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Apolipoprotein M and sphingosine-1-phosphate receptor 1 promote the transendothelial transport of HDL

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Abstract

Objective: Apolipoprotein M (ApoM) enriches sphingosine-1-phosphate (S1P) within high density lipoproteins (HDL) and facilitates the activation of the $S1P_1$ receptor by S1P,

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Author contributions

A.v.E., L.R. and J.R.N. designed the study. A.v.E, J.R.N., M.Si., K.S.P, C.C., and D.W. obtained funding. M.St., M.L., and J.S.P., provided samples from mice with knock-out or overexpression of apoM. C.C. provided human HDL depleted of apoM as well as control HDL containing apoM. F.P., R.F., M.Si., and J.R.N. generated and characterized the $S1P_1$-ECKI mice. S.V., L.R., F.P., R.F., A.H., B.H., D.P., D.W., G.P. A.P., and M.Y performed the experiments. L.R., J.R.N. and A.v.E. supervised experiments. S.V., L.R., A. L., F.P., J.R.N, and A.v.E. analyzed and interpreted data. S.V. and A.v.E. wrote the first draft of the original manuscript. A.v.E. wrote the first draft of the revision. All authors critically read and revised the manuscript.

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thereby preserving endothelial barrier function. Many protective functions exerted by HDL in extravascular tissues raise the question how S1P regulates transendothelial HDL transport.

Approach and Results: HDL were isolated from plasma of wild type mice, *Apom* knock-out mice, human apoM transgenic mice or humans and radioiodinated to trace its binding, association, and transport by bovine or human aortic endothelial cells (BAECs and HAECs, respectively). We also compared the transport of fluorescently-labeled HDL or Evan's Blue, which labels albumin, from the tail vein into the peritoneal cavity of apoE-haploinsufficient mice with ($S1P_1$-iECKI) or without (CTRL) endothelium specific knock-in of $S1P_1$. The binding, association, and transport of HDL from *Apom* knock-out mice and human apoM-depleted HDL by BAECs was significantly lower than that of HDL from wild type mice and human apoM containing HDL, respectively. The binding, uptake, and transport of ^{125}I-HDL by HAECs was increased by an $S1P_1$ agonist but decreased by an $S1P_1$ inhibitor. Silencing of scavenger receptor BI (SR-BI) abrogated the stimulation of ^{125}I-HDL transport by the $S1P_1$ agonist. Compared to CTRL, $S1P_1$-iECKI showed decreased transport of Evan's Blue but increased transport of HDL from blood into the peritoneal cavity and SR-BI expression in the aortal endothelium.

Conclusions: ApoM and $S1P_1$ promote transendothelial HDL transport. Their opposite effect on transendothelial transport of albumin and HDL indicates that HDL passes endothelial barriers by specific mechanisms rather than passive filtration.

Keywords

HDL; sphingosine-1-phosphate; apolipoprotein M; endothelium; transcytosis

Introduction

Low plasma levels of high density lipoprotein (HDL) cholesterol are associated with increased risk of coronary heart disease (CHD)[1]. HDL particles exert many effects, which may protect the organism from chemical or biological harm and thereby explain these inverse associations[2]. Nevertheless, HDL has not been as yet exploited for prevention or treatment of CHD[1]. An important reason for this shortfall is the structural and functional complexity of HDL particles, which are heterogeneous and complex macromolecules carrying hundreds of lipid species and proteins[2, 3]. Among them sphingosine-1-phosphate (S1P) has attracted considerable interest. S1P is the agonist of five G-protein coupled receptors termed $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ [4]. S1P is enriched in HDL due to the presence of apoM[5]. By providing a binding pocket [5], this lipocalin promotes efflux of S1P from erythrocytes [6, 7] and acts as a chaperone facilitating the interaction of S1P with its cognate receptors, notably $S1P_1$ [8–10]. The interaction of apoM was shown to be mandatory for the activation of $S1P_1$ by S1P in endothelial cells and the subsequent inhibition of adhesion molecule expression [6, 7]. In addition, the apoM/S1P complex stabilizes the endothelial barrier as indicated by the exudation of albumin into the extravascular space and the formation of lung edema in *Apom* knock-out mice [5, 10]. The endothelial barrier was restored by treatment with an $S1P_1$ agonist [10]. The endothelium-specific knock-out of the *S1p1* receptor in mice also led to increased endothelial permeability upon stimulation with immune complexes [11]. Moreover, *Apom* knockout mice showed a weakened blood brain barrier with increased paracellular permeability in the capillaries and enhanced vesicular

transport through the endothelium of arterioles and this phenotype was reversed by $S1P_1$-agonist treatment [12].

