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Polyunsaturated fatty acids downregulate the low density lipoprotein receptor of human HepG2 cells

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Abstract

The aim of the study was to investigate the effect of different fatty acids on the low density lipoprotein (LDL) receptor of cultured human liver HepG2 cells. Previous studies investigating the effect of fatty acids on LDL expression have reported conflicting findings and are limited to measurements of LDL receptor binding activity. Therefore, this study is unique in that the relative effects of different fatty acids on the LDL receptor were investigated at three different stages of expression: 1) functional cellular LDL binding activity, 2) amount of LDL receptor protein and 3) LDL receptor mRNA level. The HepG2 cells were incubated for 24 hr with either 100 μM palmitic, oleic, linoleic or eicosapentaenoic acid (EPA). The measurement of LDL receptor binding activity was with colloidal gold-LDL conjugates, cellular LDL receptor protein was by western blotting and LDL receptor mRNA by Southern blotting of reverse-transcribed, polymerase chain reaction-amplified cDNA. The LDL receptor binding activity, protein and mRNA levels decreased as the degree of unsaturation of the fatty acids increased (palmitic acid $\geq$ oleic acid $>$ linoleic acid $>$ EPA) and the inverse relationship held whether or not cholesterol was included in the culture media. The relative differences were very similar for the three stages of expression indicating that modulation of the LDL receptor by the fatty acids occurred at the level of gene transcription. The increased susceptibility to oxidation of the polyunsaturated fatty acids was unlikely to be a factor in the effect because EPA and linoleic acid (250 μM) still downregulated the LDL receptor in the presence of the antioxidant vitamin E (50 μM). In conclusion, the polyunsaturates, linoleic acid and EPA, effectively downregulated the LDL receptor of HepG2 cells compared to palmitic acid. The effects of these fatty acids were observed at the level of LDL receptor binding activity, protein and mRNA, strongly suggesting that the fatty acid effects were at the level of gene transcription.

Keywords: LDL receptor; HepG2 cells; Polyunsaturated fatty acids; Palmitic acid; Oleic acid; Linoleic acid; Eicosapentaenoic acid; EPA; Vitamin E; Cholesterol

1. Introduction

It has been known for over 40 years that plasma cholesterol can be altered in humans by changing the balance of the different dietary fatty acids [1,2]. The saturated fatty acids such as palmitic acid (C16:0) raise plasma and low density lipoprotein (LDL) cholesterol levels compared to carbohydrates [1–6]. In contrast, c*is* monounsaturated fatty acids such as oleic acid (C18:1) and the ω-6 polyunsaturated fatty acids such as linoleic acid (C18:2) lower plasma and LDL cholesterol levels compared to saturates [1–12].

The effect of the ω-3 polyunsaturated fatty acids on plasma and LDL cholesterol is less clear. The marine fish fatty acids eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C20:6), are highly effective in lowering plasma triacylglycerols in man but their effect on cholesterol, especially LDL cholesterol, has varied [13,14]. Like the ω-6 polyunsaturated fatty acids, the ω-3 fatty acids appear to be hypocholesterolemic relative to saturated fatty acids in that reductions in plasma and LDL cholesterol have been found in studies where

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Abbreviations: ACAT, acyl-CoA cholesterol acyltransferase; DMEM, Dulbecco's Modified Eagles Medium; ECL, enhanced chemiluminescence; EPA, eicosapentaenoic acid; FCS, fetal calf serum; LDL, low density lipoprotein; LPDFCS, lipoprotein deficient fetal calf serum; PCR, polymerase chain reaction.

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the saturated fat content was lower in the fish oil diet than in the control diet [13]. However, in studies which controlled for the saturated fatty acid content of the diets, fish oil most often had no effect or increased LDL cholesterol [13,14].

Over the years, a spectrum of studies have been undertaken to determine how dietary fatty acids modulate plasma and LDL cholesterol but their main mechanism of action still remains unclear. Some animal studies suggest that the saturated fatty acids increase blood cholesterol by down-regulating the hepatic LDL receptor, thereby decreasing the clearance of LDL cholesterol from the plasma [15,16]. Recently, however, data from humans and animals suggest that saturated fat has little effect on the clearance of LDL from the plasma and that it may increase blood cholesterol by increasing the production of LDL [17,18]. Researchers have also investigated whether PUFAs modulate the hepatic LDL receptor activity using animal models with the assumption that the effect of fatty acids on the LDL receptor in animal species is mimicked in humans [19–21]. However, there are inconsistencies in these whole animal studies which make it difficult to reach a consensus. Similarly, studies examining the effects of PUFAs on hepatic LDL receptor activity in established human cell lines have also shown extreme variability [21–23]. Furthermore, most of these studies are limited to measurements of LDL receptor binding activity. To date there has only been one *in vitro* study which describes the effect of PUFAs on LDL receptor mRNA levels [22]. Hence, it is difficult to conclude from just one study whether PUFAs exert a direct effect at the level of LDL receptor gene transcription.

