Fatty acids liberated from high-density lipoprotein phospholipids by endothelial-derived lipase are incorporated into lipids in HepG2 cells

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We previously reported that endothelial-derived lipase (EDL) efficiently hydrolyses high-density-lipoprotein-derived phosphatidycholine (HDL-PC). In the present study, we assessed the ability of EDL to supply HepG2 cells with non-esterified fatty acids (NEFA) liberated from HDL-phospholipids. For this purpose, HepG2 cells infected with adenovirus encoding human EDL (EDL-Ad), or with control β-galactosidase-expressing adenovirus (LacZ-Ad), were incubated with ^{14}C-HDL-PC. The analysis of the cellular lipids by TLC revealed that EDL overexpression led to an increase in the amount of cellular ^{14}C-lipids, whereby the label was mainly incorporated into phospholipids and triacylglycerols (TAG). Cells expressing mutant enzymically inactive EDL (MUT-EDL-Ad) contained similar amounts of ^{14}C-TAG but higher amounts of ^{14}C-phosphatidylcholine (PC) compared with LacZ-Ad-infected cells. The co-expression of CD36 augmented the EDL-mediated accumulation of ^{14}C-lipids in HEK-293 cells. The quadrupole MS analysis of the cellular lipids revealed an increased content of PC and TAG in EDL-expressing HepG2 cells compared with MUT-EDL-Ad-expressing and control cells. However, the MUT-EDL-Ad-expressing cells contained more PC than control cells. Additionally, EDL overexpression led to a 2-fold decrease in the amount of fatty acid synthase mRNA and, in turn, a slightly, but significantly, decreased rate of fatty acid (FA) synthesis in HepG2 cells. In the present study, we show for the first time that EDL efficiently supplies HepG2 cells with NEFA derived from HDL-PL, thus affecting cellular lipid composition and FA synthesis.

Key words: adenoviral vectors, CD36, fatty acid synthase, HEK-293 cells, HepG2 cells.

INTRODUCTION

The serum lipases, lipoprotein lipase (LPL) [1,2] and hepatic lipase (HL) [3,4] are members of the triacylglycerol (TAG) lipase gene family. They play a central role in lipid and lipoprotein metabolism, catalysing the hydrolysis of lipoprotein-associated lipids, thus modulating lipoprotein composition and plasma concentrations. LPL deficiency is associated with severe hypertriglyceridemia in humans [5,6] and LPL knock-out mice [7,8]. In contrast with LPL, HL deficiency in humans is associated with elevated levels of remnant lipoproteins [9] and large high-density lipoprotein (HDL) particles [10]. In addition to their function as lipolytic enzymes, LPL and HL serve as ligands that mediate the uptake of lipoproteins or lipoprotein lipids by facilitating the lipoprotein interaction with cell surface proteoglycans and receptors [11–16].

Endothelial-derived lipase (EDL) is a new member of the TAG lipase gene family [17,18]. Overexpression of EDL in the liver of mice by intravenous injection of a recombinant EDL-adenovirus resulted in dramatically reduced HDL-cholesterol and apolipoprotein A-I plasma levels, suggesting a role for EDL in HDL metabolism [18]. We have recently reported [19] that EDL facilitates HDL binding and particle uptake, as well as selective uptake of HDL-associated cholesterol esters (CE) by HepG2 cells, independent of its enzymic activity.

EDL is a serum lipase, which like LPL and HL, is anchored via the heparin-binding site [20–22] to heparan sulphate proteoglycans on the luminal endothelial surface. In contrast with LPL and HL, EDL is a phospholipase with very little TAG lipase activity [23]. Owing to its predominant phospholipase activity [23], EDL hydrolyses HDL-associated phospholipids (HDL-PL), thus generating non-esterified acids (NEFA), which might be taken up by EDL-expressing cells.

The aim of the present study was to assess the ability of EDL to supply HepG2 cells with NEFA liberated by its phospholipase activity from HDL-PL. Furthermore, we evaluated the effect of EDL overexpression on the cellular lipid composition and endogenous fatty acid (FA) synthesis in HepG2 cells.

EXPERIMENTAL

Recombinant adenovirus and plasmids

The construction of the recombinant adenoviruses used in this study coding for the human EDL (EDL-Ad), the catalytically inactive EDL (MUT-EDL-Ad) and the control β-galactosidase-expressing adenovirus (LacZ-Ad) have been described previously [19]. For transient transfection experiments, the human EDL cDNA was cloned into the expression plasmid pBK-CMV (Stratagene) cut with *BamH*I and *Kpn*I [19]. The resulting plasmid

Abbreviations used: CE, cholesterol ester; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; EDL, endothelial-derived lipase; EDL-Ad, EDL-expressing adenovirus; FA, fatty acids; FAS, FA synthase; FCS, fetal calf serum; HDL, high-density lipoprotein; HDL-PC, HDL-derived PC; HDL-PL, HDL-associated PL; HEK-293, human embryonic kidney cells; HL; hepatic lipase; HRP, horseradish peroxidase; LacZ-Ad, β-galactosidase-expressing adenovirus; LPL, lipoprotein lipase; MOI, multiplicity of infection; MUT-EDL-Ad, catalytically inactive EDL-expressing adenovirus; NEFA, non-esterified fatty acids; PC, phosphatidylcholine; PL, phospholipids; PUFA, polyunsaturated fatty acids; Q-MS, quadrupole MS; RT, reverse transcriptase; TAG, triacylgycerols.

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was called pBK-CMV-EDL. Plasmid HisMax-CD36, harbouring CD36 cDNA, was cloned in-frame with the coding sequence for His residues into plasmid HisMax (Invitrogen; provided by Dr. Karina Preiss-Landl, Institute of Molecular Biology, Biochemistry and Microbiology, University of Graz, Austria.)

