Docosahexanoic acid modifies low-density lipoprotein receptor abundance in HepG2 cells via suppression of the LXRα-Idol pathway

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Abstract. As a daily supplement, omega-3 fatty acid is confirmed to be of benefit in hypertriglyceridemia. However, the effect of omega-3 fatty acids on the low-density lipoprotein cholesterol (LDL-C) metabolism remains a controversial issue. In this study, we focused on the regulatory effect of docosahexanoic acid (DHA), one type of omega-3 fatty acid, exerted on the LDL receptor (LDLR), a determinant regulator of the LDL-C metabolism, and explored the potential mechanism. We observed that DHA increased hepatic LDLR protein in the presence of 25-hydroxycholesterol in HepG2 cells but did not alter the mRNA level. Previous studies have identified inducible degrader of the LDLR (Idol) as a novel negative post-translational modulator of LDLR and a direct transcriptional target of liver X receptor α (LXRα). Since DHA had no effect on the transcriptional level of LDLR, we speculated that the post-transcriptional pathway LXRα-Idol participated in this regulation. The results reveal that DHA downregulated the expression of LXRα and Idol in coordination with the upregulation of LDLR expression. Multiple mechanisms are involved in the regulation of LDLR by DHA, and the suppression of the LXRα-Idol pathway is one of these mechanisms.

Introduction

Low-density lipoprotein cholesterol (LDL-C) has been identified to have a crucial and causal role in the genesis of coronary heart disease and atherosclerotic cardiovascular disease (ASCVD) (1). There is notable evidence to suggest that higher LDL-C levels are correlated with greater ASCVD risk, and that lowering cholesterol levels reduces ASCVD events (2-5). Hepatic LDL receptor (LDLR) is essential for the uptake of extracellular LDL-C (6). As such, it is a determinant regulator of the LDL-C metabolism. One of the most optimal strategies to lower LDL-C is to upregulate and stabilize hepatic LDLR expression.

The abundance of LDLR is noted on the transcriptional and post-transcriptional levels. On the transcriptional level, LDLR is tightly regulated by sterol response element binding protein 2 (SREBP2) (7,8). Cellular cholesterol depletion activates the nuclear translocation of SREBP2, and subsequently SREBP2 activates LDLR and proprotein convertase subtilisin/kexin type 9 (PCSK9) gene expression (7-9). PCSK9 plays a pivotal role in the post-transcriptional regulation of LDLR, in which it binds to the extracellular domain LDLR and directs the trafficking of it to the lysosomes for degradation (10). Previous studies have identified a new significant degrader of LDLR, named inducible degrader of the LDLR (Idol), which is an E3 ubiquitin ligase that triggers ubiquitination of LDLR on its cytoplasmic domain and promotes lysosomal degradation (11). Distinct from PCSK9, Idol is regulated by another sterol-dependent nuclear receptor, liver X receptor α (LXRα), which is activated in response to cellular cholesterol excess (12).

Omega-3 fatty acids, including docosahexanoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), are also known as marine fatty acids, and are delivered from dietary fish oil. *In vivo* and *in vitro* studies confirmed that DHA and EPA are potent inhibitors of LXRα (13,14). In addition, several studies have identified that omega-3 fatty acids upregulate LDLR abundance (15,16). However, little is known about the mechanism by which fatty acids regulate LDLR. We considered that the newly identified LXRα target gene Idol may participate in this process. In the present study, we selected DHA as one type of omega-3 fatty acid to search for the possible mechanism by which these fatty acids exert their regulatory effects on LDLR. The results revealed that DHA increased hepatic LDLR abundance through the suppression of Idol expression rather than through gene expression. Furthermore, this repression of LXRα activity by DHA and the subsequent inhibition of the expression of Idol is one of multiple mechanisms.

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Materials and methods

Reagents. Palmitic acids (PA), DHA and 25-hydroxycholesterol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fatty acid-free bovine serum albumin (BSA) was obtained from MP Biomedicals (Santa Ana, CA, USA). Cell culture reagents were purchased from Gibco (Carlsbad, CA, USA). BCA protein assay was purchased from Thermo Fisher Scientific (Waltham, MA, USA). PVDF membranes and the ECL western blot system were provided by Merck Millipore (Billerica, MA, USA). Anti-LXR alpha antibody (ab176323) and anti-Idol antibody (ab74562) were obtained from Abcam (Cambridge, MA, USA); anti-LDLR antibody (10007665) from Cayman Chemical Co. (Ann Arbor, MI, USA); and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody, peroxidase-conjugated anti-mouse antibody and anti-rabbit antibody from Proteintech Group (Chicago, IL, USA). The Ultrapure RNA kit was purchased from CWBIO (Beijing, China). The RevertAid First Strand cDNA synthesis kit (K1622) was purchased from Thermo Fisher Scientific. iTaq™ Universal SYBR® Green supermix (172-5121) was purchased from Bio-Rad (Hercules, CA, USA).