The aim of the present study was therefore to investigate the effect of different fatty acids on the expression of the LDL receptor using the human liver HepG2 cell line which is known to exhibit LDL receptors amenable to regulation [25]. The approach of this study is unique in that the relative effects of different fatty acids on the LDL receptor were investigated at three different stages of expression: 1) functional cellular LDL binding activity, 2) amount of LDL receptor protein and 3) LDL receptor mRNA level. The cells were incubated with palmitic, oleic, linoleic and eicosapentaenoic acids as representative fatty acids of the saturates, monounsaturates, ω-6 polyunsaturates and ω-3 polyunsaturates, respectively. The LDL receptor binding activity, protein and mRNA were measured in order to determine whether the receptor was affected at the level of gene transcription. The possibility that the susceptibility to oxidation of EPA and linoleic acid could play a role in regulating the LDL receptor was further investigated by performing experiments in the presence and absence of α-tocopherol (vitamin E).

2. Experimental procedures

2.1. Cell culture

The HepG2 cells (American Type Culture Collection, Rockville, MD, USA) were grown [25,26] at 37°C under 5% CO_2, in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 12 μg/ml penicillin, 16 μg/ml gentamycin, 20 mM HEPES, 10 mM NaOH, 2 mM L-glutamine, and 10% (v/v) fetal calf serum (FCS) (CSL, Melbourne, Australia). Cells were grown in 175 cm^2 flasks containing DMEM supplemented with 10% FCS until confluent. Cells were then subcultured at 5 x 10^5 cells into 25 cm^2 flasks containing the same media.

2.2. Fatty acid enrichment and analysis

The fatty acid enrichment experiments were initiated when the cells were 80% confluent or 1.5–2 x 10^5 cells/cm^2. The media was first replaced, 24 h before incubation with the fatty acids, with 10 ml of fresh DMEM containing 10% FCS or 10% lipoprotein deficient fetal calf serum (LPDFCS). The LPDFCS was prepared from FCS by removing the lipoproteins by ultracentrifugation at $d = 1.215$ g/ml. The fatty acid enrichment was then accomplished using a modified procedure of Spector et al. [27]. Sodium salts of the various fatty acids (Sigma Chemical Co., Castle Hill, NSW, Australia) were added to the cell media as 5:1 fatty acid to albumin complexes [28] at concentrations up to 500 μM fatty acid. In some experiments, 50 μM α-tocopherol (Sigma-Aldrich, Castle Hill, NSW, Australia) was also added to the media. Following a 24 h incubation, the culture media was decanted and the cells were washed 3 times in cold phosphate buffered saline (PBS). The cells were then scraped off the flasks and suspended in 5 ml cold PBS and pelleted at 400 x *g* for 10 min at 4°C and finally resuspended in 250 μl of PBS.

For fatty acid analysis, cellular lipids were extracted using the procedure of Bligh and Dyer [29] and transmethylated as described by Keough and Davis [30]. The fatty acids were then analyzed on a Hewlett Packard 5890 gas chromatograph fitted with a Supelcowax 10 fused capillary column (30 m, 0.53 mm ID and 1.0 mm film thickness) operated isothermally at a temperature of 190°C with helium as the carrier gas (flow rate 15 ml/min). Heptadecanoic acid was used as the internal standard.

2.3. LDL receptor binding assay

The HepG2 cells were assayed for LDL receptor binding activity as described for mononuclear cells [31]. Human LDL, $1.025 > d > 1.050$ g/ml, was isolated from 2–4 days old blood (Red Cross, Adelaide, South Australia) by sequential ultracentrifugation [32] and conjugated to colloidal gold (LDL-gold) as previously described [31,33]. Freshly collected intact HepG2 cells, 100 μg of protein as measured by the method of Lowry et al. [34], were incubated for 1 h at room temperature with LDL-gold (20 μg protein/ml) [34] and buffer (60 mM Tris-HCL, pH 8.0, 20 mg/ml BSA) in a total volume of 300 μl either in the presence of 2 mM $Ca(NO_3)_2$ to measure total binding or 20 mM EDTA to measure calcium-independent binding. The cells were then

pelleted by centrifugation at 400 x *g* for 10 min, resuspended and washed in 300 μl of 2mM $Ca(NO_3)_2$ for total binding or 300 μl of 20mM EDTA (pH 8.0) for nonspecific binding and centrifuged at 400 x *g* for 10 min. The HepG2 cells were finally resuspended in 120 μl of 4% (w/v) gum arabic and the cell-bound LDL-gold was quantified using silver enhancement solution (IntenSE BL kit, Amersham, Castle Hill, NSW, Australia) and a Cobas Bio autoanalyser (Roche Diagnostica, Nutley, NJ). The specific binding activity of LDL to the LDL receptor (calcium-dependent binding) of HepG2 cells was calculated as the total minus the calcium-independent binding.