Cell culture, recombinant adenovirus infection and transient transfection

Cells were cultured under standard conditions (37 °C, 5 % CO_2 and 95 % humidity). HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum (FCS), non-essential amino acids and sodium pyruvate. Human embryonic kidney cells (HEK-293) were cultured in DMEM containing 10 % FCS. HepG2 were trypsinized, counted and seeded into 24-well trays (2×10^5 cells/well) 48 h before experiments were performed. Cells were infected 24 h later with a multiplicity of infection (MOI, i.e. the number of viable virus particles applied per cell) of recombinant adenovirus, as indicated in each experiment. The cells were infected in culture medium containing 2 % FCS for 60 min at 37 °C. Upon infection, fresh medium with 10 % FCS was added to the cells. For some experiments, HEK-293 cells were plated into 24-well plates (5×10^5 cells/well) and transiently transfected by calcium phosphate co-precipitation [24] with 6 μg of pBK-CMV (control), pBK-CMV-EDL and pBK-CMV (3 μg of each), HisMax-CD36 and pBK-CMV (3 μg of each) and pBK-CMV-EDL and HisMax-CD36 (3 μg of each). Cell culture studies were performed 24 h after infection or transfection.

Western blot analysis

Western blot was performed essentially as described previously [19]. Briefly, cells were washed with PBS, collected with a hot loading buffer [20 % (w/v) glycerol, 5 % (w/v) SDS, 0.15 % (w/v) Bromophenol Blue, 63 mmol/l Tris/HCl, pH 6.8] and boiled for 10 min before loading. The cell-lysate aliquots were fractionated by SDS/PAGE (12 % polyacrylamide gels) for 1.5 h at 150 V, blotted on to nitrocellulose, followed by the detection of the EDL bands using our anti-EDL antibody, described previously [19], and a horseradish peroxidase (HRP)-labelled mouse anti-rabbit antiserum as a second antibody. The bands were visualized using an enhanced chemiluminescent substrate for HRP (SuperSignal, Pierce, Rockford, IL, U.S.A.). To analyse the cell-surface-bound EDL, cells were washed with PBS and afterwards incubated with DMEM and heparin (2 units/ml). The heparin-releasable EDL fractions were mixed 1 : 1 (v/v) with loading buffer, incubated for 10 min at 95 °C and analysed as described for cell lysates.

Isolation and characterization of human HDL

HDL was isolated from normolipidaemic human plasma by sequential ultracentrifugation as described previously [25]. Protein concentrations were measured by the method of Lowry [26] using BSA as a standard. Total cholesterol was determined using an enzymic procedure (CHOD-PAP, Boehringer Mannheim).

Lipoprotein labelling procedure

Labelling of HDL with [^{14}C]dipalmitoyl-phosphatidylcholine (NEN, Boston, MA, U.S.A.) was performed as follows: 2 μCi of [^{14}C]dipalmitoyl-phosphatidylcholine (PC) was dried under nitrogen and then incubated with 1 ml of HDL (60 mg of total cholesterol/ml). The incubation was performed under nitrogen at 37 °C for 15 h in the dark. HDL was separated from the labelling mixture by exclusion chromatography using PD10 columns (Pharmacia). To confirm incorporation of the label into the PL fraction of HDL, the radiolabelled HDL was further subjected to ultracentrifugation, followed by extraction with hexane/isopropanol (3 : 2, v/v) and TLC analysis with $CHCl_3$/MeOH/CH_3COOH : H_2O (50 : 30 : 8 : 4, by vol.) to separate the PL. Subsequent exposure to a ^{14}C-screen and visualization on the STORM imager revealed ^{14}C-PC as the radioactive component of the labelled HDL. The protein content of ^{14}C-HDL-derived PC (HDL-PC) was determined and the appropriate amount was used for the experiments. Labelled lipoproteins were stored at 4 °C under argon atmosphere and used within 1 week.

TLC analysis of ^{14}C-lipids in cell supernatants and cells expressing EDL, MUT-EDL and LacZ

HepG2 cells were seeded into 24-well trays and infected with LacZ-Ad, EDL-Ad or MUT-EDL-Ad, each at an MOI of 60, as described above. HEK-293 cells were also seeded into 24-well trays and transiently transfected as described above. At 24 h post-infection/transfection, the medium was aspirated and the cells were incubated with the indicated concentrations of ^{14}C-HDL-PC in DMEM containing 3 % BSA at 37 °C under cell culture conditions. After incubation (time indicated in each experiment) the supernatant was collected and the cells were incubated with DMEM containing 100 units/ml heparin to remove ^{14}C-HDL-PC bound to the cell surface via EDL. Afterwards, cells were washed extensively with PBS. The lipids were extracted twice from the supernatant and cells with hexane/isopropanol (3 : 2, v/v), dried in the speed vac and redissolved in chloroform before they were applied on to TLC plastic sheets. Hexane/isopropanol/CH_3COOH (70 : 29 : 1, by vol.) was used as the mobile phase. The lipids were visualized with I_2, and lipid spots were cut out of the TLC plate, mixed into a scintillation cocktail and measured in the β-counter. The cellular protein content was measured by the method of Bradford [26a].

Phospholipase activity assay

The assay was done exactly as described previously [19]. PC substrate was made by mixing [^{14}C]dipalmitoyl-PC (NEN), lecithin (1 mg/ml) and substrate buffer Tris/TCNB [100 mM Tris/HCl, pH 7.4, 1 % (v/v) Triton X-100, 5 mM $CaCl_2$, 200 mM NaCl, 0.1 % (w/v) FA-free BSA] and subsequently dried under nitrogen. The dried PL was reconstituted in the substrate buffer. To determine EDL-phospholipase activity in the cell supernatants from EDL-overexpressing and control cells, 190 μl of the cell supernatants was added to the substrate and incubated at 37 °C for 1 h. The reaction was terminated by addition of 1 ml of 0.2 M HCl and extraction with hexane/isopropanol (3 : 2, v/v, 0.1 % HCl). Portions (500 μl) of the upper phase were dried in a speed vac and reconstituted in 100 μl of hexane/isopropanol (3 : 2, v/v). After separation by TLC (hexane/diethyl ether/acetic acid, 70 : 29 : 1, by vol.) the liberated ^{14}C-NEFA were quantitated in a scintillation counter (Beckman). In order to determine the capacity of the MUT-EDL to hydrolyse the ester bonds in the HDL-associated ^{14}C-PC, HepG2 cells were infected with EDL-Ad, MUT-EDL-Ad or LacZ-Ad, each at an MOI 60. After 48 h, cells were washed with PBS and incubated with DMEM containing 2 units/ml heparin at 37 °C for 30 min. Aliquots of 190 μl of the heparin-releasable fraction were used for the assay.

