Endothelial cell-derived lipase mediates uptake and binding of high-density lipoprotein (HDL) particles and the selective uptake of HDL-associated cholesterol esters independent of its enzymic activity

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Endothelial cell-derived lipase (EDL) is a new member of the lipase gene family with high sequence homology with lipoprotein lipase (LPL). EDL is a phospholipase with very little triacylglycerol lipase activity. To investigate the effects of EDL on binding and uptake of high-density lipoprotein (HDL), as well as on the selective uptake of HDL-derived cholesterol esters (CEs), HepG2 cells were infected with adenovirus coding for EDL. For comparison, cells were also infected with LPL and with lacZ as a control. Both HDL binding and particle uptake were increased 1.5-fold and selective HDL-CE uptake was increased 1.8-fold in EDL-infected HepG2 cells compared with controls. The effect of LPL was less pronounced, resulting in 1.1-fold increase in particle uptake and 1.3-fold increase in selective uptake. Inhibition of the enzymic activity with tetrahydrolipstatin (THL) significantly enhanced the effect of EDL, as reflected by a 5.2-fold increase in binding, a 2.6-fold increase in particle uptake and a 1.1-fold increase in CE selective uptake compared with incubations without THL. To elucidate the mechanism responsible for the effects of THL, we analysed the abundance of heparin-releasable EDL protein from infected HepG2 cells upon incubations with THL, HDL and free (non-esterified) fatty acids (FFAs). In the presence of THL, vastly more EDL protein remained bound to the cell surface. Additionally, HDL and FFAs reduced the amount of cell-surface-bound EDL, suggesting that fatty acids that are liberated from phospholipids in HDL release EDL from the cell surface. This was substantiated further by the finding that, in contrast with EDL, the amount of cell-surface-bound enzymically inactive mutant EDL (MUT-EDL) was not reduced in the presence of HDL and foetal calf serum. The increased amount of cell-surface-bound MUT-EDL in the presence of THL suggested that the enzymic inactivity of MUT-EDL, as well as an augmenting effect of THL that is independent of its ability to inactivate the enzyme, are responsible for the increased amount of cell-surface-bound EDL in the presence of THL. Furthermore, in cells expressing MUT-EDL, binding and holoparticle uptake were markedly higher compared with cells expressing the active EDL, and could be increased further in the presence of THL. Despite 1.7-fold higher binding and 1.8-fold higher holoparticle uptake, the selective CE uptake by MUT-EDL-expressing cells was comparable with EDL-expressing cells and was even decreased 1.3-fold with THL. Experiments in CLA-1 (CD-36 and LIMPII analogous 1, the human homologue of scavenger receptor class B type I)-deficient HEK-293 cells demonstrated that EDL alone has the ability to stimulate HDL-CE selective uptake independently of CLA-1. Thus our results demonstrate that EDL mediates both HDL binding and uptake, and the selective uptake of HDL-CE, independently of lipolysis and CLA-1.

Key words: adenoviral vector, HEK-293 cell, HepG2 cell, scavenger receptor class B Type I (SR-BI).

INTRODUCTION

High-density lipoprotein (HDL) has long been considered as an anti-atherogenic lipoprotein because of its central role in reverse cholesterol transport [1]. In the first step of this process, small discoidal HDL particles receive cholesterol from cell membranes, followed by the esterification of cholesterol by lecithin:cholesterol acyltransferase [2]. In some species, cholesterol esters (CEs) from large CE-rich HDL particles (HDL3s) can be exchanged for triacylglycerols (TAGs) and transported in TAG-rich lipoproteins by CE transfer protein (CETP) [3]. CEs from HDL can also be taken up by some cells, predominantly hepatocytes and cells of steroidogenic tissues, via a mechanism called selective uptake [4,5]. Acton et al. [6] have demonstrated that CD36-related scavenger receptor class B Type I (SR-BI) mediates selective uptake of HDL-derived CE. Selective uptake of HDL-CE proceeds in two steps: an initial step involving incorporation of HDL-CE into the plasma membrane, followed by the transfer of CE into cytosol in the second step. Recently, Silver et al. [7] provided evidence that implicates HDL recycling in the process of selective uptake.

In addition to SR-BI, lipoprotein lipase (LPL) mediates selective HDL-CE uptake by macrophages [8] and hepatic cells *in vitro* [9]. LPL associates with lipoproteins and anchors them to the cell surface of a variety of cells, as a consequence of its high affinity for cell surface proteoglycans, especially heparan sulphate

Abbreviations used: CE, cholesterol ester; CETP, CE transfer protein; CLA-1, CD-36 and LIMPII analogous 1; DMEM, Dulbecco's modified Eagle's medium; EDL, endothelial cell-derived lipase; EDL-Ad, EDL-expressing adenovirus; FCS, foetal calf serum; FFA, free fatty acid; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HDL, high-density lipoprotein; hEDL, human EDL; HL, hepatic lipase; HSPG, heparan sulphate proteoglycans; LPDS, lipoprotein-deficient serum; LPL, lipoprotein lipase; MOI, multiplicity of infection; PC, phosphatidylcholine; SR-BI, scavenger receptor class B Type I; TAG, triacylglycerol; TCA, trichloroacetic acid; THL, tetrahydrolipstatin; VLDL, very-low-density lipoprotein.

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proteoglycans (HSPG), which have a bridging function [10]. Thus LPL brings lipoproteins in close proximity to the cell membrane and increases the binding and degradation of lipoproteins, as well as selective CE uptake from lipoproteins [11–14]. Alternatively, LPL–lipoprotein complexes that are bound to HSPG can be taken up as a consequence of internalization of cell surface proteoglycans [15]. Hepatic lipase (HL) [16], like LPL, facilitates the interaction of lipoproteins with cell surface proteoglycans and receptors, because of its bridging and/or ligand function, leading to enhanced binding and uptake of different lipoproteins [12–14,17]. HL also enhances HDL-CE selective uptake [8] by a variety of cultured cells.

Most recently, a new member of the lipase gene family has been cloned from endothelial cells, and named endothelial cell-derived lipase (EDL) [9,18]. Northern blot analysis of human tissues demonstrated high expression of EDL in the placenta, thyroid, liver, lung, kidney, testis and ovary. In addition to that of endothelial cells, EDL expression has been detected in hepatocytes and macrophages [9,18]. In contrast with LPL and HL, EDL primarily exhibits phospholipase activity with relatively little TAG lipase activity [9,18]. Overexpression of EDL in mice by a recombinant adenovirus approach dramatically lowered HDL cholesterol and apolipoprotein A-I levels [9].

In the present study, we demonstrate that EDL can increase HDL binding and holoparticle uptake, as well as selective HDL-CE uptake, in HepG2 cells, independent of its catalytic activity.

EXPERIMENTAL

Cloning of human EDL (hEDL) cDNA

Total RNA was isolated from cultured human umbilical-vein endothelial cells and was used for the reverse transcriptase-PCR amplification of hEDL cDNA by using the following primers: EDL-*Bgl*II, 5′-CGAGGGC**AGATCT**CGTTCTGG-3′ (nt 34–54) and EDL-*Kpn*I, 5′-CGG**GGTACC**CCGTTGGATAGCA-GGAAGTCTTGC-3′ (nt 1807–1786). The full-length cDNA (1.7 kb), including 200 bp of the 5′ region, was cloned into the TA cloning vector pCR2.1 (Invitrogen, Groningen, The Netherlands) and sequenced. Subsequently, the hEDL cDNA was isolated from pCR2.1 by cleaving with *Bgl*II and *Kpn*I and cloned into pAvCvSv [19]. For transient transfection experiments, the hEDL cDNA was cloned into expression plasmid pBK-CMV (Stratagene, La Jolla, CA, U.S.A.) cut with *Bam*HI and *Kpn*I.

Site-directed mutagenesis of hEDL in the catalytic site

Human EDL cDNA was subcloned into pUC19 plasmid, and subsequently the mutation $Gly^{826} \rightarrow Ala$ was introduced using a PCR-based site-specific mutagenesis kit (GibcoBRL, Groningen, The Netherlands) and the primers: ^{193}N-EDL, 5′-GCCCGGC-AGGATTCAAACCTGTGATTC-3′ and *Sca*I control selection primer (from the kit), 5′-CTGTGACTGGTGACGCGGTCA-ACCAAGTC-3′. This resulted in a $Asp^{193} \rightarrow Asn$ mutation. The mutated hEDL cDNA was then subcloned into pAvCvSv cut with *Bgl*II and *Kpn*I.