2.4. LDL receptor protein assay

The mass of LDL receptor protein in the HepG2 cells was measured essentially as previously described for rat liver [35,36]. The HepG2 cells were solubilized by incubation for 12 h in a solution of 1.5% (w/v) Triton X-100 containing 50 mM Tris-maleate (pH 6), 2 mM $CaCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 mM n-ethylmaleamide. Solubilized cell proteins (100 μg) [34] were separated by electrophoresis on 2–15% sodium dodecyl sulfate (SDS)-polyacrylamide gradient gels at 30 mA for 5 h. Separated proteins were electrotransferred at 45 V for 12 h onto 0.45 μm nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and the membranes were blocked for 1 h at room temperature in 10 mM Tris-HCL buffer, pH 7.4, containing 150 mM NaCl and 10% (w/v) skim milk powder.

After washing in 10 mM Tris-HCL buffer, pH 7.4, containing 150 mM NaCl and 1% (w/v) skim milk powder, the membranes were incubated with a rabbit polyclonal anti-LDL receptor antibody (3.7 μg protein/ml in 10 mM Tris-HCL buffer, pH 7.4, containing 150 mM NaCl and 1% (w/v) skim milk powder) [35,36]. The membranes were then incubated with anti-rabbit IgG linked to horseradish peroxidase (Amersham, Castle Hill, NSW, Australia), diluted 1:5000 in 10 mM Tris-HCL buffer, pH 7.4 containing 150 mM NaCl and 1% (w/v) skim milk powder and subsequently washed twice with 10 mM Tris-HCL buffer, pH 7.4, containing 150 mM NaCl and 2 mM $CaCl_2$. The membranes were then soaked in enhanced chemiluminescence (ECL) detection kit solution and exposed to hyper-film ECL (Amersham, Castle Hill, NSW, Australia) for 1 to 5 min. The images were scanned to determine the intensity of the LDL receptor protein bands using an LKB Ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, North Ryde, Australia) and the measurements in arbitrary absorbance units were taken as the relative mass of LDL receptor protein in the HepG2 cells.

2.5. LDL receptor mRNA assay

To assay for the LDL receptor mRNA [37], total cellular RNA was first isolated using the procedure of Chomocznski and Sacchi [38]. The LDL receptor mRNA was then measured using reverse transcription and the polymerase chain reaction to incorporate a nucleotide conjugated with digoxygenin into an amplified LDL receptor sequence [39].

The isolated HepG2 cell total RNA was reversed transcribed into cDNA along with an internal standard, AW109 (Perkin-Elmer Cetus Instruments, Norwalk, CT), a synthetic piece of cRNA which contains primer site sequences unique to the LDL receptor, using the Moloney Murine Leukemia Virus reverse transcriptase (50 U/ml, Perkin-Elmer Cetus Instruments, Norwalk, CT). The transcription was done by sequentially incubating the transcription mixture at 23°C for 10 min, 45°C for 15 min and 95°C for 5 min in a Thermal Cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT) and finally chilling on ice.

An LDL receptor sequence was then amplified using the polymerase chain reaction (PCR) with AW125 and AW126 (Perkin Elmer Cetus, Norwalk, CT) as the downstream and upstream primers, respectively. During the amplification a modified nucleotide, dUTP conjugated to digoxygenin (DIG), was incorporated into the LDL receptor sequence. The amplification was done with a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) for 27 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min. After 27 cycles, a further extension period of 10 min at 72°C was done.

The amplified sequences were size fractionated by electrophoresis for 90 min at 90 V in 3% (w/v) agarose gels with 0.8 mM Tris acetate, pH 8.5, and 0.04 mM EDTA as running buffer and the DNA was transferred onto positively charged nylon membranes (Boehringer Mannheim, Rose Park, Australia) by blotting for 4 hr in 0.15 M Na_3Citrate, pH 7.6 and 1.5 M NaCl. The sequences were then visualized by exposing the nylon membranes to hyper-film ECL (Amersham, North Ryde, Australia) after incubation with an anti-digoxygenin-IgG antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and an ECL alkaline phosphatase substrate solution containing AMPPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2-(5-chloro) tricyclo [3.3.1.1] decan}-4-y) phenyl phosphate) (Boehringer Mannheim, Rose Park, Australia).

The films were then scanned using the LKB Ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, North Ryde, Australia) to determine the intensity of the two bands corresponding to 1) cellular LDL receptor mRNA at 258 bp and 2) synthetic AW109 internal standard RNA at 301 bp. The amount of LDL receptor mRNA in the HepG2 cells was calculated relative to the intensity of the band for the known amount of AW109 RNA added as internal standard and was expressed as copies per mg of cellular RNA originally reverse transcribed.

Table 1
Fatty acid composition of HepG2 cells incubated with various fatty acids.