^{14}C-PC-labelled HDL was used as a substrate and incubated for 1 h at 37 °C with 190 μl of the heparin-releasable fraction, and the assay was performed exactly as described above. The released ^{14}C-FA were separated by TLC and quantified in a scintillation counter. Total cellular protein was measured by the method of Bradford. The results represent means ± S.D. of two independent experiments performed in duplicate dishes.

Determination of PL and TAG content of the cells by quadrupole MS (Q-MS)

HepG2 cells were seeded into 24-well trays and infected with LacZ-Ad, EL-Ad or MUT-EDL-Ad, each at an MOI of 60, as described above. At 24 h post-infection, the medium was aspirated, and the cells were incubated for a further 24 h with DMEM containing 10 % FCS. After incubation, the medium was aspirated, heparin release was performed, cells were washed extensively with 1 × PBS and lipids were extracted twice with hexane/isopropanol (3 : 2, v/v). Cells were dissolved in 0.5 ml of 0.2 M NaOH and aliquots were assayed for protein by the method of Lowry et al. [26]. Aliquots of the lipid extracts corresponding to identical amounts of the total cell protein were dried in the speed vac and redissolved in chloroform for the Q-MS determination.

Quantification of mRNA by reverse transcriptase (RT)-PCR

The relative amounts of mRNAs encoding human fatty acid synthase (FAS) and β-actin were measured by sequential RT-PCR. Total RNAs from LacZ-Ad- and EDL-Ad-infected HepG2 cells were extracted using the Qiagen RNeasy system (Qiagen, Vienna, Austria) according to the manufacturer's instructions. Total RNA (1.5 μg) was treated with RQ1 RNase-free DNase I (Promega, Mannheim, Germany) for 15 min at 37 °C and subsequently used as a template for first-strand cDNA synthesis in a 30 μl reaction. The reaction mix contained 0.5 mM dNTPs (Pharmacia, Vienna, Austria), 15 units of RNAguard (Pharmacia), 3.3 μM random hexamer primers (Pharmacia), 10 mM dithiothreitol (Life Technologies, Vienna, Austria), 1 × First Strand Buffer (Life Technologies) and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies). Reactions were incubated for 1 h at 37 °C, heated to 75 °C for 10 min, and 2.5 μl of the completed reactions were used as template for PCR. PCR amplifications for FAS and β-actin were performed in parallel reactions. Gene-specific oligonucleotide primers based on sequences published in GenBank® human DNA database were purchased from MWG-Biotech (Ebersberg, Germany). The primer sequences were as follows: for FAS, forward primer, 5′-AGCTGCCAGAGTCGGAGAACTTG-3′ (nucleotides 35–57), and reverse primer, 5′-CAAGAACTG-CACGGAGGTGTTGG-3′ (nucleotides 580–603); and for β-actin, forward primer, 5′-GCAAGAGAGGCATCCTCACC-3′, and reverse primer, 5′-GCACAGCCTGGATAGCAACG-3′. PCR mixtures of 50 μl contained 0.2 mM dNTPs, appropriate oligonucleotide primers at 10 μM, 1 × PCR buffer (Finnzymes Oy, Vienna, Austria) and 1 unit of Finnzyme DyNAzyme II DNA polymerase (Finnzymes Oy). The reaction mix was heated to 94 °C for 4 min, and subsequently amplification was carried out for 30 cycles (or 26 cycles in the case of β-actin): denaturation, 30 s at 94 °C, annealing, 30 s at 60 °C and extension, 1 min at 72 °C. PCR products (15–20 μl) were separated by electrophoresis through 2 % agarose at 80 V for 60 min in Tris/acetate/EDTA (TAE) buffer containing 0.05 μl/ml ethidium bromide (10 mg/ml). The relative amounts of mRNA were determined visually by densitometric scanning of gels using the E.A.S.Y. Win32 photo-documentation system (Herolab, Germany).

Measurement of FA synthesis

HepG2 cells (5×10^6 cells/plate) were seeded into 100 mm plates and infected with LacZ-Ad or EDL-Ad, each at an MOI of 60. After infection, each plate was incubated with 6 ml of DMEM plus 10 % FCS supplemented with ^3H_2O (1 mCi). At 24 h post-infection, medium was collected and cells were washed with PBS. Lipids were extracted from the medium and cells using hexane/isopropanol (3 : 2, v/v) as described above, and lipid-depleted cells were used for protein measurement. Dried lipid extracts were mixed with ethanolic KOH solution (final concentration 10 %) and incubated for 1 h at 70 °C [27]. Afterwards, the mixture was extracted 3 times with hexane to remove non-saponifiable lipids. The water phase was acidified with 6 M HCl and extracted three times with hexane/diethyl ether (1 : 1, v/v). The extract containing ^{3}H-FA was washed with water to remove glycerol and water soluble compounds. After drying over solid Na_2SO_4, radioactivity of the extract containing ^{3}H-FA was determined by scintillation counting. The ^{3}H-FA from the cell supernatant was obtained using the same method.

Statistics

Data are expressed as means ± S.D. Significance of differences was examined using Student's *t* test.