Construction and purification of the recombinant adenovirus

The recombinant adenovirus coding for hEDL was prepared by co-transfection of pAvCvSv [19] containing the 1.7 kb EDL cDNA and pJM17 [19,20] into HEK-293 cells. The 1.7 kb *Bgl*II/*Kpn*I hEDL cDNA fragment was subcloned into *Bgl*II/*Kpn*I-digested pAvCvSv. The resulting shuttle plasmid (5 μg) was co-transfected with 5 μg of pJM17 into HEK-293 cells using the calcium phosphate co-precipitation method [21]. At 2 weeks after transfection, recombinant plaques were picked and propagated on HEK-293 cells, followed by screening for EDL enzyme activity. Positive clones were subcloned twice again by plaque assay on HEK-293 cells, and large-scale production of high-titre recombinant EDL-expressing adenovirus (EDL-Ad) was performed as described previously [22]. EDL-Ad coding for the mutant hEDL was generated as described for EDL-Ad, except that in the first step, pAvCvSv containing the mutant hEDL cDNA was co-transfected with pJM17 into HEK-293 cells. The resulting virus was designated MUT-EDL-Ad. LPL-Ad, CLA-1 (CD-36 and LIMPII analogous 1, the human homologue of SR-BI)-Ad and lacZ-Ad have been described elsewhere [23,24].

Northern blot analysis

Total RNA was prepared from either EDL-Ad- or lacZ-Ad-infected HepG2 cells by using an RNeasy kit (Qiagen, Vienna, Austria). The RNAs were quantified by spectrophotometry, and 15 μg of total cellular RNA was size-fractionated on a 1% formaldehyde/agarose gel in Mops buffer, transferred on a Biodyne® B nylon membrane (Pall Life Sciences, Vienna, Austria), incubated for 6 h in prehybridization buffer (0.15 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS and 1% BSA) and then incubated further with radiolabelled EDL cDNA as a probe. After 16 h of incubation at 65 °C, the filters were washed in 0.5% (w/v) SDS and 2 × SSC (0.3 M NaCl/0.03 M sodium citrate), pH 7.0, for 10 min at 23 °C, and then in 0.5% SDS/0.3 × SSC for 10–15 min at 65 °C before exposing to Cronex medical X-ray film (Sterling Diagnostic Imaging, Inc., Newark, DE, U.S.A.). In addition, the blots were stripped and re-probed with labelled glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA (Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.) that served as a control for RNA loading.

Preparation of anti-EDL antiserum

In order to prepare a specific polyclonal antiserum against hEDL, a polypeptide containing the N-terminal amino acids 18–76 (N-$EDL_{18–76}$) was expressed using an established *Escherichia coli* expression system. DNA sequence coding for this region was amplified by PCR from EDL cDNA using two synthetic oligonucleotides: A, 5′-gct aga att cat cac cat cat cac cat gcg ggg agc ccc gta cct ttt-3′ and B, 5′-gtc agg atc ctt atc agt ctt cta agg gct ggc tgt-3′.

To permit the cloning of N-$EDL_{18–76}$ sequence into the expression vector pT7-7, an *Eco*RI restriction site was included in the 5′ primer and a *Bam*HI and two stop codons were included in the 3′ primer. A His_6 tag sequence was also included into the 5′ primer in order to enable a one-step purification of recombinant N-$EDL_{18–76}$ using Talon™ metal affinity chromatography (Clontech Laboratories). Conditions for PCR (in 50 μl) were as follows: 10 pmol each of oligonucleotides A and B, 150 ng of dNTPs, 0.6 unit of *Taq* polymerase (Finnzymes, Espoo, Finland) and 50 ng of hEDL cDNA as a template. The reaction conditions were as follows: denaturation, 94 °C, 60 s; annealing, 70 °C, 60 s; extension, 72 °C, 90 s; 25 cycles. The PCR product (215 bp) was gel-purified, digested with *Eco*RI and *Bam*HI and ligated into a *Eco*RII/*Bam*HI-cleaved pT7-7 vector. This construct was used to transform *E. coli* DH5α competent cells in order to amplify recombinant plasmid. Positive clones, confirmed by restriction analysis and DNA sequencing, were finally trans-

formed into *E. coli* BL-21 (DE3) cells (Novagen, Madison, WI, U.S.A.). Expression of N-$EDL_{18–76}$ and subsequent purification by Talon™ metal affinity chromatography were prepared as described previously [25]. Specific polyclonal antiserum against hEDL was prepared by immunizing a rabbit with three 500 μg portions of purified N-$EDL_{18–76}$ for 3 weeks. At 2 weeks after the third injection, 50 ml of blood was collected and centrifuged at 1500 ***g*** for 15 min at room temperature (25 °C) to eliminate blood cells. Plasma was incubated with 600 mg/l $CaCl_2$ for 1 h at room temperature (25 °C) and the sample was centrifuged at 45000 ***g*** for 10 min; the supernatant was collected, supplemented with 1 mg/ml each of EDTA and NaN_3, and stored at −20 °C in 200 μl aliquots. An antiserum dilution of 1:1000 was found to be optimal for immunoblotting.

Western blot analysis

Heparin-releasable EDL fractions were mixed 1:1 with 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 incubated for 10 min at 95 °C. The cells were washed with PBS, collected with a hot loading buffer and boiled for 10 min before loading on to the gel. Heparin-releasable fractions and cell-lysate aliquots were fractionated by SDS/PAGE (12% gels) for 1.5 h at 150 V, transferred on to nitrocellulose, and bands were visualized with an enhanced chemiluminescence assay (ECL®; Amersham Biosciences, Uppsala, Sweden) after incubation with a specific antibody against hEDL. As a second antibody, horseradish peroxidase-labelled mouse anti-rabbit antiserum was used at a dilution of 1:1000.

Phospholipase activity assay

The assay is on the basis of the lipolysis of phospholipids labelled in the fatty acid moiety, whereby the amount of liberated free fatty acids (FFAs) is a measure of phospholipase activity in the sample. Phosphatidylcholine (PC) substrate was made by mixing [^{14}C]dipalmitoyl-PC (NEN, Boston, MA, U.S.A.), lecithin (1 mg/ml) and substrate buffer Tris-TCNB [100 mM Tris/HCl, pH 7.4, 1% Triton X-100, 5 mM $CaCl_2$, 200 mM NaCl, 0.1% fatty acid-free BSA (Sigma, St. Louis, MO, U.S.A.)], and was subsequently dried under nitrogen. The dried phospholipid was reconstituted in the substrate buffer. The heparin-releasable fraction from the cell surface of EDL-infected cells 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 extracted with hexane/propan-2-ol [3:2 (v/v); 0.1% HCl]. A 500 μl aliquot of the upper phase was dried in a Speed Vac (Savant GMI, Inc., Albertville, MN, U.S.A.) and reconstituted in 100 μl of hexane/propan-2-ol (3:2, v/v). After separation by TLC [hexane/diethyl ether/acetic acid 70:29:1, by vol.], the liberated [^{14}C]FFAs were quantified in a scintillation counter (Beckman Instruments, Palo Alto, CA, U.S.A.). In order to test the capacity of EDL to cleave HDL-associated phospholipids, 40 μl of ^{14}C-PC was dried under nitrogen and then incubated under an argon atmosphere with HDL (60 mg of cholesterol/ml) for 20 h at 37 °C. The labelled HDL was separated by Sephadex chromatography. Indicated concentrations of [^{14}C]PC-labelled HDL were used as a substrate for the heparin-released EDL and a phospholipase assay was performed as described above.

In order to determine the concentration of the active site inhibitor tetrahydrolipstatin (THL) [26,27], required for the inhibition of EDL, an activity phospholipase assay was performed in the presence of increasing concentrations of THL ranging from 0 to 25 μg/ml.

Isolation and characterization of human HDL

HDL was isolated from normolipidaemic human plasma by sequential ultracentrifugation, as described previously [28]. Protein concentrations were measured by the Lowry method [29] using BSA as standard. Total cholesterol was determined using an enzymic procedure (CHOD-PAP, Boehringer Mannheim, Mannheim, Germany).