Cell cultures and treatments. The human hepatoma HepG2 cell line, obtained from Laboratory Animal Center (Sun Yat-sen University, Guangzhou, China), was cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37˚C in a humidified atmosphere of 5% CO_2. For experiments, cells were seeded at a density of $1x10^6$ cells per well and allowed to adhere for 24 h. Each fatty acid was dissolved in ethanol and mixed with fatty acid-free BSA at 2.5:1 molar ratios, working as a fatty acid/BSA complex. 25-hydroxycholesterol was dissolved in ethanol and used at a concentration of 10 μmol/l. Following the replacement of serum-free medium at 24 h, fatty acid/BSA complexes were added to the culture dishes at a concentration of 100 μmol/l fatty acid and 0.25% BSA, with or without 25-hydroxycholesterol. Control cells were treated with BSA vehicle with or without 25-hydroxycholesterol. For dose-response experiments, 25-hydroxycholesterol and increasing concentrations (50, 100 and 200 μmol/l) of DHA were added to the culture media. After 24 h, cells were either harvested for protein extraction or RNA isolation.

Western blot analysis. Following the treatments, cells were washed with phosphate-buffered saline, lysed in cell lysis buffer and incubated at 4˚C for 20 min, then centrifuged at 10,000 x g for 10 min at 4˚C. Protein concentration was measured using the BCA method. Twenty micrograms of total proteins from each extract were separated by 8% or 10% SDS-polyacrylamide gels and transferred onto PVDF membranes in a cooling system at 100 V for 2 h. Membranes were blocked by 5% non-fat dried milk in Tris-buffered saline with 0.1% Tween (TBS-T) for 1 h at room temperature. Membranes were then incubated with anti-GAPDH (diluted 1:20,000), anti-LDLR (diluted 1:200), anti-LXRα (diluted 1:1,000) or anti-Idol (diluted 1:1,000) overnight at 4˚C, washed three times with TBS-T and incubated with peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit, respectively, diluted 1:20,000) for 1 h at room temperature. Specific bands were then detected by the ECL western blot system. Antibodies against GAPDH were used as the normalizing control.

RNA isolation, cDNA synthesis and quantitative polymerase chain reaction (qPCR). Total RNA was isolated with the Ultrapure RNA kit and cDNA synthesized with the RevertAid First Strand cDNA synthesis kit from 1 μg total RNA. Primers for mRNA detection were designed and synthesized by Sangon Biotech (Shanghai, China). qPCR was carried out on a CFX96 real-time machine (Bio-Rad) using the SYBR Green polymerase by the $\Delta\Delta$Ct method. Values were normalized to GAPDH levels.

Statistical analysis. Duplicates were used in all experiments and experiments were repeated at least three times. Significant differences between the control and treatment groups were assessed by one-way ANOVA with a Bonferroni post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DHA increases the amount of LDLR protein in a dose-dependent manner. To examine the effect of DHA on the expression of LDLR *in vitro*, the abundance of LDLR in HepG2 cells was detected by western blot analysis following exposure to the respective fatty acid for 24 h. Compared with HepG2 cells cultured with DMEM, LDLR protein decreased by 77% ($P<0.05$) when treated with 25-hydroxycholesterol (10 μmol/l; Fig. 1A). In the presence of 25-hydroxycholesterol, HepG2 cells were treated with BSA vehicle, PA and DHA, respectively. BSA plus 25-hydroxycholesterol had no significant effect on LDLR protein compared with 25-hydroxycholesterol alone. With co-treatment of 25-hydroxycholesterol, DHA, but not PA, upregulated LDLR protein levels 1.4-fold compared with control cells treated with BSA vehicle (i.e., DHA significantly attenuated the suppressive effects of 25-hydroxycholesterol on LDLR protein abundance; Fig. 1A). However, there was no significant difference in LDLR expression between the cells treated with DHA and with DMEM only (data not shown).

Experiments were performed to determine LDLR expression in response to various concentrations of DHA with 25-hydroxycholesterol co-treatment. At a concentration of 50 μmol/l, the amount of LDLR protein did not differ from that observed in control cells treated with BSA vehicle. A dose-dependent increase in LDLR protein abundance was only observed in DHA-treated cells above concentrations of 100 μmol/l, confirming its inductive effect on LDLR expression (Fig. 1B).