Fatty Acid	Control	Palmitic (16:0)	Oleic (18:1)	Linoleic (18:2)	EPA (20:5)
14:0	2.0 ± 0.3	1.6 ± 0.1	1.5 ± 0.9	1.5 ± 0.8	2.2 ± 0.5
14:1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
16:0	19.0 ± 1.2	33.5 ± 2.3	14.4 ± 1.7	15.0 ± 2.1	20.8 ± 2.3
16:1	4.4 ± 0.1	6.5 ± 1.1	2.8 ± 0.8	2.7 ± 0.6	2.9 ± 0.4
18:0	11.3 ± 1.1	9.4 ± 0.8	9.7 ± 1.3	8.4 ± 1.9	10.1 ± 1.4
18:1	27.0 ± 1.8	21.0 ± 1.8	43.1 ± 3.2	20.0 ± 2.2	24.0 ± 3.1
18:2	4.0 ± 0.5	4.0 ± 0.5	3.1 ± 0.5	20.7 ± 2.4	2.9 ± 1.1
20:3	1.5 ± 0.8	1.4 ± 0.1	1.0 ± 0.2	11.3 ± 0.2	0.6 ± 0.5
20:4	13.7 ± 1.7	10.3 ± 0.9	10.3 ± 1.3	6.2 ± 2.1	5.3 ± 1.5
20:5	0.90 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	13.6 ± 0.9
22:5	0.8 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.8 ± 0.2	4.1 ± 0.7
22:6	3.7 ± 0.3	2.4 ± 0.7	3.1 ± 0.4	3.7 ± 0.8	1.6 ± 0.7

Values are expressed as percentages relative to the total amount of fatty acids and are mean ± SEM for triplicate cell incubations determined in duplicate.

3. Results

3.1. Cell fatty acid composition

When HepG2 cells were incubated for 24 h in the presence of the various fatty acids (100 μM), added as BSA complexes, analysis by gas chromatography confirmed that the fatty acids added to the media were incorporated into the cells to a similar extent during the incubation (Table 1). Compared to control cells incubated in the absence of added fatty acids, cells increased their percentage of the treatment fatty acid by 14.5 (from 19% to 33.5%) for palmitic acid, 16.1 (from 27% to 43.1%) for oleic acid, 16.7 (from 4% to 20.7%) for linoleic acid and 12.7 (from 0.9% to 13.9%) for EPA.

3.2. Fatty acids and the LDL receptor

The effects of the different fatty acids on LDL receptor activity, LDL receptor protein and LDL receptor mRNA are shown in Figures 1, 2 and 3, respectively. The cells incubated with palmitic and oleic acid, in a background media of DMEM plus 10% LPDFCS, showed no significant difference in LDL receptor activity, protein mass and mRNA levels compared to cells incubated in the absence of added fatty acids. However, LDL receptor activity, protein and mRNA levels for cells incubated in a background media of DMEM plus 10% LPDFCS were suppressed by approximately 40 and 70% in the presence of linoleic acid and EPA, respectively, compared to cells incubated with palmitic or oleic acid or without fatty acids.

In the absence of added fatty acids, the LDL receptor activity, protein and mRNA levels, for HepG2 cells incubated in background media that contained cholesterol (DMEM plus 10% FCS), were significantly decreased (-75%) compared to cells incubated in background media that was deficient in cholesterol (DMEM plus 10% LPDFCS). When cholesterol was present in the background media (DMEM plus 10% FCS), palmitic, oleic, and linoleic acid increased LDL receptor activity, protein and mRNA levels compared to cells incubated in the absence of added fatty acids. However, when cells were enriched with EPA, the LDL receptor activity, protein and mRNA remained relatively unchanged compared to the control cells.

Relative to palmitic acid however, linoleic acid and EPA decreased LDL receptor activity, protein and mRNA to the same extent in the background media containing FCS as they did in the media containing lipoprotein-deficient serum. In contrast to oleic acid in the DMEM + 10% LPDFCS, where oleic acid had no effect compared to palmitic acid, oleic acid in the DMEM + 10% FCS background media significantly decreased LDL receptor activity, protein and mRNA compared to palmitic acid in either cholesterol-

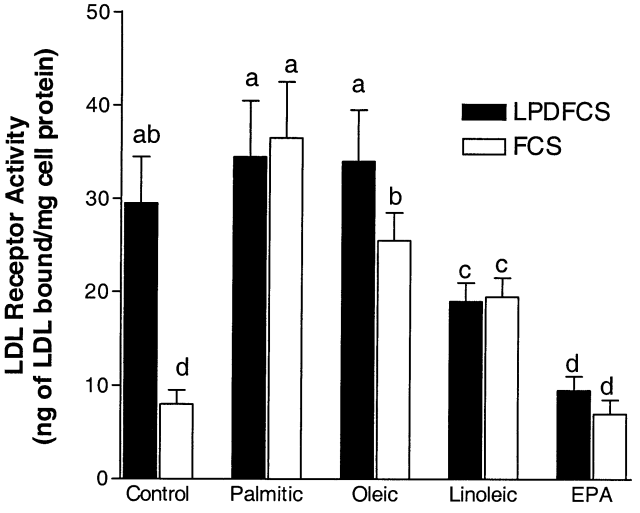

Fig. 1. The effect of fatty acids on LDL receptor binding activity. The HepG2 cells were incubated for 24 h with no added fatty acids as a control or 100 μM palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) or EPA (C20:5) as indicated either in the absence (DMEM + 10% LPDFCS, solid bars) or presence (DMEM + 10% FCS, open bars) of cholesterol. The LDL receptor binding activity was measured in triplicate as the calcium-dependent binding of LDL-gold to the intact cells. Values are means ± SEM of 4 experiments and those not sharing a common letter are significantly different at $P < 0.01$.