Lipoprotein labelling procedure

Labelling of lipoproteins with cholesteryl [1,2,6,7-^{3}H]linoleate (DuPont NEN, Boston, MA, U.S.A.) was performed by the CETP-catalysed transfer from donor liposomes to lipoproteins, as described previously [30]. Briefly, 50 μl of cholesteryl [1,2,6,7-^{3}H]linoleate and 20 μg of egg-yolk lecithin (Sigma) were dried under nitrogen and sonicated in 1 ml of 10 mM PBS on ice. A 1 ml volume of the liposomes was added to 3 ml of lipoprotein-deficient serum (LPDS) containing HDL [0.5 mg of total cholesterol/ml]. The incubation was performed under nitrogen at 37 °C for 15 h in the dark. The labelled lipoprotein was isolated as described above. HDL was desalted by exclusion chromatography using PD10 columns (Amersham Biosciences). Iodination of HDL was performed using *N*-bromosuccinimide as the coupling reagent [31]. For the labelling of 5 mg of lipoprotein, 1 mCi of $Na^{125}I$ (Amersham Biosciences) was used, before desalting using exclusion chromatography, and this was dialysed extensively against PBS containing 0.01% (w/v) EDTA. Labelled lipoproteins were stored at 4 °C and used within 1 week.

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 150 mm × 25 mm dishes (Corning, New York, NY, U.S.A.) in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal-calf serum (FCS), non-essential amino acids and sodium pyruvate. HEK-293 cells were cultured in DMEM containing 10% FCS. Cells were trypsinized, counted and seeded into 24-well trays at 2×10^5 HepG2 cells/well or 5×10^5 HEK-293 cells/well, 48 h before experiments. The cells were infected 24 h later with the particular multiplicity of infection (MOI; i.e. the number of viable virus particles applied per cell) of lacZ-Ad, EDL-Ad, LPL-Ad or CLA-1-Ad, as indicated in each experiment. The cells were infected in culture medium containing 2% (v/v) FCS for 60 min at 37 °C. Upon infection, fresh medium with 10% (v/v) FCS was added to the cells. For some experiments, HEK-293 cells were plated into 24-well plates (5×10^5 cells/well) and transfected transiently with 1 μg of pBK-CMV-EDL or pBK-CMV (mock vector) respectively, with the calcium phosphate co-precipitation method. Cell-culture studies were performed 24 h after infection or transfection. For Western blot analysis of the heparin-releasable fraction, EDL-Ad-infected cells were incubated 21 h after infection, in either the presence or absence of 25 μg/ml THL, in order to block the active site and thus the lipolytic activity of EDL for 3 h. Afterwards, the cells were washed extensively with PBS and incubated with 100 units/ml heparin in a total volume of 100 μl at 37 °C for 30 min. The aliquots were analysed by Western blotting as described above.

Release of EDL by HDL and fatty acids

Cells were trypsinized, counted and seeded into 24-well trays (2×10^5 cells/well) 48 h before the experiments. After 24 h, the cells were infected with MOI 60 of EDL-Ad in culture medium containing 2 % (v/v) LPDS for 90 min at 37 °C. Upon infection, fresh medium with 10 % LPDS was added to the cells. After a further 24 h, cells were incubated with 300 μl of DMEM containing 3 % (v/v) fatty-acid-free BSA and increasing concentrations of HDL (0, 25 and 50 μg of HDL protein/ml). To study the effect of FFAs, cells were incubated with 300 μl of medium containing 3 % (v/v) fatty-acid-free BSA and increasing concentrations of oleic acid (0, 0.88 and 1.76 mM; Sigma). All incubations were performed for 2 h, in either the presence or absence of 25 μg/ml THL. Afterwards, the cells were washed extensively with PBS and incubated with 100 units/ml heparin in a total volume of 150 μl at 37 °C for 30 min. The samples were either concentrated from 150 μl to 30 μl in the Speed Vac or analysed, without concentrating, by Western blotting, as described above.

Determination of HDL cell association, selective CE uptake and apolipoprotein degradation

To determine cell association (heparin-non-releasable binding and internalization), and degradation of HDL as well as selective HDL-CE uptake, cells were incubated with 25 μg of HDL-protein labelled with ^{125}I in the protein moiety and with [^{3}H]CE in the lipid moiety in separate incubations. Labelled lipoproteins were added to infected cells in DMEM containing 10 % LPDS and incubated for 6 h under standard conditions. In some experiments, cells were pre-incubated for 3 h with 25 μg/ml THL in order to inhibit the enzymic activity of both EDL and LPL. Following the 6 h incubation period with labelled HDL, cells were placed on ice, washed twice with PBS containing 0.5 % BSA and then twice with PBS alone. If not otherwise stated, surface-bound HDL was removed by incubating cells with 100 units of heparin/ml of DMEM for 1 h at 4 °C with constant shaking. To determine cell-associated (i.e. heparin-non-releasable) and internalized ^{125}I-HDL and [^{3}H]CE-HDL radioactivity, cells were washed and solubilized in 0.3 M NaOH at room temperature (25 °C) for 4 h. The ^{125}I radioactivity represents cell-associated HDL and ^{3}H radioactivity is a measure of cell-associated HDL-CE. Cell protein was measured using the bicinchoninic acid (BCA) protein reagent assay (Pierce, Rockford, IL, U.S.A.).

Degradation of ^{125}I-labelled apolipoproteins was determined by measuring the trichloroacetic acid (TCA) non-precipitable radioactivity in the medium after precipitation of free iodine with $AgNO_3$ [32]. Briefly, 0.25 ml of medium was mixed with 50 μl of BSA (20 mg/ml) and 0.5 ml of ice-cold TCA (50 %, w/v) at 4 °C. After 30 min, 125 μl of $AgNO_3$ (0.7 M) was added, and the mixture was then vortex-mixed and centrifuged for 15 min at 5500 ***g*** at 4 °C. A 0.5 ml volume of the supernatant was aspirated and radioactivity was measured in the γ-counter. To determine non-cell-mediated degradation, control experiments were performed without cells.

To simplify the comparison of results obtained with ^{125}I-labelled and [^{3}H]CE-labelled HDL, the uptake of radiolabelled HDL is expressed as the apparent HDL particle uptake [33]. Apparent particle uptake is expressed as HDL protein (calculated from the specific radioactivity of the corresponding [^{3}H]CE-HDL or ^{125}I-HDL) that is required to deliver the observed amount of tracer. This is done to normalize the uptake of both tracers (^{125}I and ^{3}H respectively). Apparent HDL holoparticle uptake represents cell-associated ^{125}I-HDL (heparin-non-releasable and internalized) and degraded ^{125}I-HDL. It is characterized by an equal uptake of both tracers. Apparent selective uptake is calculated as the difference between apparent [^{3}H]CE-HDL cell association and apparent ^{125}I-HDL holoparticle uptake.

Statistics

Results are expressed as means $\pm$ S.D. The significance of differences was examined using Student's t test.

RESULTS

Expression and activity of EDL in HepG2 cells infected with EDL-Ad

The efficiency of adenovirus-mediated expression of EDL in HepG2 cells was analysed by examining RNA and protein levels. Total RNA was isolated from cells separately infected with MOI 30 of EDL-Ad and lacZ-Ad. Northern-blot analysis showed EDL-mRNA expression in EDL-Ad-infected cells but not in lacZ-Ad-infected cells (Figure 1A). To assess whether EDL protein is synthesized by the infected cells, cell lysates of EDL-Ad- and lacZ-Ad-infected cells were analysed by Western blotting. EDL-specific bands could be detected in the cell lysate of EDL-Ad-infected cells. Weak EDL-specific bands were also present in the cell lysate of lacZ-Ad-infected cells due to endogenous EDL expression by HepG2 cells (Figure 1B). The EDL expressed by adenovirus-infected cells efficiently cleaved fatty acids from HDL-associated phospholipids, as demonstrated by using [^{14}C]PC-labelled HDL as a substrate (Figure 2A). Furthermore, we demonstrated that a THL concentration of

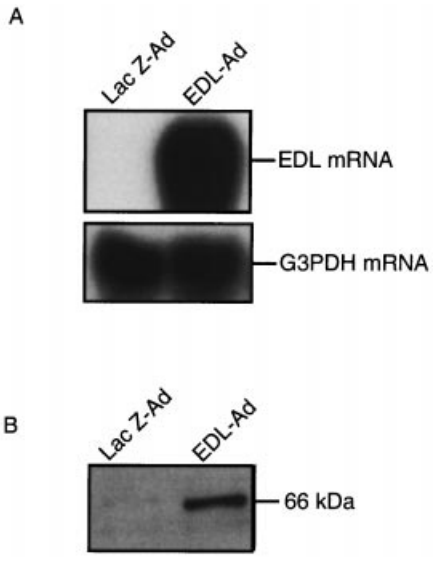

Figure 1 Northern blot (A) and Western blot (B) analysis of EDL expression by HepG2 cells infected with EDL-Ad and lacZ-Ad

(**A**) Total RNA (15 μg) was isolated from EDL-Ad- and lacZ-Ad-infected cells (MOI 30) and analysed on a 1% formaldehyde/agarose gel followed by blotting on a nylon membrane and incubation with radiolabelled EDL-cDNA as a probe. After 16 h of incubation at 65 °C, the filter was extensively washed and exposed to X-ray film. The blots were stripped and re-probed with labelled G3PDH-cDNA. (**B**) For Western blotting, the cells were infected as described for (**A**). Cells were washed with PBS, 24 h after infection, lysed with a boiling loading buffer containing 5% 2-mercaptoethanol and boiled for an additional 10 min before analysis by SDS/12% PAGE. EDL bands were visualized by an ECL® assay after incubation with an anti-EDL antiserum and horseradish peroxidase-labelled mouse anti-rabbit antiserum.