DHA has no significant effect on the regulation of LDLR mRNA. To study whether the increase in LDLR protein levels by DHA was due to the upregulation of LDLR gene expression, the amount of LDLR mRNA was quantified by qPCR. Compared with HepG2 cells cultured with DMEM, LDLR mRNA decreased by 72% ($P<0.05$) when treated with 25-hydroxycholesterol (10 μmol/l; Fig. 2). With co-treatment of 25-hydroxycholesterol, neither DHA nor PA had a significant effect on LDLR mRNA compared with control cells treated with BSA vehicle (Fig. 2). Despite a DHA-induced moderate increase in LDLR protein abundance, DHA did not signifi-

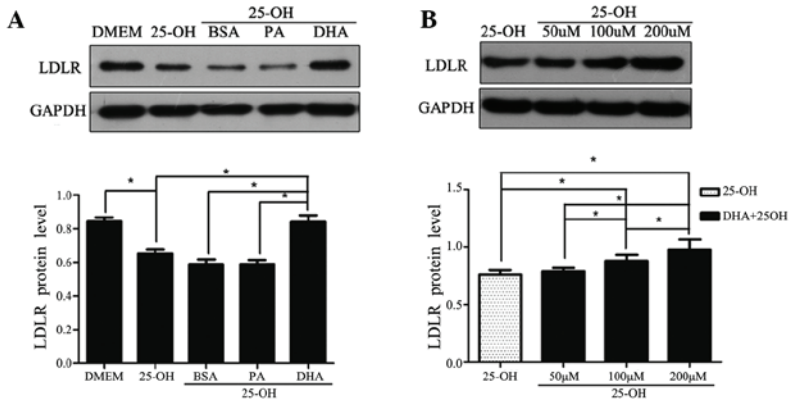

Figure 1. Regulatory effects of LDLR protein on fatty acid treatment. Following overnight culture in a serum-free medium, HepG2 cells were exposed to 25-hydroxycholesterol (10 μmol/l) with or without fatty acid/BSA complexes (100 μmol/l) for 24 h (A). HepG2 cells were exposed to 25-hydroxycholesterol (10 μmol/l) with DHA at various concentrations (50, 100 and 200 μmol/l) (B). At the end of the treatment, total cell lysates were isolated for western blot analysis for LDLR and GAPDH. The data shown are the mean (± SEM) of at least three separate experiments (*P<0.05). LDLR, low-density lipoprotein receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; 25-OH, 25-hydroxycholesterol; BSA, bovine serum albumin; PA, palmitic acid; DHA, docosahexanoic acid.

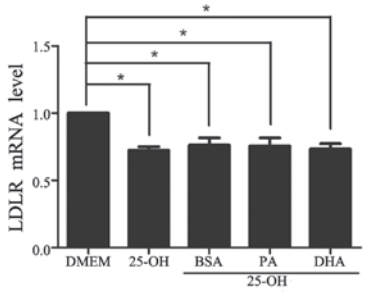

Figure 2. Regulatory effects of LDLR mRNA on fatty acid treatment. Following overnight culture in a serum-free medium, HepG2 cells were exposed to 25-hydroxycholesterol (10 μmol/l) with or without fatty acid/BSA complexes (100 μmol/l) for 24 h. LDLR mRNA levels were analyzed by quantitative polymerase chain reaction and were normalized to glyceraldehyde 3-phosphate dehydrogenase. The data shown are the mean (± SEM) of at least three separate experiments (*P<0.05). LDLR, low-density lipoprotein receptor; DMEM, Dulbecco's modified Eagle's medium; 25-OH, 25-hydroxycholesterol; BSA, bovine serum albumin; PA, palmitic acid; DHA, docosahexanoic acid.