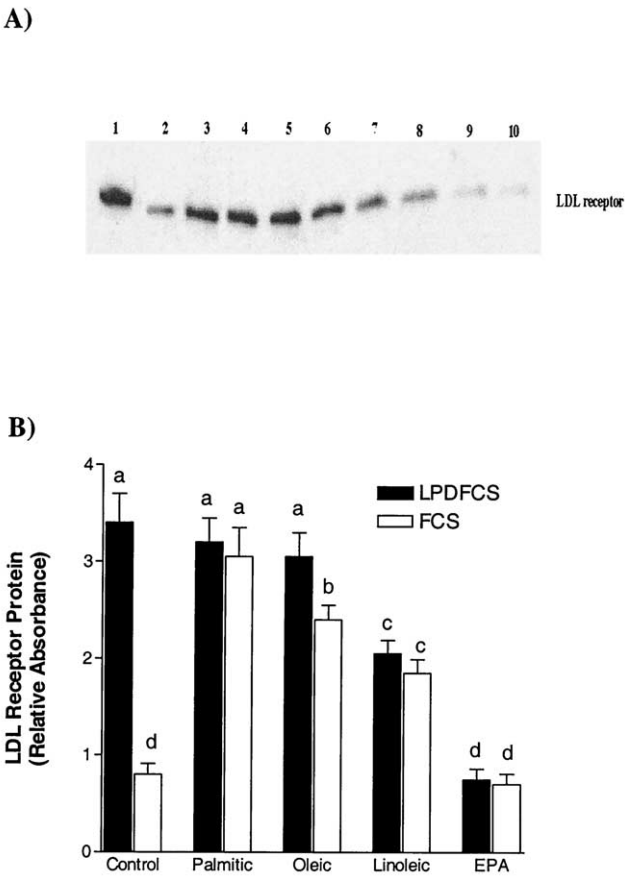

Fig. 2. The effect of fatty acids on LDL receptor protein. The HepG2 cells were incubated for 24 h with no added fatty acids as controls or 100 μM palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) or EPA (C20:5) either in the absence (DMEM + 10% LPDFCS, solid bars in **B**) or presence (DMEM + 10% FCS, open bars in **B**) of cholesterol. The relative mass of LDL receptor protein in the cells was determined in duplicate by western blotting and ECL detection using a specific polyclonal antibody against the LDL receptor. A representative chemiluminescence pattern is shown in **A).** The lanes represent LDL receptor protein bands from cells incubated in DMEM with (1) LPDFCS and no added fatty acids, (2) FCS and no added fatty acids, (3) LPDFCS and palmitic acid, (4) FCS and palmitic acid, (5) LPDFCS and oleic acid, (6) FCS and oleic acid, (7) LPDFCS and linoleic acid, (8) FCS and linoleic acid, (9) LPDFCS and EPA and (10) FCS and EPA. The results in **B)** are expressed as arbitrary absorbance units and are means ± SEM of 4 experiments. Values not sharing a common letter are significantly different at $P < 0.01$.