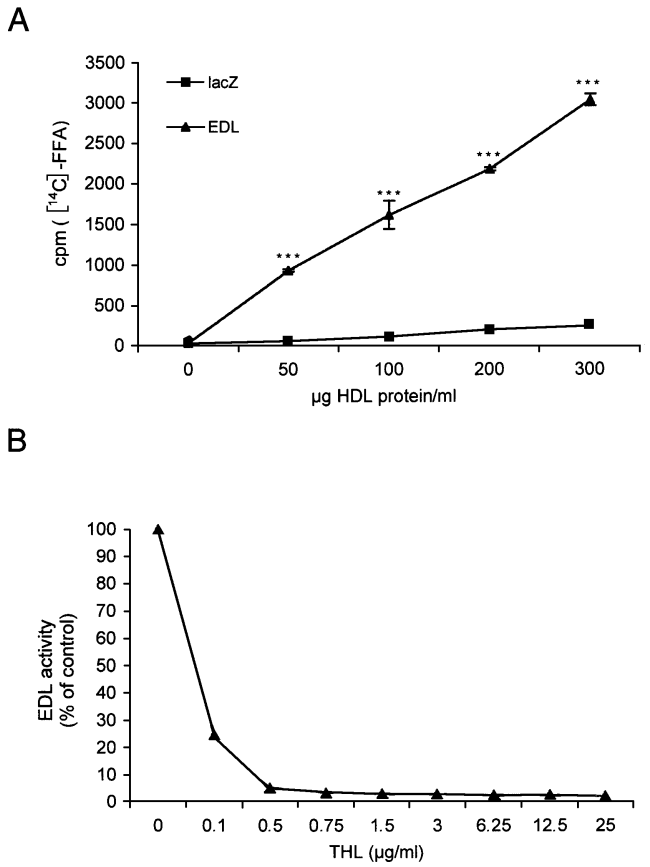

Figure 2 Heparin-releasable EDL activity (A) and THL inhibition (B) of EDL activity in HepG2 cells infected with EDL-Ad and lacZ-Ad

HepG2 cells were infected with MOI 30 of EDL-Ad and lacZ-Ad. After 24 h, the cells were washed and EDL was released from the cells with 100 μl of DMEM containing 2 units/ml heparin, at 37 °C for 30 min. The heparin-releasable fraction was mixed with (**A**) increasing concentrations of [^{14}C]PC-labelled HDL as a substrate and incubated at 37 °C for 1 h. After extraction with hexane/propan-2-ol, the upper phase was dried and reconstituted in 100 μl of hexane/propan-2-ol and separated by TLC. The liberated [^{14}C]FFAs were quantified in a scintillation counter. Each datum is the mean ± S.D. for two experiments performed in duplicate dishes. ***, $P \leqslant 0.001$ compared with lacZ-infected cells. (**B**) The heparin-releasable fraction from EDL-Ad-infected HepG2 cells was mixed with a substrate ([^{14}C]dipalmitoyl-PC) and incubated at 37 °C for 1 h with increasing concentrations of THL. The EDL activity was expressed as a residual activity of the incubation without THL. The results represent the means for two independent experiments performed in triplicate. cpm, counts per minute.

more than approx. 3 μg/ml completely inhibited phospholipase activity of expressed EDL (Figure 2B).

HDL binding, holoparticle uptake and selective HDL-CE uptake by HepG2 cells infected by EDL-Ad and LPL-Ad

To assess the effects of EDL on HDL binding, particle uptake and selective HDL-CE uptake in comparison with LPL, HepG2 cells separately infected with MOI 30 of EDL-Ad, LPL-Ad and lacZ-Ad were incubated with ^{125}I- and [^{3}H]CE-labelled HDL in parallel experiments. A concentration of 25 μg of HDL protein/ml medium was used because, in dose–response experiments, this concentration was found to be in a range where HDL binding, uptake and selective HDL-CE uptake increased linearly with the HDL concentration (results not shown). The results presented in Table 1 demonstrate an increased binding of ^{125}I-HDL at 37 °C by EDL-Ad (1.5-fold; $P < 0.001$), but not by LPL-Ad-infected cells, compared with lacZ-Ad infection. The uptake of ^{125}I-HDL holoparticles was also elevated after EDL-Ad infection (1.5-fold; $P < 0.001$) and LPL-Ad infection (1.2-fold; $P < 0.05$), compared with lacZ-Ad-infected cells (Table 1). In EDL-Ad-infected cells, selective uptake of [^{3}H]CE-HDL was increased by 1.8-fold ($P < 0.001$), whereas a 1.3-fold increase ($P < 0.05$) was observed in LPL-Ad-infected cells when compared with lacZ-Ad-infected controls (Table 1).

Table 1 HDL heparin-releasable binding, HDL holoparticle uptake and selective HDL–CE uptake by HepG2 cells overexpressing EDL and LPL in the presence and absence of THL

Cells were infected separately with EDL-Ad, LPL-Ad and LacZ-Ad with MOI 30. Cells were treated, 24 h after infection, in parallel incubations with 25 μg of HDL (protein), labelled with ^{125}I in the protein moiety or with [^{3}H]CE labelled in the lipid moiety, per ml of DMEM + 10% LPDS for 6 h at 37 °C. To inactivate EDL in some incubations, before the addition of labelled HDL, cells were pre-incubated for 3 h with 25 μg/ml THL. THL was also present in the incubations with labelled HDL for 6 h at 37 °C. To determine heparin-releasable binding the cells were incubated with 100 units of heparin/ml of DMEM at 4 °C for 1 h. HDL holoparticle uptake was calculated from cell-associated ^{125}I radioactivity (heparin-non-releasable + internalized) in cell lysate, obtained by solubilization with 0.3 M NaOH and TCA-non-precipitable ^{125}I radioactivity in the medium. Selective uptake was calculated as the difference between cell-associated [^{3}H]CE-HDL and ^{125}I holoparticle uptake. To allow the comparison of cellular uptake of ^{125}I- and ^{3}H-labelled HDL tracers, uptake is shown as apparent HDL particle uptake and is expressed in HDL protein that would be required to deliver the observed tracer uptake. Results are means ± S.D. for four independent experiments performed in triplicate dishes. *$P < 0.001$ compared with LacZ-infected cells; †$P < 0.001$ compared with infection without THL; ‡$P < 0.01$ compared with LacZ-infected cells; §$P < 0.01$ compared with infection without THL; ‖$P < 0.05$ compared with LacZ-infected cells; ¶$P < 0.05$ compared with infection without THL.