RESULTS

EDL expression in HepG2 cells infected with EDL-Ad and LacZ-Ad, as well as in HEK-293 cells transiently transfected with pBK-CMV-EDL and pBK-CMV (control), was analysed by Western blot. As shown in Figure 1(A), strong EDL-specific bands were detected in cell lysates of EDL-Ad-infected HepG2 cells, as well as in HEK-293 cells transfected with pBK-CMV-EDL. Weak endogenous EDL expression in HepG2 and HEK-293 cells was found, as demonstrated by a faint band in the cell lysates of LacZ-Ad-infected HepG2 cells and mock-transfected HEK-293 cells. Western blot analysis of the heparin-releasable fractions revealed substantial amounts of EDL bound to the cell surface of EDL-overexpressing HepG2 and HEK-293 cells (Figure 1B). A profoundly higher phospholipase activity in the cell supernatants from EDL-overexpressing HepG2 and HEK-293 cells compared with control cells indicated secretion of EDL into the medium (Figure 1C).

Our previous experiments have shown that EDL releases ^{14}C-NEFA from ^{14}C-HDL-PC very efficiently [19]. To examine the ability of EDL to supply cells with NEFA released from HDL-PL, cells were infected with EDL-Ad or LacZ-Ad, each at an MOI of 60, in parallel dishes and incubated with ^{14}C-HDL-PC for 8 h. The TLC analysis of the cellular lipids revealed that EDL-Ad infection led to a profound increase in ^{14}C-lipids in HepG2 cells. The amount of ^{14}C-PL was increased 1.6-fold and that of ^{14}C-TAG 2-fold compared with LacZ-Ad-infected cells (Figure 2). In contrast with PL and TAG in both types of cells, low percentages of label were associated with diacylglycerol (DAG), NEFA and CE fractions (results not shown). From these results it appeared that ^{14}C-NEFA liberated from ^{14}C-HDL-PC by EDL were taken up by cells and incorporated efficiently into PL and TAG. Since ^{14}C-HDL-PC could also be imported into HepG2 cells as a consequence of the HDL particle uptake [19] and selective uptake of HDL-PL [28], processes that might be enhanced by

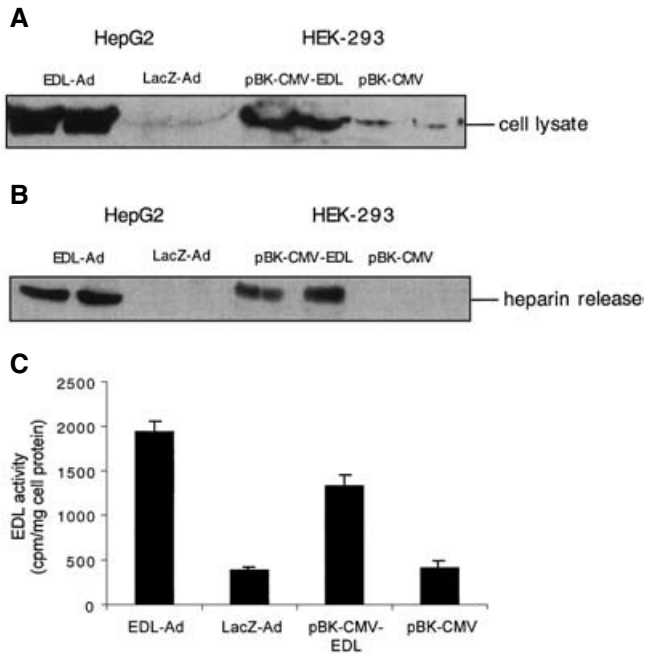


Figure 1 Western blot analysis of the EDL fraction in the cell lysate (A) and bound to the cell surface (B) of HepG2 cells infected with EDL-Ad and LacZ-Ad, and of HEK-293 cells transfected with pBK-CMV-EDL and pBK-CMV, (C) EDL phospholipase activity in the media of cells obtained as described in (B)

(**A**) HepG2 cells were plated on to 24-well trays and infected with EDL-Ad and LacZ-Ad at an MOI of 60. Cells were washed with PBS 24 h after infection, lysed with a boiling loading buffer containing 5 % (v/v) 2-mercaptoethanol and boiled for an additional 10 min before analysis by SDS/PAGE (12% gels). EDL bands were visualized by an enhanced chemiluminescence assay after incubation with an anti-EDL antiserum and HRP-labelled mouse anti-rabbit antiserum. HEK-293 cells were plated on to 24-well plates and transiently transfected with pBK-CMV-EDL (3 μg) and pBK-CMV (3 μg). Cell lysates were prepared and analysed as described for HepG2 cells. (**B**) Cells were infected and transfected as in (**A**). After 24 h, cell medium was collected, cells were washed with PBS and incubated with 2 units/ml heparin for 30 min at 37 °C. The heparin-releasable fraction was collected, centrifuged to remove cell debris and concentrated in a speed vac. Concentrated samples were mixed 1 : 1 with loading buffer and analysed by Western blot as described in (**A**). The blots (**A**) and (**B**) show samples from representative experiments performed in duplicate dishes. (**C**) EDL phospholipase activity was determined in 24 h cell media obtained as described in (**B**). After collecting the media, cells were washed with PBS containing 0.5 M NaOH, and cellular proteins were measured by the method of Bradford. Results (c.p.m./mg of cell protein) are means $\pm$ S.D. of two independent experiments performed in duplicate dishes.