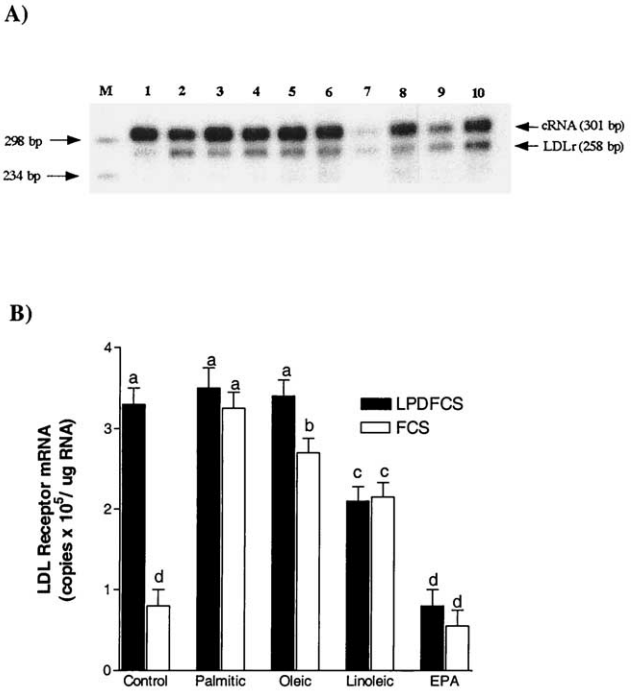

Fig. 3. The effect of fatty acids on LDL receptor mRNA. The HepG2 cells were incubated for 24 h with no added fatty acids as controls or 100 μM palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) or EPA (C20:5) either in the absence (DMEM + 10% LPDFCS, solid bars in **B**) or presence (DMEM + 10% FCS, open bars in **B**) of cholesterol. After reverse transcription of the cellular mRNA, a cDNA sequence of the LDL receptor was amplified and dioxygenin-dUTP was inserted into the sequence by PCR. The LDL receptor sequence was then quantified relative to an internal standard sequence in plasmid AW109 by blotting and ECL detection using an antibody against deoxygenin-dUTP. A representative chemiluminescence pattern is shown in **A).** The lanes represent the bands visualized for the AW109 internal standard (301 base pairs) and the LDL receptor mRNA (258 base pairs) from cells incubated with (1) FCS and no added fatty acids, (2) LPDFCS and no added fatty acids, (3) FCS and palmitic acid, (4) LPDFCS and palmitic acid, (5) FCS and oleic acid, (6) LPDFCS and oleic acid, (7) FCS and EPA, (8) FCS and linoleic acid, (9) LPDFCS and EPA and (10) LPDS and linoleic acid. Bands in lane (M) are markers of 239 and 239 base pairs. The results in **B)** are expressed as the number of copies of LDL receptor mRNA per μg total RNA relative to the 301 base pair cRNA internal standard and are means ± SEM of 4 experiments. Values not sharing a common letter are significantly different at $P < 0.01$.

containing or cholesterol-deficient media. The LDL receptor activity, protein and mRNA measurements were also significantly lower when the cells were incubated with oleic acid in DMEM + 10% FCS compared to oleic acid in DMEM + 10% LPDFCS and were halfway between the levels observed with palmitic and linoleic acid in either background media.

Regression analysis of the whole data set (Fig. 1–3) revealed a very high correlation between: 1) LDL receptor activity and LDL receptor protein ($r = 0.946$, $P < 0.01$), 2) LDL receptor activity and LDL receptor mRNA ($r = 0.975$, $P < 0.01$) and 3) LDL receptor protein and LDL receptor mRNA ($r = 0.982$, $P < 0.01$). This implied that the effects of the various treatments on the LDL receptor were likely to be at the level of gene transcription.

3.3. EPA, linoleic acid,α-tocopherol and the LDL receptor

From these results, it appeared that the expression of the LDL receptor progressively decreased as the number of double bonds of the fatty acid increased, especially when the background media contained cholesterol in the form of fetal calf serum. As double bonds render polyunsaturated fatty acids highly susceptible to oxidation, the HepG2 cells were exposed to EPA and linoleic acid in the presence or

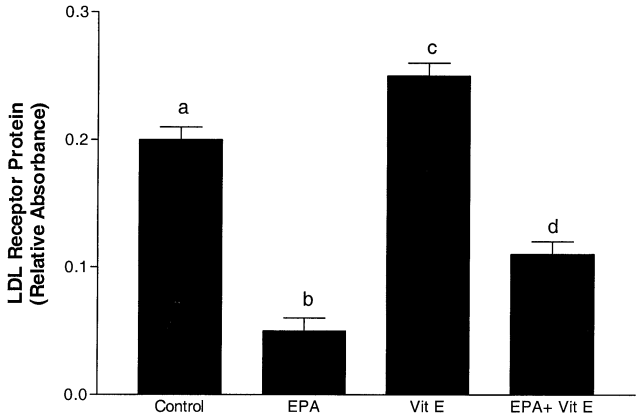

Fig. 4. The effect of EPA on LDL receptor protein in the presence and absence of vitamin E. The HepG2 cells were incubated for 24 h with either no fatty acid or vitamin E as control, 250 μM EPA only, 50 μM vitamin E only or 250 μM EPA + 50 μM vitamin E. The relative mass of LDL receptor protein in the cells was determined in duplicate by western blotting and ECL detection using a specific polyclonal antibody against the LDL receptor. Results are expressed as arbitrary absorbance units and are means ± SEM of 2 experiments. Values not sharing a common letter are significantly different at $P < 0.01$.

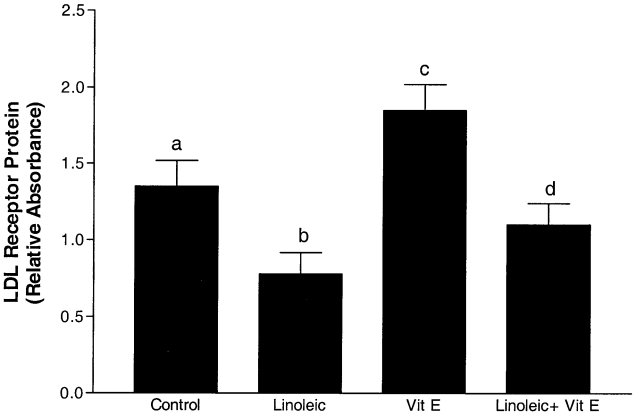

Fig. 5. The effect of linoleic acid on LDL receptor protein in the presence and absence of vitamin E. The HepG2 cells were incubated for 24 h with either no fatty acid or vitamin E as control, 250 μM linoleic acid only, 50 μM vitamin E only or 250 μM linoleic acid + 50 μM vitamin E. The relative mass of LDL receptor protein in the cells was determined in duplicate by western blotting and ECL detection using a specific polyclonal antibody against the LDL receptor. Results are expressed as arbitrary absorbance units and are means ± SEM of 2 experiments. Values not sharing a common letter are significantly different at $P < 0.01$.

absence of α-tocopherol (vitamin E), the main antioxidant which protects fatty acids from oxidation *in vivo* [34]. For these experiments, 250 μM fatty acid and 50 μM α-tocopherol were used and only the effect on LDL receptor protein was determined.