	HDL protein bound or taken up (μg/mg of cell protein)		
	HDL binding	HDL particle uptake	Apparent selective CE uptake
LacZ − THL	0.03 ± 0.001	0.032 ± 0.002	0.93 ± 0.07
EDL − THL	0.046 ± 0.001*	0.047 ± 0.002*	1.71 ± 0.016‡
LPL − THL	0.029 ± 0.001	0.038 ± 0.001‖	1.21 ± 0.035‖
LacZ + THL	0.032 ± 0.006	0.043 ± 0.001	0.88 ± 0.006
EDL + THL	0.246 ± 0.005*†	0.122 ± 0.005*§	1.923 ± 0.044*§
LPL + THL	0.05 ± 0.006¶	0.055 ± 0.002‖	1.22 ± 0.03‖

Effect of enzymic activity of EDL and LPL on HDL binding, holoparticle uptake and selective uptake

To determine whether enzymic activity of either EDL or LPL is required for HDL binding and holoparticle uptake as well as HDL-CE selective uptake, a similar experiment was performed as described above, but in the presence of 25 μg/ml THL. From the results presented in Table 1, it is evident that EDL-Ad-infected cells exhibited an increased ^{125}I-HDL-particle binding at 37 °C in the presence of THL either compared with lacZ-Ad-infected cells with THL (7.7-fold; $P < 0.001$) or with EDL-Ad-infected cells in the absence of THL (5.2-fold; $P < 0.001$). EDL-mediated ^{125}I-HDL holoparticle uptake with THL and selective [^{3}H]CE-HDL uptake with THL were increased 2.8-fold ($P < 0.001$) and 2.2-fold ($P < 0.001$) respectively, compared with lacZ-Ad infection with THL, and 2.6-fold ($P < 0.001$) and 1.1-fold ($P < 0.01$) respectively, when compared with EDL-Ad infection without THL. In contrast with EDL, LPL-mediated binding, uptake and selective uptake were not affected by THL.

Evidence exists that fatty acids released by LPL-mediated hydrolysis of very-low-density lipoprotein (VLDL)–TAG can release LPL from the surface of endothelial cells [34]. To determine whether this was a plausible explanation for the

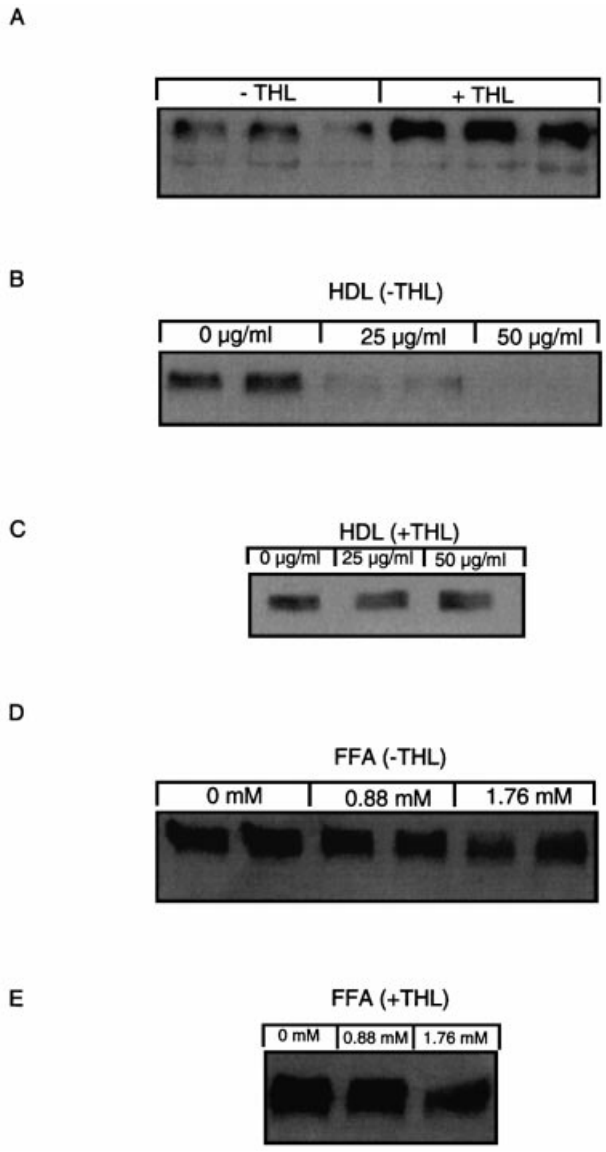

Figure 3 Western blot analysis of the EDL fraction bound to the cell surface of EDL-Ad-infected cells upon incubations with (A) THL, (B) HDL, (C) HDL + THL, (D) FFAs and (E) FFAs + THL

(**A**) HepG2 cells were infected with EDL-Ad (MOI 30). After infection, cells were incubated for 21 h under standard conditions in DMEM + 10% LPDS, followed by incubation for a further 3 h in the presence (+) or absence (−) of THL (25 μg/ml). Cells were washed extensively with PBS and incubated with 100 units/ml heparin for 30 min at 37 °C. Aliquots of heparin-releasable fractions were mixed 1:1 with loading buffer, fractionated by SDS/PAGE (12% gels) and EDL-specific bands were detected using EDL polyclonal antiserum and ECL® assay. The blot shows samples from triplicate dishes from one representative experiment out of three. (**B**) HepG2 cells were infected with EDL-Ad (MOI 60). The cells were incubated, 24 h after transfection, with increasing concentrations of HDL (protein) in DMEM + 3% fatty acid-free BSA in the absence of THL for 2 h. The blot of heparin-releasable fractions shows samples from duplicate dishes from one representative experiment out of three. (**C**) Experiments were performed as for (**B**), but in the presence of 25 μg/ml THL. (**D**) Experiments were performed as for (**B**), but cells were incubated with increasing concentrations of FFAs. The blot shows concentrated samples of heparin-releasable fractions from duplicate dishes of one representative experiment. (**E**) Experiments were performed as for (**D**), but in the presence of THL.

differences in binding and uptake in the presence and absence of THL, we performed Western blot analyses of the heparin-releasable EDL protein fraction of EDL-Ad-infected cells pre-incubated for 3 h either with or without THL (Figure 3A). The intensity of the EDL-specific bands in the lanes with THL was drastically stronger compared with the bands without THL, suggesting release of EDL from the cell surface by the products of EDL lipolytic activity. Furthermore, the amount of heparin-releasable EDL decreased gradually upon a 2 h incubation of EDL-Ad-infected cells with increasing concentrations of HDL (Figure 3B). The effect of HDL was abolished when incubations were performed in the presence of THL (Figure 3C), suggesting requirement of EDL phospholipase activity for the release of EDL from the cell surface.

The capacity of FFAs to release EDL from the cells was also confirmed by the finding that oleic acid decreased the amount of heparin-releasable EDL from EDL-Ad-infected cells in a dose-dependent manner (Figure 3D). THL had no effect on the release of EDL mediated by FFAs (Figure 3E). In contrast with FFAs, lysoPC failed to release EDL from the cell surface (results not shown). These findings clearly demonstrated that enzymically active EDL releases fatty acids from HDL-associated phospholipids, which in turn detach EDL from the cell surface. To prove further that the EDL enzymic activity is a determinant of cell-surface-bound EDL, we infected HepG2 cells with EDL-Ad and MUT-EDL-Ad. Cells were incubated in a medium containing 10% (v/v) LPDS, either with or without 25 μg/ml HDL, to imitate cell-culture conditions applied in the binding and uptake experiments. The MUT-EDL ($Asp^{193} \rightarrow Asn$ mutation) expressed by infected cells showed undetectable phospholipase activity in our phospholipase assay (results not shown). Western blot analysis of the heparin-releasable fractions showed a decrease in the amounts of the catalytically active EDL bound to the cell surface in the presence of HDL (Figure 4A). In contrast, the presence of HDL failed to decrease the amounts of cell-surface-bound MUT-EDL (Figure 4A). To substantiate these findings, we incubated EDL- and MUT-EDL-expressing cells in the presence of low (0.5%) and high (10%) concentrations of FCS. Western blot analysis of the heparin-releasable fractions revealed decreased amounts of the catalytically active EDL on the cell surface in the presence of high substrate concentrations (10% FCS) compared with low substrate concentrations (0.5% FCS) (Figure 4B). A phospholipase activity assay showed that decreased amounts of the active EDL on the cell surface in the presence of 10% FCS was accompanied by a slight, but significant, increase in the EDL activity in the supernatant of the cells (Figure 4C). In contrast with the active EDL, the amounts of MUT-EDL on the cell surface were unchanged or slightly increased upon incubations with 10% FCS compared with incubations with 0.5% FCS (Figure 4B). These results confirmed that FFAs liberated from HDL- and FCS-associated phospholipids by the EDL-phospholipase activity have an ability to regulate the abundance of cell-surface-bound EDL. We then tested the ability of THL to increase the amounts of EDL on the cells independently of its ability to inactivate the enzyme. For this, EDL- and MUT-EDL-expressing cells were incubated in the presence and absence of THL. As shown in Figure 5, the presence of THL in the media of infected cells led to a substantial increase in the amounts of both EDL and MUT-EDL on the cell surface. From these results, we conclude that THL has the capacity to increase the amounts of the cell-surface-bound EDL, independent of its ability to inactivate the enzyme, by a so far unknown mechanism.

