to result from the ingestion of diets rich in or supplemented with n-3 PUFAs could be explained by a decrease in cholesterol absorption due to down-regulation of NPC1L1 expression by EPA and DHA. The variable effect of n-3 PUFAs on cholesterol levels reported in other studies may be explained by differences in the amount of n-3 PUFAs used and the manner in which they are administered, the type and amount of fat in the background diet and the lipoprotein profiles of the subjects.

Individuals considered to be hyperabsorbers, like apoE4 carriers, are hypercholesterolemics [20], and those participants in the 4S study who had higher cholesterol absorption rates had an increased number of cardiovascular events [21]. Thus, our results may have important clinical implications.

In this report, we also demonstrated that LXR and LXR+RXR agonists decreased *NPC1L1* gene expression and that FAs decreased *SREBPs* mRNA levels. Thus, the inhibitory effect of EPA and DHA on NPC1L1 expression could be mediated by either an LXR- or an SREBP-dependent mechanism, or by a combination of both. Using a bioinformatics approach (TFexplorer 2.0, Korean Bioinformation Center), we were able to identify two recognition elements for LXR/RXR transcription factors in the promoter region of the NPC1L1 gene. Moreover, two putative SREs have also been identified in the human NPC1L1 promoter [36,37]. It has also been shown that PUFAs decrease expression of promoters with SREs by decreasing levels of mature SREBPs [47]. Furthermore, other authors have shown that PUFAs can lower the levels of *SREBP-1c* mRNA and inhibit the activation process of SREBPs in intestinal cells and other cellular models [48–50]. Moreover, in a recent report, it has been demonstrated that human NPC1L1 expression and promoter activity are modulated by cholesterol via a SREBP-2-dependent mechanism [37]. Another nuclear receptor recently involved in NPC1L1 regulation is PPARα [34]. However, the findings, to date, have been contradictory and are subject to debate [51]. Our results, together with other published findings, show that SREBPs and LXR/RXR pathways are involved in the down-regulation of *NPC1L1* gene expression by EPA and DHA, although our results do not confirm the exact mechanism underlying this effect. The inhibitory effect of DHA on NPC1L1 expression had already been evaluated by Mathur et al. [52]. Our results extend the observations of Mathur et al. [52] and show that EPA is also able to inhibit NPC1L1 gene and protein expression and show a decrease in NPC1L1 mRNA levels by LXR+RXR agonists.

Several lines of evidence suggest that intestinal cholesterol absorption is the result of cooperation among several membrane transporters. Among these, we have not observed any significant effect of FAs on the gene expression of SR-BI, ABCG5, ABCG8, CAV1 or ANX2. However, *ABCA1* mRNA levels were reduced in enterocytes by n-6 and n-3 PUFAs. Although the down-regulation effect of PUFAs on ABCA1 gene expression has been shown in macrophages and hepatocytes, this is, to our knowledge, the first time that these results are presented in intestinal cells. ABCA1 is the key protein mediating cholesterol efflux to lipid-free apoA-1 and, thus, it is critical in regulating HDL cholesterol levels. High levels of free fatty acids have been shown in patients with diabetes mellitus who, interestingly, tend to exhibit low levels of HDL cholesterol that, in part, appear to be related to an increased risk of atherosclerosis. The mechanism by which PUFAs decrease the promoter activity of ABCA1 is not clear.

It has been shown that a 35% down-regulation in *NPC1L1* gene expression in mice reduces cholesterol absorption by 43% [35]. Other authors have demonstrated in human studies that a 50% reduction in cholesterol absorption with ezetimibe is associated with a decrease in plasma LDL cholesterol of approximately 22% [27]. Moreover, a 36% reduction in cholesterol absorption with an intake of 2.5 g/d of phytosterols is associated with a decrease in total cholesterol and LDL cholesterol by 10% and 14%, respectively [53]. Our results show that

n-3 PUFAs down-regulated *NPC1L1* gene expression by 35–60%. These results may explain, the lipid-lowering effects of n-3 fatty acids and also suggest the mechanism may involve a decrease in cholesterol absorption due to a down-regulation of NPC1L1. As we have shown recently with butyrate and propionate [54], neither can we rule out an inhibitory effect of FAs on the enterocyte cholesterol synthesis pathway.

In conclusion, we have shown that n-3 PUFAs down-regulate NPC1L1 expression, and this could play a role in the observed lipid-lowering effects of n-3 PUFAs though more studies are needed to clarify the role of FAs on intestinal cholesterol absorption and the role of SREBPs, LXR and RXR activators on NPC1L1 regulation.

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