In experiments not shown here, the inhibitory response was found to be dose-responsive up to 500 μM. Consistent with this, 250 μM EPA decreased the LDL receptor protein by about 80% (Fig. 4) compared to 100 μM EPA which lowered it 70% (Fig. 2). Similarly, 250 μM linoleic acid decreased it by 50% (Fig. 5) compared to 100 μM linoleic acid which lowered it by 40% (Fig. 2).

It was also observed that the amount of LDL receptor protein was decreased in cells incubated with 250 μM EPA whether 50 μM vitamin E was present or not (Fig. 4). Similarly, LDL receptor protein mass in cells incubated with 250 μM linoleic acid was also depressed regardless of the presence or absence of the antioxidant vitamin (Fig. 5). This suggests that the suppression of the LDL receptor by EPA or linoleic acid was unlikely to be the result of fatty acid oxidation. It was also observed, however, that cells incubated in the presence of α-tocopherol consistently had significantly higher ($p < 0.05$) amounts of the LDL receptor protein than cells not incubated with the vitamin whether EPA or linoleic acid were present or not. The α-tocopherol therefore upregulated the LDL receptor in its own right as we have previously reported [37].

4. Discussion

This study is unique in that the relative effects of different fatty acids on the LDL receptor were investigated at three different stages of expression: 1) functional cellular LDL binding activity, 2) amount of LDL receptor protein and 3) LDL receptor mRNA level. Incubation of HepG2 cells with the different fatty acids clearly demonstrated that the LDL receptor expression decreased progressively as the degree of unsaturation of the fatty acid increased and this was consistent across the three measurements of LDL receptor binding activity, protein and mRNA. The inverse relationship mostly held with or without cholesterol in the culture media although a decrease in receptor expression was only seen for oleic acid when the media contained cholesterol in the form of fetal calf serum. The inverse relationship was however unlikely to be due to oxidation of the unsaturated fatty acids because EPA and linoleic acid still downregulated the LDL receptor in the presence of vitamin E.

Downregulation of the LDL receptor by unsaturated fatty acids was most likely due to effects on transcription of the LDL receptor gene. Overall, the LDL receptor activity, measured as the calcium-dependent binding of gold-labeled LDL to the cells, went from highest to lowest as follows: palmitic acid $\geq$ oleic acid $>$ linoleic acid $>$ EPA. The reductions in the LDL receptor activity appeared to be due entirely to reductions in the relative number of LDL receptors because the total amounts of LDL receptor protein in the cells, as measured by western blotting, paralleled the reductions in the calcium-dependant binding of LDL-gold. In turn, the reductions in the number of LDL receptors were most likely due to reductions in the transcription of the LDL receptor gene because the decrease in LDL receptor mRNA was similarly affected by the unsaturated fatty acids relative to palmitic acid.

These observations are consistent with the accepted the-

ory that the LDL receptor is only regulated at the level of gene transcription [40–42]. They also mirror the recent findings that there is an inverse relationship between the level of unsaturation of fatty acids and the expression of the sterol regulatory element binding proteins (SREBPs) [43–45], transcription factors known to regulate the LDL receptor [42]. Separate studies in HepG2 cells had previously found that EPA suppressed the binding of LDL to the cells compared with oleic acid [42] and decreased the LDL receptor activity and mRNA level when the cells were incubated with EPA-enriched LDL from human subjects fed dietary fish oil [23]. However, the results from the present study are novel in that linoleic acid and oleic acid, as well as EPA, were found to decrease the LDL receptor at three levels of expression, mRNA, protein and functional activity.

The relative effects of the different fatty acids on the LDL receptor mostly held whether or not cholesterol was included in the incubation media. In line with the classical Brown and Goldstein theory [40], the LDL receptor was downregulated when cells were incubated in cholesterol-containing media (DMEM + 10% FCS) compared to cholesterol-deficient media (DMEM + 10% LPDFCS). However, as previously observed by Rumsey et al. [24] in cultured fibroblasts, the suppression of the HepG2 cell LDL receptor by extracellular cholesterol was overridden by the addition of fatty acids in the growth media, especially palmitic acid. Oleic acid was slightly less effective than palmitic acid and linoleic acid was about half as effective in overriding the downregulatory effects of cholesterol but EPA had no overriding effect. As suggested by Rumsey et al. [24], it appears that the effects of extracellular cholesterol may not be as important as the effects of the media fatty acids in controlling LDL receptor levels in cultured cells.