To examine the effect of THL on binding and uptake of HDL independent of its ability to inactivate the enzyme, we infected HepG2 cells with lacZ-Ad, EDL-Ad and MUT-EDL-Ad in the presence and absence of THL for 6 h at 37 °C. To determine the effect of THL on selective HDL-CE uptake, cells were infected

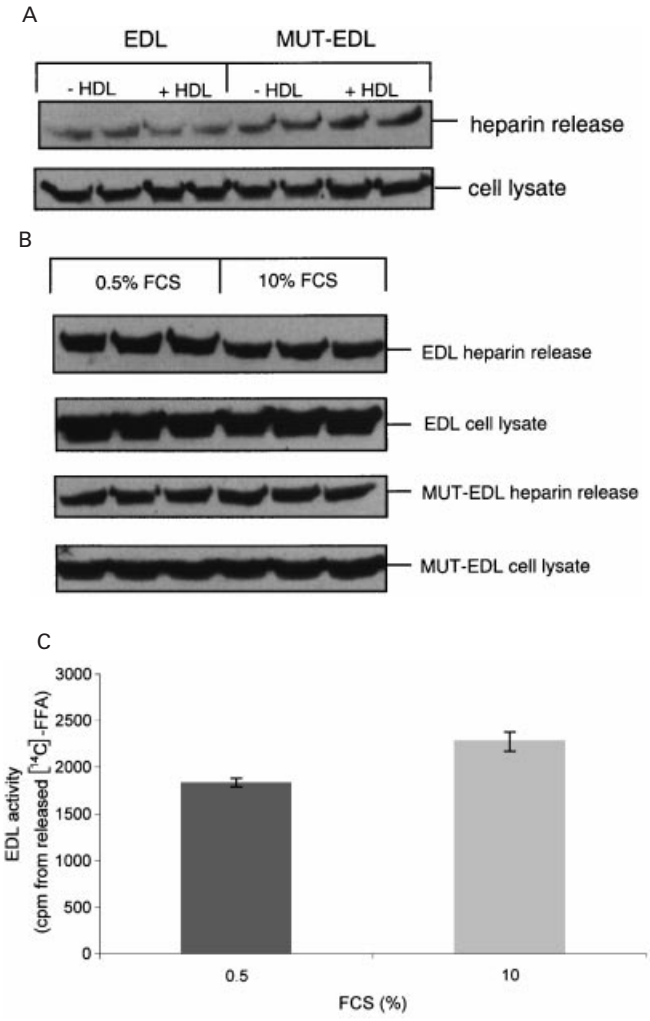

Figure 4 Western blot analysis of the EDL fraction bound to the cell surface and in the cell lysate of EDL-Ad- and MUT-EDL-Ad-infected cells upon incubations with (A) HDL, (B) 0.5 and 10% FCS; (C) EDL phospholipase activity in the media of cells described in (B)

(**A**) HepG2 cells were infected with EDL-Ad and MUT-EDL-Ad at MOI 60. After infection, cells were incubated for 24 h under standard conditions in DMEM + 10% LPDS, followed by incubation for 2 h with the same medium either without (−HDL) or with (+HDL) 25 μg/ml HDL (protein). After incubation, cells were extensively washed with PBS and incubated with 100 units/ml heparin for 30 min at 37 °C. Aliquots of heparin-releasable fractions were mixed 1:1 with loading buffer, fractionated by SDS/PAGE (12% gels) and EDL-specific bands were detected using EDL polyclonal antiserum and ECL® assay. After heparin release, cells were washed with PBS and lysed by Western blot loading buffer and analysed as the heparin-releasable fraction by Western blot. The blots show concentrated samples of one representative experiment performed in duplicate dishes. (**B**) Cells were infected as in (**A**) and, after infection for 24 h, were incubated with either 0.5 or 10% FCS. Cells were washed and the heparin-releasable fraction and cell lysates were obtained and analysed as described for (**A**). The blots show concentrated samples of one representative experiment performed in triplicate dishes. (**C**) Cell media were collected from cells that had been infected and incubated as described for (**B**). EDL phospholipase activity was determined in cell media from EDL-Ad-infected cells. Results are means ± S.D. for two independent experiments performed in triplicate dishes. cpm, counts per minute.

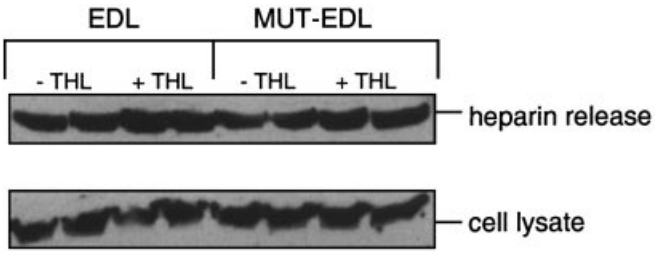

Figure 5 Western blot analysis of the EDL fraction bound to the cell surface and in the cell lysate of EDL-Ad- and MUT-EDL-Ad-infected cells upon incubation with THL

HepG2 cells were infected separately at MOI 60 with EDL-Ad and MUT-EDL-Ad. After infection, cells were incubated for 22 h in DMEM + 10% FCS, followed by incubation for a further 4 h in the same medium in the presence (+THL) or absence (−THL) of THL (25 μg/ml). After incubation, cells were washed with PBS and the heparin-releasable fraction was obtained by incubation with DMEM + 100 units/ml heparin. Upon washing with PBS, cells were lysed with Western blot loading buffer. Heparin-releasable EDL fractions (Speed Vac-concentrated) and cell lysates were analysed by Western blot after SDS/PAGE (12% gels) and EDL-specific bands were detected using EDL polyclonal antiserum and the ECL® assay. The blot shows samples from duplicate dishes from one representative experiment out of three.

and incubated in parallel experiment with [^{3}H]CE-HDL. As shown in Figure 6(A), the binding of ^{125}I-HDL to EDL-Ad-infected cells was 1.5-fold greater ($P < 0.001$) compared with lacZ-Ad-infected cells. In the presence of THL, the ^{125}I-HDL binding to EDL-Ad-infected cells was further increased 5.2-fold ($P < 0.001$). This represented a 7.7-fold increase ($P < 0.001$) compared with lacZ-Ad-infected cells (+THL). MUT-EDL-Ad-infected cells (−THL) bound 1.7-fold more ($P < 0.001$) ^{125}I-HDL compared with EDL-Ad-infected cells (−THL), and THL could increase further the binding of ^{125}I-HDL by MUT-EDL-Ad-infected cells (2-fold; $P < 0.001$). As for binding, the ^{125}I-HDL uptake by MUT-EDL-Ad-infected cells was significantly higher compared with EDL-Ad-infected cells (1.8-fold; $P < 0.001$), and could be increased further 1.3-fold ($P < 0.05$) by THL (Figure 6B). Figure 6(C) shows a 1.1-fold increase ($P < 0.001$) in selective uptake of HDL-CE by EDL-Ad-infected cells in the presence of THL (+THL), compared with cells infected with EDL-Ad (−THL) and MUT-EDL-Ad (−THL) respectively. Interestingly, selective uptake by MUT-EDL-infected cells was decreased 1.3-fold ($P < 0.001$) in the presence of THL.