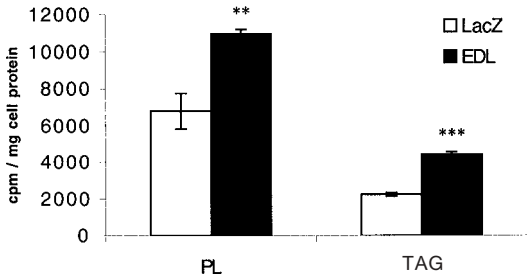


Figure 2 Quantification of ^{14}C-lipids in EDL overexpressing HepG2 cells upon incubation with ^{14}C-HDL-PC

HepG2 cells were plated on to 24-well trays and infected with EDL-Ad or LacZ-Ad, each at an MOI of 60. At 24 h post-infection, cells were incubated with ^{14}C-HDL-PC (50 μg of HDL protein/ml of medium) in 300 μl of DMEM containing 3 % (w/v) BSA at 37 °C for 8 h. ^{14}C-HDL-PC contains [^{14}C]palmitate at the positions sn1 and sn2. After incubation, medium was removed and cells were incubated with DMEM containing 100 units/ml heparin. After 30 min, cells were washed extensively with PBS and extracted twice with 300 μl of hexane/isopropanol (3 : 2, v/v). Dried lipids were redissolved in chloroform and analysed by TLC. Lipid spots were visualized by I_2-staining and subsequently cut out from the TLC plate and quantified by scintillation counting. Figure 2 shows PL- and TAG-c.p.m./mg of cell protein. The cellular proteins were measured in lipid-depleted cells by the method of Bradford. Results (c.p.m./mg cell protein) are means $\pm$ S.D. of three independent experiments performed in triplicate dishes. ***, $P \leqslant 0.001$ (compared with LacZ) ; **, $P \leqslant 0.01$ (compared with LacZ) ; *, $P \leqslant 0.05$ (compared with LacZ).

EDL bridging function, we next tested the contribution of the non-enzymic function of EDL to the accumulation of ^{14}C-lipids in HepG2 cells.

For these experiments we employed MUT-EDL-Ad, an adenovirus encoding catalytically inactive EDL [19]. First, we tested the capability of MUT-EDL to hydrolyse the ester bonds in the HDL-associated ^{14}C-PC *in vitro*. The amounts of ^{14}C-NEFA liberated from HDL-associated ^{14}C-PC by the heparin-releasable fractions obtained from MUT-EDL-Ad (920 $\pm$ 120 c.p.m.) was comparable with that of the heparin-releasable fraction from LacZ-Ad-infected cells (890 $\pm$ 109 c.p.m.), and profoundly lower than by the heparin-releasable fraction from EDL-Ad-infected cells (10120 $\pm$ 875 c.p.m.). From these results, we concluded that MUT-EDL expressed by infected cells is completely incapable of hydrolysing the ester bonds in the HDL-associated ^{14}C-PC. To test the contribution of the non-enzymic function of EDL to the accumulation of ^{14}C-lipids, HepG2 cells were infected with EDL-Ad, MUT-EDL-Ad and LacZ-Ad and incubated with ^{14}C-HDL-PC. The TLC analysis of the cell supernatants after 5 h of incubation with increasing concentrations of ^{14}C-HDL-PC demonstrated a dose-dependent increase in the amount of ^{14}C-NEFA in the media of EDL-Ad-, MUT-EDL-Ad- and LacZ-Ad-infected cells (Figure 3A). The increase was most pronounced in the supernatant of EDL-Ad-infected cells. The TLC analysis of the cell extracts showed that EDL-Ad-infected cells accumulated profoundly higher amounts of both ^{14}C-TAG and ^{14}C-PL compared with MUT-EDL-Ad- and LacZ-Ad-infected cells (Figures 3B and 3C). The amount of ^{14}C-TAG was similar in MUT-EDL-Ad- and LacZ-Ad-infected cells (Figure 3B). Importantly, MUT-EDL-Ad-infected cells contained significantly higher amounts of ^{14}C-PL than did LacZ-Ad-infected cells (Figure 3C). From these results, we concluded that the accumulation of ^{14}C-lipids in EDL overexpressing HepG2 cells was mainly due to the EDL-mediated release of ^{14}C-NEFA from ^{14}C-HDL-PC and their subsequent incorporation into newly synthesized lipids. The fact that MUT-EDL-Ad-infected cells contained significantly higher amounts of ^{14}C-PL than LacZ-Ad-infected cells indicated that the non-enzymic function ('bridging function') of EDL is in part responsible for the increase in ^{14}C-PL in EDL-infected cells.

To further confirm that EDL overexpression leads to cellular accumulation of PL and TAG as revealed in experiments with ^{14}C-labelled HDL, we performed the Q-MS analysis of the cellular lipids extracted from EDL-Ad-, MUT-EDL-Ad- and LacZ-Ad-infected cells. As shown in Figures 4(A), 4(B) and 4(C), the PC and TAG content of EDL-Ad-infected cells were profoundly higher compared with MUT-EDL-Ad- and LacZ-Ad-infected cells. The finding that MUT-EDL-Ad-infected cells contained more PC than LacZ-Ad-infected, control cells (Figures 4B and 4C) corroborated the results from the ^{14}C-tracer experiments, showing that the non-enzymic function of EDL contributes to the PL accumulation in EDL-overexpressing HepG2 cells.

Considering CD36 as a FA translocase [29], we assessed whether co-expression of EDL and CD36 might enhance the effect of EDL on PL and TAG accumulation in EDL-overexpressing cells. For this purpose we transiently transfected HEK-293 cells to obtain CD36-, EDL- and CD36 plus EDL-overexpressing cells. Transfected cells were incubated for 5 h with ^{14}C-HDL-PC