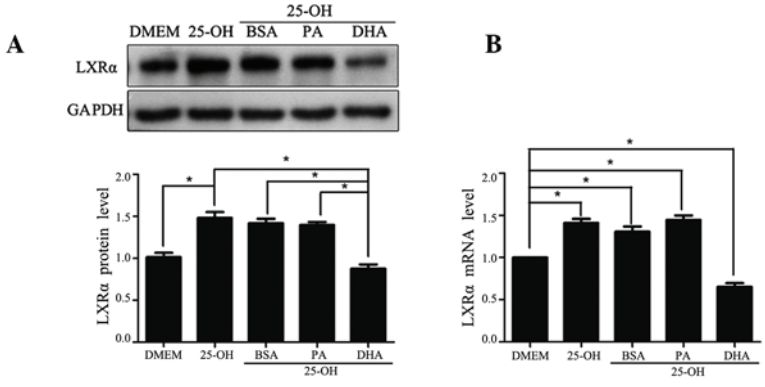

Figure 3. Regulatory effects of LXRα expression on fatty acid treatment. Following overnight culture in a serum-free medium, HepG2 cells were exposed to 25-hydroxycholesterol (10 μmol/l) with or without fatty acid/BSA complexes (100 μmol/l) for 24 h. At the end of the treatment, total cell lysates were isolated for western blot analysis for LXRα and GAPDH (A), and total RNA was isolated for quantitative polymerase chain reaction analysis of target genes (B). The data shown are the mean (± SEM) of three separate experiments (*P<0.05). LXRα, liver X receptor α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; 25-OH, 25-hydroxycholesterol; BSA, bovine serum albumin; PA, palmitic acid; DHA, docosahexanoic acid.

cantly antagonize the inhibition of 25-hydroxycholesterol on LDLR mRNA, even at the highest dose applied.

DHA exerts a downregulatory effect on the expression of LXRα. The expression of LXRα was detected on the mRNA and protein levels. Compared with HepG2 cells cultured with DMEM, LXRα protein was increased 1.4-fold (P<0.05) when treated with 25-hydroxycholesterol (10 μmol/l; Fig. 3A). With co-treatment of 25-hydroxycholesterol, DHA, but not PA, significantly downregulated the protein level of LXRα by 50%

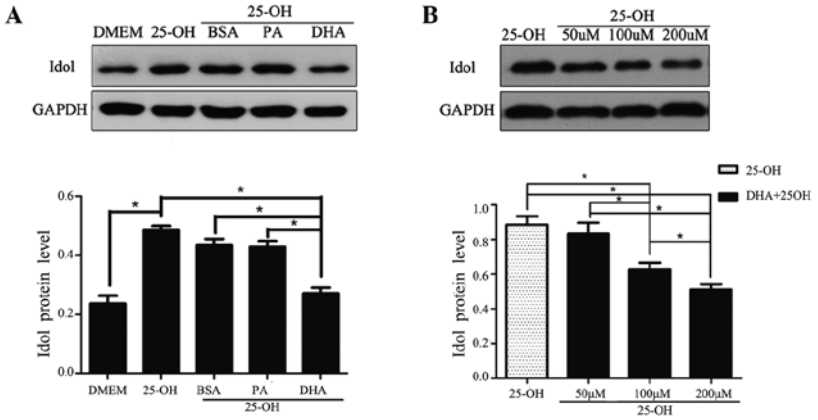

Figure 4. Regulatory effects of Idol protein on fatty acid treatment. Following overnight culture in a serum-free medium, HepG2 cells were exposed to 25-hydroxycholesterol (10 μmol/l) with or without fatty acid/BSA complexes (100 μmol/l) for 24 h (A). HepG2 cells were exposed to 25-hydroxycholesterol (10 μmol/l) with DHA at various concentrations (50, 100 and 200 μmol/l) (B). At the end of the treatment, total cell lysates were isolated for western blot analysis for Idol and GAPDH. The data shown are the mean (± SEM) of at least three separate experiments (*P<0.05). Idol, inducible degrader of the low-density lipoprotein receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; 25-OH, 25-hydroxycholesterol; BSA, bovine serum albumin; PA, palmitic acid; DHA, docosahexanoic acid.

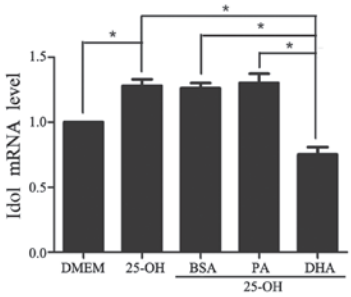

Figure 5. Regulatory effects of Idol mRNA on fatty acid treatment. Following overnight culture overnight in a serum-free medium, HepG2 cells were exposed to 25-hydroxycholesterol (10 μmol/l) with or without fatty acid/BSA complexes (100 μmol/l) for 24 h. Idol mRNA levels were analyzed by quantitative polymerase chain reaction and were normalized to glyceraldehyde 3-phosphate dehydrogenase. The data shown are the mean (± SEM) of three separate experiments (*P<0.05). Idol, inducible degrader of the low-density lipoprotein receptor DMEM, Dulbecco's modified Eagle's medium; 25-OH, 25-hydroxycholesterol; BSA, bovine serum albumin; PA, palmitic acid; DHA, docosahexanoic acid.

compared with control cells treated with BSA vehicle. Parallel alteration was observed with the LXRα mRNA levels (Fig. 3B).