Selective uptake of HDL-CE by HEK-293 cells overexpressing EDL and CLA-1

Considering an increase in selective uptake of [^{3}H]CE from HDL by EDL-Ad-infected HepG2 cells almost twice that of control cells, and the fact that HepG2 cells express high amounts of CLA-1 endogenously, we attempted to assess whether EDL alone can mediate the selective CE uptake from HDL independently of CLA-1. HEK-293 cells, which do not produce CLA-1 endogenously, were infected in parallel experiments with lacZ-Ad (MOI 4), EDL-Ad + lacZ-Ad (MOI 2 each), CLA-1-Ad + lacZ-Ad (MOI 2 each), and EDL-Ad + CLA-1-Ad (MOI 2 each) and incubated for 6 h at 37 °C with ^{125}I- and [^{3}H]CE-labelled HDL (25 μg of HDL protein/ml). Compared with control lacZ-Ad-infected cells, CLA-1-Ad infection resulted in a 4.8-fold increase ($P < 0.001$) in HDL-CE selective uptake (Figure 7A). The selective uptake by cells infected with EDL-Ad was 1.3-fold greater ($P < 0.05$) than that of lacZ-Ad-infected cells. Co-expression of EDL and CLA-1 in HEK-293 cells resulted in selective uptake of HDL-CE, similar to that observed by cells expressing only CLA-1. Western blot analysis and phospholipase

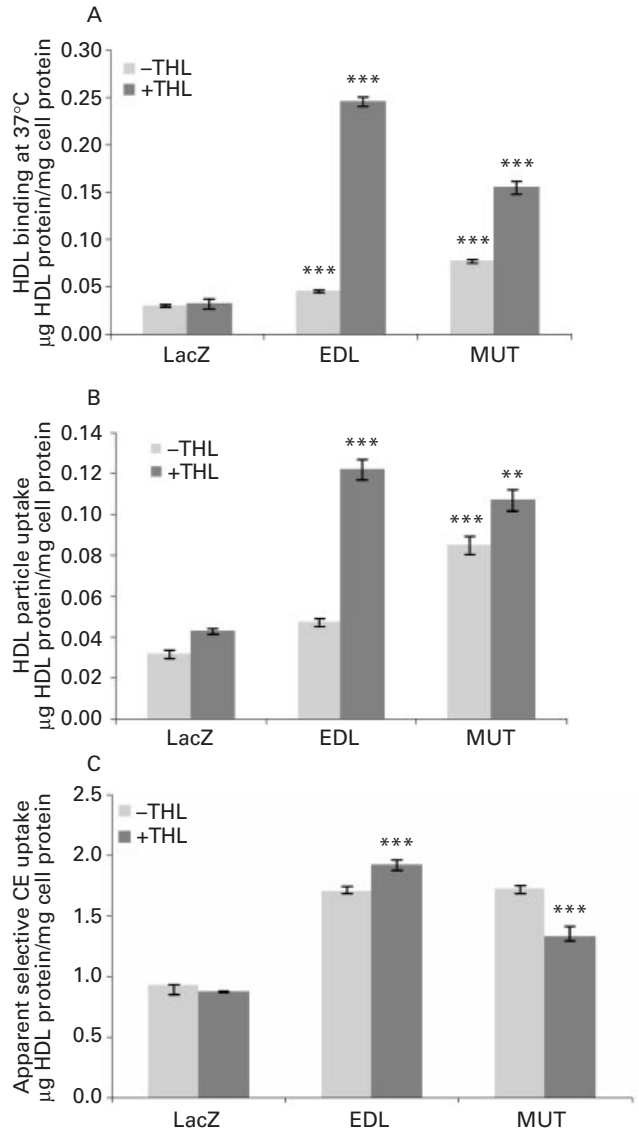

Figure 6 HDL heparin-releasable binding (A), HDL holoparticle uptake (B) and selective HDL-CE uptake (C) by HepG2 cells overexpressing EDL and MUT-EDL in the presence and absence of THL

Cells were infected separately with MOI 30 of EDL-Ad, MUT-EDL-Ad and lacZ-Ad. Cells, 24 h after infection, were treated in separate incubations with 25 μg of HDL (protein), labelled with ^{125}I in the protein moiety or with [^{3}H]CE in the lipid moiety, per ml of DMEM + 10% LPDS for 6 h at 37 °C. Heparin-releasable binding, holoparticle uptake and selective uptake were determined in the presence or absence of THL as described in Table 1. Results are means ± S.D. for three (lacZ-Ad and EDL-Ad) or four (MUT-EDL-Ad) independent experiments performed in triplicate dishes. (**A**) All values are means ± S.D. ***, $P \leqslant 0.001$ [compared with lacZ (−THL), EDL (−THL) and MUT (−THL)]. (**B**) All values are means ± S.D. ***, $P \leqslant 0.001$ [compared with EDL (−THL)] **, $P \leqslant 0.01$ [compared with MUT (−THL)]. (**C**) All values are means ± S.D. ***, $P \leqslant 0.001$ [compared with EDL (−THL) and MUT (−THL)].

assay showed that CLA-1 co-expression did not alter the EDL expression and activity in EDL-Ad and CLA-1-Ad co-infected HEK-293 cells (results not shown). To confirm further that EDL overexpression can enhance selective uptake of HDL-CE independently of CLA-1, HEK-293 cells were transiently transfected with EDL-expressing plasmid (pBK-CMV-EDL) or pBK-CMV alone (Figure 7B). In EDL-transfected HEK-293 cells,

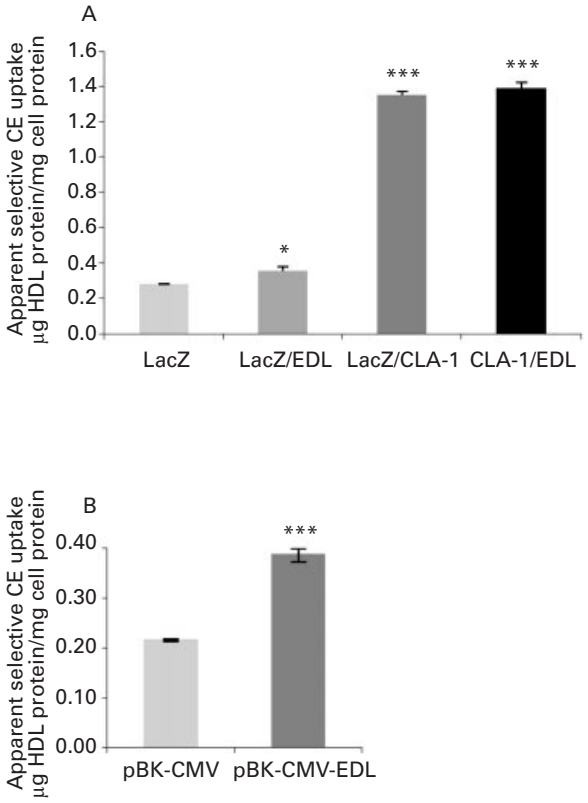

Figure 7 Selective uptake of HDL-CE by HEK-293 cells overexpressing EDL, CLA-1 or CLA-1 + EDL

(**A**) HEK-293 cells were infected separately with lacZ-Ad (MOI 4), CLA-1-Ad + lacZ-Ad (MOI 2 each), EDL-Ad + lacZ-Ad (MOI 2 each) and EDL-Ad + CLA-1-Ad (MOI 2 each) and incubated for 6 h at 37 °C with ^{125}I- and [^{3}H]CE-labelled HDL (25 μg of HDL protein/ml) in parallel experiments. The selective uptake was calculated as the difference between cell-associated [^{3}H]CE-HDL and ^{125}I holoparticle uptake. Results are means ± S.D. for triplicate dishes from one representative experiment out of three; ***, $P \leqslant 0.001$; *, $P \leqslant 0.05$ (compared with lacZ). (**B**) HEK-293 cells were transiently transfected with 1 μg of expression plasmid pBK-CMV-EDL or pBK-CMV (mock vector) by using the calcium phosphate co-precipitation method. Cells, 24 h after transfection, were incubated with ^{125}I- and [^{3}H]CE-labelled HDL (25 μg of HDL protein/ml) in parallel experiments for 6 h at 37 °C. The selective uptake was determined as described for (**A**). Results are means ± S.D. for three independent experiments performed in triplicate. ***, $P \leqslant 0.001$ (compared with pBK-CMV).

selective uptake was increased 1.8-fold ($P < 0.01$) compared with mock-transfected cells.

DISCUSSION

EDL is a new member of the lipase gene family, with substantial phospholipase and very little TAG lipase activity [9,18]. Adenovirus-mediated overexpression of EDL in mice led to a dramatic decrease in HDL and apolipoprotein A-I plasma levels, and a slight decrease in the concentration of apolipoprotein B-containing lipoproteins [9]. EDL is expressed in various tissues and cell types, including HepG2 cells [9]. In the present study, we aimed to assess the impact of EDL on the binding and cell association of HDL, as well as the selective uptake of HDL-CE by HepG2 cells infected with an adenovirus coding for hEDL (EDL-Ad). EDL overexpression in HepG2 cells caused a significant increase in binding and uptake of HDL particles. In addition, the selective uptake of HDL-CE was markedly elevated.