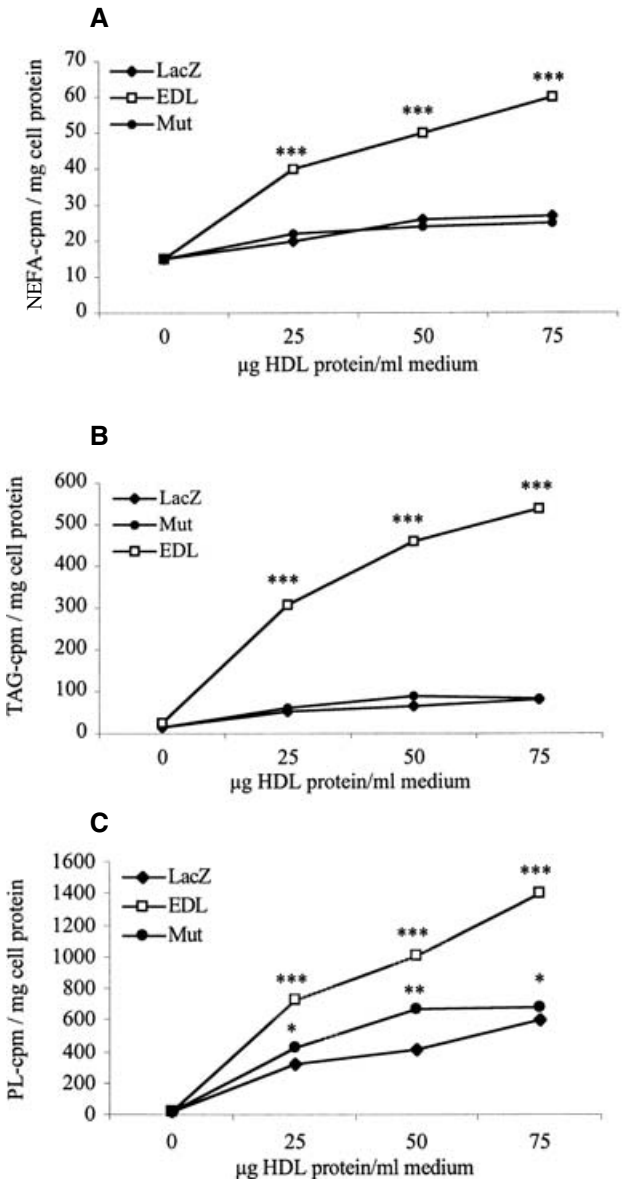
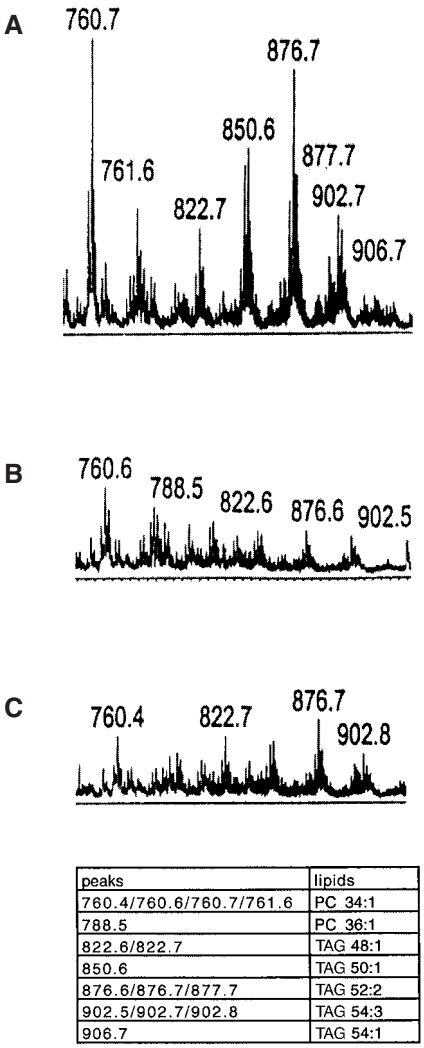


Figure 3 Quantification of ^{14}C-NEFA in cell supernatant (A), ^{14}C-TAG (B) and ^{14}C-PL (C) in cell extracts of HepG2 cells infected with EDL-Ad, MUT-EDL-Ad and LacZ-Ad incubated with increasing concentrations of ^{14}C-HDL-PC

HepG2 cells were plated on to 24-well trays and infected with EDL-Ad (EDL), MUT-EDL-Ad (MUT) or LacZ-Ad (LacZ) at an MOI of 60. At 24 h post-infection, cells were incubated with increasing concentrations of ^{14}C-HDL-PC (μg of HDL protein/ml medium) in 300 μl of DMEM containing 3 % (w/v) BSA at 37 °C for 5 h. After incubation, cell medium was collected and lipids were extracted with hexan/isopropanol (3 : 2, v/v). After heparin release and extensive PBS washing, cellular lipids were extracted and analysed by TLC, as described in the legend for Figure 1. Results (c.p.m./mg of cell protein) represent means ± S.D. (S.D.s are so small that they are within the symbols) from two independent experiments performed in duplicate dishes. ***, $P \leqslant 0.001$ (compared with LacZ); **, $P \leqslant 0.01$ (compared with LacZ); *, $P \leqslant 0.05$ (compared with LacZ).

followed by the TLC analysis of cellular lipids. As shown in Figures 5(A) and 5(B), EDL-overexpressing cells contained a 1.5-fold higher amount of ^{14}C-PL and a 2-fold higher amount of ^{14}C-TAG compared with mock-transfected cells. Co-expression of CD36 and EDL led to an additive 1.4-fold increase in the amounts of ^{14}C-PL and ^{14}C-TAG respectively, compared with EDL-overexpressing cells (Figures 5A and 5B).



peaks	lipids
760.4/760.6/760.7/761.6	PC 34:1
788.5	PC 36:1
822.6/822.7	TAG 48:1
850.6	TAG 50:1
876.6/876.7/877.7	TAG 52:2
902.5/902.7/902.8	TAG 54:3
906.7	TAG 54:1

Figure 4 Determination of PL and TAG content of HepG2 cells infected with (A) EDL-Ad, (B) MUT-EDL-Ad and (C) LacZ-Ad by Q-MS

HepG2 cells were plated on to 24-well trays and infected with EDL-Ad, MUT-EDL-Ad or LacZ-Ad (each at an MOI of 60) and incubated for 48 h in DMEM containing 10 % (v/v) FCS. After incubation, cell supernatant was aspirated, and cells were washed with DMEM containing 100 units/ml heparin for 30 min at 37 °C. After extensive washing with PBS, lipids were extracted as described in the legends for Figures 1 and 2. Aliquots of the lipid extracts corresponding to identical amounts of the total cell protein, determined as described in the Experimental section, were dried in the speed vac and redissolved in chloroform for the Q-MS analysis. Results for each type of infection represent relative amounts of PL and TAG in the pooled samples from three wells from one of two representative experiments. Identified peaks representing PC and TAG in (**A**), (**B**) and (**C**) are presented in the Table.