The observed differential effects of the unsaturated fatty acids compared to palmitic acid may however be mediated through effects on the intracellular cholesterol concentration. There are numerous studies showing that the polyunsaturated fatty acids, linoleic acid and EPA, can decrease intracellular cholesterol esterification compared to palmitic and oleic acid [46–49]. It is also well documented that EPA and linoleic acid can inhibit the secretion of lipoproteins from cells including HepG2 cells [22,48,50,51]. Interestingly, the inverse relationship between the level of unsaturation of the fatty acids and the LDL receptor expression in the present study is very similar to the relative effects of the same fatty acids on cholesterol esterification and secretion observed by Schafer and Kattermann, also in HepG2 cells [48]. The inhibition of cholesterol esterification and secretion is likely to cause an accumulation of unesterified cholesterol within the cell, thereby suppressing LDL receptor expression through the SREBP gene transcription system [42–45].

In the presence of the antioxidant vitamin E, EPA and linoleic acid were still able to downregulate the LDL receptor. The high susceptibility to peroxidation of these two long chain polyunsaturated fatty acids, did not therefore appear to be a factor in their effects on the LDL receptor. Hence, the polyunsaturated fatty acids themselves, rather than fatty acid oxidation products that may have formed during the incubation period, were most likely to have downregulated the LDL receptor.

The finding that EPA downregulates the LDL receptor of HepG2 cells is consistent with evidence that the hepatic LDL receptor is downregulated in animals fed fish oils as we have previously found in rats [52] and others have observed in hamsters [53,54] mice [45] pigs [55] and rabbits [56]. Human studies have demonstrated that LDL receptor plays an important role in the regulation of plasma LDL concentrations [40,57]. It follows then that downregulation of the LDL receptor by EPA in hepG2 cells observed in our study is consistent with the rise in plasma LDL that is often observed in humans fed fish oil [13,14,58] and may explain the increase in plasma cholesterol seen in some animals studies with marine oils [53,56]. On the other hand, ω-3 polyunsaturates, especially EPA and DHA, have been shown to consistently lower plasma triglyceride levels in virtually all human and animal experiments [13,14]. Therefore, these fatty acids are especially useful when fed to individuals with hypertriglyceridemia. The mechanism of triglyceride lowering appears to be an inhibition of secretion of VLDL triglycerides as the fatty acids interfere with the hepatic triglyceride synthesis [22] but not apo B synthesis [50]. Fish oil feeding has been also shown to diminish lipogenesis, increase ketogenesis and fatty acid oxidation, which can also contribute to the overall reduction in triglyceride secretion by the liver and lower plasma VLDL.

The finding that linoleic acid also downregulated the LDL receptor relative to palmitic acid are also consistent with the recent observations that linoleic acid can decrease SREBP levels compared to saturated fatty acids or oleic acid [43,45]. They are also consistent with findings that the hepatic LDL receptor activity was reduced in rats fed safflower oil [20] and in rabbits fed sunflower oil [56]. Hepatic LDL receptor mRNA levels have also been observed to be lower in hamsters fed a diet enriched in linoleic acid compared to palmitic acid [59]. However, if ω-6 PUFAs have the same effect on the LDL receptor *in vivo* as *in vitro,* then a decreased LDL receptor can not be the reason why plasma LDL cholesterol is lowered by these fatty acids. In this case, there must be other pathways affected by ω-6 PUFAs, which cause the cholesterol lowering. For example, the secretion of VLDL would have a major impact on plasma LDL cholesterol as this lipoprotein is the precursor of LDL. In light of this, one can assume that the effect of ω-6 PUFAs on VLDL in humans is more likely to be the mechanism by which LDL levels are decreased rather than their effects on LDL receptor activity. It has been documented that linoleic acid decreases VLDL synthesis and secretion from liver cells [51,60]. This effect of linoleic acid may be the cause the reduction in VLDL levels observed *in vivo.* Thus, the reduction of VLDL secretion by ω-6 PUFAs could be one of

the major mechanisms whereby LDL cholesterol is lowered, even when LDL receptor activity is reduced by these fatty acids.

Taken together, the present results and those of others lend support to the suggestion by Hayes and co-workers [17,43], that the clearance of LDL from the circulation may not be the predominant factor in the cholesterol-modulating effects of fatty acids. Rather, as they suggest, the relative effects the different fatty acids have on the production of LDL may be more determinate of the plasma cholesterol concentration in humans. Conversely, these results are not consistent with the hypothesis that ω-6 polyunsaturated fatty acids reduce plasma cholesterol by upregulating the hepatic LDL receptor [15,16]. Similarly, the downregulatory effects oleic acid had on the LDL receptor in the present study and on SREBPs in other studies [43] is also not consistent with monounsaturated fatty acids lowering plasma cholesterol through upregulation of the LDL receptor.

In conclusion, LDL receptor expression measured as binding activity, protein and mRNA in cultured human liver HepG2 cells decreased as the degree of unsaturation of the fatty acid increased. The fatty acids therefore appeared to regulate the LDL receptor primarily at the level of gene transcription. This inverse association was observed either in the presence or absence of cholesterol in the culture media. The susceptibility of the polyunsaturated fatty acids, EPA and linoleic acid to oxidation did not appear to be a factor as they had similar effects on the receptor whether or not the antioxidant vitamin E was included in the culture media.

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