DHA decreases the expression of Idol in a dose-dependent manner. Since DHA exerted a downregulatory effect on the expression of LXRα, we evaluated the change in Idol, which is the downstream protein of the nuclear receptor LXRα. As expected, the expression of Idol was increased by 25-hydroxycholesterol and decreased by DHA on the mRNA and protein levels, by 60% and 62%, respectively, in coordination with the change in LXRα expression (Figs. 4 and 5). In addition, a dose-dependent decrease in the amount of Idol protein was observed with the various doses of DHA (Fig. 4B).

Discussion

Idol has been identified as a novel post-transcriptional regulator of LDLR abundance, as its full name implies. Containing a unique C-terminal RING domain, Idol is an E3 ubiquitin ligase that triggers ubiquitination of LDLR and promotes its internalization and degradation (11,17). Distinct from LDLR and PCSK9 genes, Idol is directly regulated by LXRα, which is activated in response to cellular cholesterol excess (12). Conversely, the expression of SREBP2 is responsive to cellular cholesterol depletion (7). Therefore, the LXRα-Idol-LDLR pathway and the SREBP2-PCSK9-LDLR pathway are complementary but independent pathways in the response to cellular sterol status.

As one type of oxysterol, 25-hydroxycholesterol strongly represses the SREBP2 process and slightly activates the LXRα pathway (18). Since LDLR is the downstream protein in the two pathways, the net effect of 25-hydroxycholesterol is the downregulation of LDLR abundance. Therefore, in the present study, HepG2 cells were initially treated with 25-hydroxycholesterol to decrease basal levels of the LDLR protein as previously described (16). In the presence of 25-hydroxycholesterol, DHA significantly increased LDLR protein level in a dose-dependent manner over 100 μmol/l.

Although numerous *in vivo* and *in vitro* studies have been conducted, the mechanism by which omega-3 fatty acids exerted their effect on LDLR expression remained unclear. Previous studies have indicated that multiple mechanisms are involved in regulating the LDLR gene independently of SREBP1 (16,19). Conversely, a number of studies demonstrated that DHA inhibited lipogenic gene transcription by suppressing the expression of SREBP1, possibly at the post-transcriptional level (20,21). There was no evidence that LDLR played a role in this; however, DHA downregulated the hepatic mRNA of SREBP2, and LDLR was observed in hamsters fed a high cholesterol diet (22).

The present study revealed that DHA had no effect on LDLR mRNA levels even at the highest dose applied, suggesting that DHA may affect LDLR via mechanisms other than gene expression. In addition, a number of studies demonstrated that DHA inhibits the activity of LXRα (13,14). Furthermore, LXRα controls the activation of the transcription of Idol (11). Therefore, we speculated that the unclear mechanism by which DHA increased LDLR abundance is most likely mediated by suppression of the LXRα-Idol pathway.

When delivered with the appropriate treatment, we observed that 25-hydroxycholesterol activated LXRα expression since it is an agonist of LXRα. In line earlier findings, DHA significantly repressed the expression of LXRα at the mRNA and protein levels when administered with 25-hydroxycholesterol (16). As expected, the LXRα target gene Idol was significantly decreased on DHA treatment. Consistent with the change in mRNA levels, DHA reduced Idol protein. Moreover, the reduction of Idol abundance presented in a dose-dependent manner, corresponding with the alteration of LDLR protein but in the opposite manner.

All of these results confirmed that DHA suppressed the LXRα-Idol pathway, and in turn lowered the Idol-induced degradation of LDLR protein, leading to the upregulation of LDLR.

However, there was no significant difference in the LDLR abundance between DHA treatment and BSA vehicle or PA treatment when 25-hydroxycholesterol was absent. We therefore speculated that DHA possibly exerted an upregulatory effect of LDLR abundance under the condition of high cholesterol levels. In our study, DHA significantly attenuated the suppression effect of 25-hydroxycholesterol on LDLR abundance, as well as the LXRα-Idol pathway. That is, DHA modified LDLR abundance via suppression of the LXRα-Idol pathway. The findings of the present study suggest that in addition to the suppression of the LXRα-Idol pathway, there are multiple mechanisms participating in the regulation of LDLR by DHA treatment, and further exploration is required.

In conclusion, we identified that DHA increased hepatic LDLR abundance in the presence of 25-hydroxycholesterol. Multiple mechanisms are involved in DHA regulating the LDLR abundance, and the suppression of LXRα-Idol pathway is one such mechanism.

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