Taking into account the ability of EDL to supply cells with NEFA, including polyunsaturated fatty acids (PUFA) derived from HDL-PL [30] and the capacity of PUFA to suppress the expression of genes encoding enzymes involved in lipid synthesis [31], we hypothesized that EDL overexpression might affect FA synthesis in HepG2 cells. To address this, we first analysed the abundance of FAS mRNA in HepG2 cells infected with EDL-Ad or LacZ-Ad by RT-PCR. Results presented in Figure 6 show that EDL overexpression led to a 2-fold (at an MOI of 30) and 2.3-fold (at an MOI of 60) decreased FAS mRNA level compared with controls. We further assessed whether decreased FAS mRNA levels are accompanied by a decreased rate of FA synthesis in EDL-Ad-infected HepG2 cells. HepG2 infected with EDL-Ad at an MOI of 60 synthesized significantly less FA over 24 h

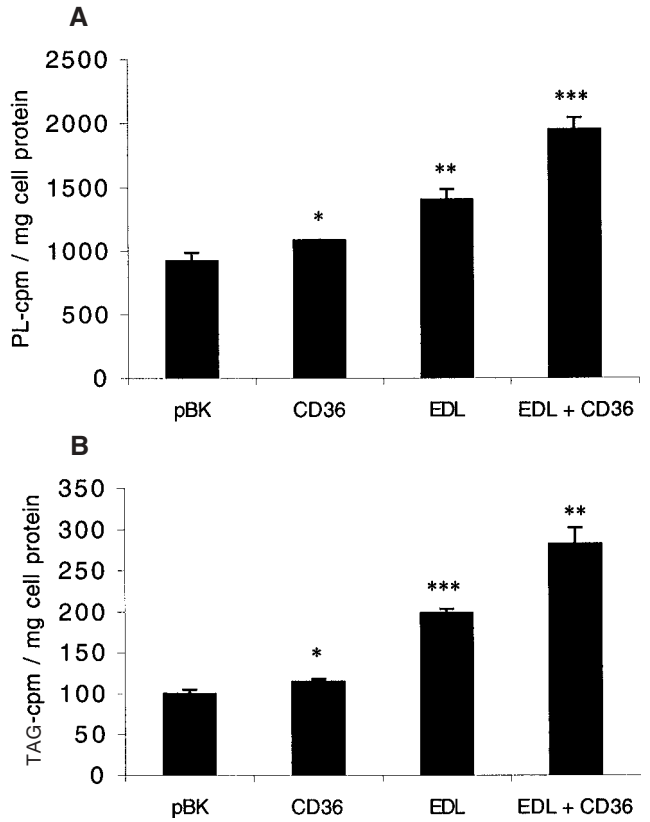


Figure 5 Quantification of (A) ^{14}C-PL and (B) ^{14}C-TAG in HEK-293 cells expressing CD36, EDL or CD36 plus EDL after incubation with ^{14}C-HDL-PC

HEK-293 cells were seeded into 24-well trays and transiently transfected by calcium phosphate co-precipitation with 6 μg of pBK-CMV (mock), pBK-CMV-EDL plus pBK-CMV (3 μg of each), HisMax-CD36 plus pBK-CMV (3 μg of each) and pBK-CMV-EDL plus HisMax-CD36 (3μg of each). At 24 h post-transfection, cells were incubated with ^{14}C-HDL-PC (50 μg of HDL protein/ml of medium) in DMEM containing 3 % (w/v) BSA at 37 °C for 5 h. After incubation, cell lipids were analysed by TLC as described in the legends for Figures 1 and 2. Results (c.p.m./mg of cell protein) represent means $\pm$ S.D. from two independent experiments performed in triplicate dishes. (**A**) ***, $P \leqslant 0.001$ (compared with EDL); **, $P \leqslant 0.01$ (compared with LacZ); *, $P \leqslant 0.05$ (compared with LacZ). (**B**) ***, $P \leqslant 0.001$ (compared with LacZ); **, $P \leqslant 0.01$ (compared with EDL); *, $P \leqslant 0.05$ (compared with LacZ).

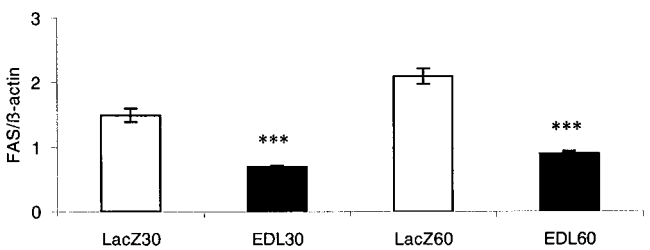


Figure 6 FAS mRNA quantification by RT-PCR

HepG2 cells were infected with EDL-Ad and LacZ-Ad at an MOI of 30 and 60 respectively. At 24 h post-infection, RNA was isolated from infected cells and used for RT-PCR-based quantification of FAS mRNA (30 cyles) and β-actin mRNA (26 cycles) in parallel reactions: initial denaturation, 4 min at 94 °C; denaturation, 30 s at 94 °C; annealing, 30 s at 60 °C; synthesis, 1 min at 72 °C. PCR products were separated by electrophoresis on 2 % agarose gels and stained with ethidium bromide. The relative amounts of mRNA were determined by densitometric scanning of gels, as described in the Experimental section. Results (relative amount of FAS mRNA normalized to β-actin mRNA) are means of four independent experiments performed in triplicate dishes. ***, $P \leqslant 0.001$ (compared with LacZ).

compared with LacZ-Ad-infected control cells (results not shown).