To investigate whether the EDL-mediated effect is dependent on its enzymic activity as a phospholipase, the experiments for HDL binding and particle uptake, as well as selective CE-uptake in HepG2 cells, were also performed in the presence of the lipase inhibitor, THL. THL led to greatly increased binding and moderately elevated uptake of HDL particles. The results of a previous study [34], demonstrating the release of LPL from the cell surface by FFAs generated by the hydrolysis of TAG in chylomicrons and VLDL, prompted us to assess whether a similar mechanism is operative for EDL in our experimental model. Accordingly, EDL might release fatty acids from phospholipids in HDL, which in turn cause the release of EDL from the cell surface. Four lines of evidence support this assumption. First, a marked increase in cell-associated EDL protein was observed in the presence of THL. Secondly, a marked decrease of cell-associated EDL protein was found upon incubations with increasing concentrations of HDL. Thirdly, incubation with increasing concentrations of oleic acid decreased the cell-surface-bound EDL protein. Fourthly, unlike EDL, the amount of cell-surface-bound MUT-EDL remained unchanged upon incubations with HDL and FCS respectively; additionally, like EDL, MUT-EDL could be released efficiently from the cell surface with oleic acid (results not shown). Taken together, these observations provide evidence that FFAs that are liberated by the EDL phospholipase activity can regulate the abundance of cell-surface-associated EDL protein. A decrease in the amount of cell-surface-bound EDL in the presence of 10% FCS compared with that in 0.5% FCS was accompanied by a slightly increased EDL activity in the media. From our initial experiments (results not shown), we discovered that the presence of increasing concentrations of FCS or unlabelled HDL interferes with the phospholipase assay. This might be due to the fact that HDL and FCS–lipoproteins serve as an alternative substrate acting as a competitive inhibitor of the radiolabelled substrate. Accordingly, we have assumed that EDL activity released into media with 10% FCS might be greater than was detected by the phospholipase assay. Of utmost interest was the finding that THL has the ability to increase the cell-surface abundance of both EDL and MUT-EDL. Taken all together, we conclude that elevated amounts of EDL on the cell surface in the presence of THL are due to both inhibition of EDL phospholipase activity and an augmenting effect of THL that is independent of its ability to inactivate the enzyme. The mechanism responsible for this latter augmenting effect of THL remains to be determined. In contrast with EDL, the effect of LPL on the binding and uptake of HDL, as well as the selective uptake of HDL-CE, was not affected by THL. This is probably due to the fact that neither active nor THL-inactivated LPL can liberate substantial amounts of FFAs from HDL. Therefore HDL has only limited ability to release LPL from the cells [34]. Furthermore, we demonstrated an increased heparin-releasable HDL binding and holoparticle uptake by cells expressing MUT-EDL, compared with cells expressing enzymically active EDL, which is most probably due to a higher amount of MUT-EDL on the cell surface compared with EDL (Figure 4A). Importantly, the heparin-releasable binding and holoparticle uptake by MUT-EDL-expressing cells could be increased further in the presence of THL. This is in part due to elevated amounts of EDL on the cell surface in the presence of THL and might be additionally due to an increased affinity of EDL for HDL in the presence of THL. The latter explanation is in line with previous studies demonstrating an increased LPL affinity for chylomicrons [35] and β-VLDL [36] in the presence of THL. The augmenting effect of THL on HDL binding by MUT-EDL was less pronounced than that of the active enzyme (Figure 6A). Considering a similar augmenting effect of THL on the cell surface abundance of EDL and MUT-EDL, a possible explanation for the less pronounced augmenting effect of THL on HDL binding by MUT-EDL might be a lower affinity of THL for the mutated active site.

In contrast with the increased heparin-releasable binding and holoparticle uptake, the selective uptake of CE from HDL was similar in cells expressing the active or catalytically inactive EDL. These results suggest that selective CE uptake by EDL-expressing cells is largely unrelated to either the amounts of EDL protein and HDL present on the cell surface or EDL enzymic activity. A possible explanation for the discrepancy between the abundance of cell-surface-bound HDL and the extent of selective CE uptake might be that the phospholipase activity of EDL is required for maximally efficient selective uptake of HDL-CE. Accordingly, cells that express the active EDL had less cell-surface-bound EDL and, in turn, less heparin-releasable HDL, but, owing to phospholipase activity, a more efficient selective uptake of HDL-CE. In contrast, cells expressing the inactive EDL had more cell-surface-bound EDL and, accordingly, more heparin-releasable HDL, but, due to the lack of phospholipase activity, a less efficient selective CE uptake from the bound HDL. This was in line with previous studies that demonstrated that the hydrolysis of phospholipids from HDL surface by HL [37] and by secretory phospholipase A_2 [38] modifies HDL structure, thus enhancing the selective uptake of CE from HDL. In addition, both a 3.2-fold-greater amount of HDL particles bound to the EDL-expressing cells (+THL) than to the MUT-EDL-expressing cells (−THL) (Figure 6A), and an almost identical selective uptake of CE from HDL by these cells, suggest that THL might exert a diminishing effect on the selective uptake of HDL-CE. This could be confirmed further by the finding that the selective uptake by MUT-EDL-expressing cells was decreased 1.3-fold in the presence of THL.

Considering the high-level expression of CLA-1 [6,39,40] in HepG2 cells, we speculated that the apparent increase in selective uptake of HDL-CE in EDL-infected cells might be due to an enhancing effect of EDL on CLA-1-mediated selective uptake of HDL-CE. Previous studies have demonstrated that the selective uptake of HDL-CE in different hepatoma cell lines [14,41,42] or Chinese-hamster ovary cells [17,41], both known to synthesize SR-BI, is enhanced by expression of HL. In contrast, HL expression in HEK-293 cells, which lack endogenous SR-BI, failed to enhance selective uptake of CE from HDL [37]. Most recently, Rinninger et al. [43] have demonstrated that LPL mediates an increase in selective uptake of HDL-CE, independently of SR-BI. In the present study, EDL also mediated an increase in HDL-CE selective uptake in EDL-overexpressing HEK-293 cells, indicating the capacity of EDL to mediate selective uptake of HDL-CE independently of CLA-1. Although EDL transfection and EDL-Ad infection resulted in similar levels of EDL expression, as determined by Western blotting and activity assay (results not shown), a more pronounced effect of EDL on selective uptake was consistently found in all experiments performed with EDL-transfected, compared with EDL-Ad-infected, HEK-293 cells. A possible explanation for this could be the fact that recombinant adenovirus has the ability to replicate in HEK-293 cells, which might in turn affect some cellular processes, including selective uptake. The infection of HEK-293 cells with MOI 4 did not lead to any morphological changes or cytopathic effects during experiments (results not shown). The co-expression experiments (EDL-Ad + CLA-1-Ad) failed to demonstrate any enhancing effect of EDL on CLA-1-mediated selective uptake of HDL-CE in adenovirus-infected cells (Figure 7A) or in transiently transfected cells (results not shown). The ability of EDL to enhance selective CE uptake was also observed

in EDL-Ad-infected Cos-7 cells that lacked CLA-1 (a 1.5-fold increase compared with mock-infected controls; results not shown).

In conclusion, we demonstrate for the first time that EDL facilitates the binding and holoparticle uptake of HDL as well as the selective uptake of HDL-CE by HepG2 cells. The binding and uptake were dramatically increased in the presence of THL. We found that THL increases amounts of EDL on the cell surface in two ways: first, THL inactivates the enzyme, preventing the release of EDL from cells by FFAs liberated from HDL-associated phospholipids; secondly, THL increases amounts of cell-surface-bound EDL independently of its ability to inactivate the enzyme by an unknown mechanism. The increased HDL binding and holoparticle uptake in the presence of THL are primarily due to an augmenting effect of THL on the amounts of cell-surface-bound EDL. Similar selective uptake of HDL-CE by cells expressing the active EDL, THL-inactivated EDL and MUT-EDL, despite vastly different amounts of cell-surface-bound EDL and HDL, suggests that selective CE uptake by EDL-expressing cells is largely unrelated to the amounts of cell-surface-bound EDL and HDL as well as to enzymic activity of EDL. EDL is able to increase the selective CE uptake from HDL in cells lacking CLA-1, but it fails to enhance CLA-1-mediated selective uptake. Further studies are required to determine whether the facilitating effect of EDL on HDL binding, uptake and selective CE uptake from HDL observed *in vitro* has a physiological relevance *in vivo*.

This work was supported by grants SFB-F702 (to G.M.K.), SFB-F713 (to R.Z.) and SFB-F717 (to S.F.) of the Austrian Fonds zur Förderung der Wissenschaftlichen Forschung. We thank Michaela Tritscher for technical assistance.

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Received 20 February 2002/31 July 2002; accepted 6 August 2002
Published as BJ Immediate Publication 6 August 2002, DOI 10.1042/BJ20020306

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