DISCUSSION

EDL is a member of the TAG lipase gene family [17,18]. Adenovirus-mediated EDL overexpression in mice dramatically reduced HDL and apoliprotein A-I plasma levels [17]. Cell culture experiments showed that EDL very efficiently mediates HDL particle binding and uptake, as well as selective uptake of HDL-associated CE, independent of its enzymic activity [19]. Additionally, it has been demonstrated that EDL very efficiently releases NEFA from HDL-PL [19,23]. In the present study, we aimed to assess the ability of EDL to supply NEFA derived from HDL-PL to cells and to determine the effect of liberated NEFA on the cellular lipid composition and FA synthesis. For these purposes, we expressed human EDL in HepG2 cells and HEK-293 cells by a recombinant adenovirus or transient transfection approach. In both EDL-overexpressing cell types, EDL was detected in the cell lysate, on the cell surface bound to the heparin sulphate proteoglycans and in the cell medium. EDL overexpression in HepG2 cells led to a pronounced increase in the amounts of ^{14}C-PL and ^{14}C-TAG upon incubation with ^{14}C-HDL-PC labelled in the FA moiety. From this finding we concluded that ^{14}C-NEFA derived from ^{14}C-HDL-PC by EDL phospholipase activity are taken up by cells and incorporated into newly synthesized lipids. These results are in line with a previous study, demonstrating that the capacity of HepG2 cells for PL and TAG synthesis from exogenously derived FA was higher than from endogenously synthesized FA [32]. The low amount of ^{14}C-NEFA in the cell lysates of both LacZ-Ad- and EDL-Ad-infected cells might be explained by the fact that [^{14}C]palmitic acid released from labelled HDL-PC represents a preferential substrate for the acyl transferases which catalyse its rapid incorporation into endogenous PL and TAG [33]. The low amount of ^{14}C-DAG detected in EDL-Ad-infected cells upon incubation with the ^{14}C-HDL-PC is most probably due to a rapid conversion of DAG into TAG owing to the high rate of the TAG synthesis in HepG2 cells. The fact that acyl CoA :cholesterol acyltransferase 1 (ACAT1) in HepG2 cells utilizes preferentially oleoyl- and linolenoyl-CoA as substrates for cholesterol esterification [34] might conceivably explain the low amount of ^{14}C-CE in HepG2 cells incubated with the ^{14}C-HDL-PC. To assess whether the effect of EDL is dependent only on its enzymic function, we employed an adenovirus encoding the enzymically inactive EDL. The finding that cells expressing enzymically inactive EDL accumulated profoundly lower amounts of ^{14}C-PL and ^{14}C-TAG than cells expressing enzymically active EDL confirmed that the effect of EDL on the accumulation of ^{14}C-lipids is strongly dependent on EDL enzymic activity. However, the amount of ^{14}C-PL was significantly higher in cells expressing mutant EDL compared with control cells. This finding indicated that EDL has the ability to enhance the uptake of ^{14}C-HDL-PC by HepG2 cells independent of its enzymic activity. One possible explanation for this finding could be that enzymically inactive EDL anchors HDL to the cell surface [19], thus facilitating the scavenger receptor class B Type I (SR-BI) [35,36] -mediated selective import of the lipoprotein-associated PL into cells [28]. Accordingly, ^{14}C-PL in HepG2 cells that express enzymically active EDL represent a mixture of both endogenously synthesized ^{14}C-PL containing ^{14}C-NEFA released from ^{14}C-HDL-PC by EDL phospholipase activity and ^{14}C-PC taken up from ^{14}C-HDL-PC, mediated by the non-enzymic (bridging) function of EDL. Results obtained from experiments with ^{14}C-labelled HDL could further be confirmed by

Q-MS analysis of cellular lipids obtained from EDL-Ad-, MUT-EDL-Ad- and LacZ-Ad-infected HepG2 cells. These experiments again showed that the highest amounts of PL and TAG were found in cells expressing enzymically active EDL. Importantly, cells expressing enzymically inactive EDL contained more PL than LacZ-Ad-infected control cells. To examine a possible impact of CD36 on EDL action, we performed co-expression experiments (EDL plus CD36), which clearly demonstrated that CD36 expression augmented EDL-mediated accumulation of ^{14}C-PL and ^{14}C-TAG in HEK-293 cells. This augmenting effect of CD36 might be ascribed to its role as a FA translocase [29]. Accordingly, NEFA released from HDL-PL by EDL phospholipase activity could be imported into cells expressing CD36 more efficiently, in addition to EDL. Alternatively, or additionally, due to its high affinity for HDL [37], CD36 might concentrate HDL on the cell surface thus supplying it to EDL for cleavage. Considering the ability of EDL to supply cells with FA, including PUFA [30], and the fact that PUFA suppress the expression of lipogenic genes, including FAS [31], a central enzyme in the *de novo* lipogenesis, via down-regulation of sterol regulatory element binding protein-1 (SREBP-1) [38–43], we assumed that EDL expression might have an impact on FA synthesis in HepG2 cells. Our assumption was confirmed by the finding that the amount of FAS mRNA, as well as the rate of FA synthesis, were decreased in EDL-expressing cells compared with controls. Additionally, these observed effects of EDL overexpression might be due to a PUFA-mediated suppression of the FAS promoter activity in HepG2 cells [44].

Taken together, our results demonstrate that EDL efficiently cleaves NEFA from HDL-PL, supplying NEFA for HepG2 cells, where they are incorporated into endogenous lipids, leading to an increase in the amounts of PL and TAG. This effect of EDL could be augmented by EDL/CD36 co-expression in HEK-293 cells. By its non-enzymic function, EDL enhances the uptake of HDL-PL in HepG2 cells. Additionally, the overexpression of EDL in HepG2 cells lowers the amount of FAS mRNA and the rate of FA synthesis.

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