The classical anti-atherogenic function of HDL, namely the removal of cholesterol from macrophages for reverse transport of cholesterol to the liver, requires both entry into and exit from the arterial wall, and hence two passages through endothelial cells[13]. By recording the binding, uptake, degradation, and resecretion of radioiodinated HDL as well as by fluorescence microscopy and electron microscopy of labeled HDL, our laboratory showed that human and bovine aortic endothelial cells (HAECs and BAECs, respectively) internalize HDL without degrading it [14, 15]. By using a transwell cell culture system and applying siRNAs or pharmacological inhibitors against candidate genes and proteins, respectively, we found scavenger receptor BI (SR-BI), ATP binding cassette transporters (ABC) A1 and G1, endothelial lipase (LIPG), and the ecto-ATPase/purinergic P2Y-receptor axis to be rate limiting for the transendothelial transport of apoA-I or HDL [16–19]. Moreover, SR-BI facilitates the transport of HDL from extravascular tissues into lymphatic vessels as well as for reverse cholesterol transport [20]. However, how HDL passes the elastin barrier in the arterial wall is not known.

Any general stabilization of intercellular junctions and general inhibition of transendothelial vesicular transport by HDL-bound apoM and S1P would interfere with the exit of HDL from the blood stream into extravascular compartments and thereby counteract the protective functions exerted by HDL therein [2, 21]. We hypothesized that S1P does not interfere with transendothelial transport of HDL and therefore investigated the effects of apoM and the $S1P_1$ receptor on the binding, uptake, and re-secretion of HDL by bovine or human aortic endothelial cells as well as transendothelial transport of HDL in vivo.

Materials and Methods

The authors declare that all supporting data are available within the article and its online supplementary files.

Details on animals, cells, antibodies, primers, and small interfering RNA's are provided in the Major Resource Table.

Mouse models

ApoM-free/S1P-poor and apoM/S1P-enriched HDL were isolated from pooled plasmas of male and female *Apom* knock-out mice and male and female human *APOM* overexpressing mice, respectively, which were described previously [20, 22, 23].

Experiments on the effects of the $S1P_1$ receptor agonist SEW 2871 on the transport of HDL and Evan' Blue were performed in 10 – 12 weeks old female *C57Bl/6J* mice (Charles River Laboratories, Sulzfeld, Germany)) fed with a regular laboratory diet.

Triple transgenic mice overexpressing murine $S1P_1$ exclusively in endothelial cells were developed by crossing two lines. The *C57Bl/6J-Gt(ROSA)26Sor*$^{tm1(S1pr1)Geno}$ (referred to as *S1pr1* ^{LSL} line), generated by genOway (Lyon, France) using their patented Rosa26 locus Quick knock-in™ technology, carries a transgenic cassette in the Rosa26 locus, which

harbors $S1P_1$ cDNA. It is separated from the synthetic cytomegalovirus early enhancer/chicken β-actin (*CAG*) strong promoter by a *LoxP-STOP-LoxP (LSL)* insert (Supplemental Figure Ia). *S1pr1*^{LSL} mice were crossed to *Apoe*$^{-/-}$*Cdh5-CreER*^{T2} mice, provided by Dr. Christian Weber (Ludwig Maximillian University, München, Germany). They express Cre recombinase under control of the VE-cadherin promoter, which is in turn induced by tamoxifen treatment [24]. In triple transgenic mice, the *LSL* insert is hence excised only in Cre-expressing cells, which induces cDNA expression driven by the *CAG* promoter only in these lineages. Transcription thus occurs exclusively in the endothelial cell lineage. These mice are henceforth referred to as $S1P_1$-inducible endothelial cell knock-in $S1P_1$-iECKI mice. Double heterozygous *Apoe*$^{+/-}$*Cdh5-CreER*$^{T2\,+/-}$ mice were used as controls (CTRL). Genotyping was performed by classical PCR (Supplemental Figure Ib). The experiments on transport of lipoproteins and Evan's Blue as well as en face immunostaining of $S1P_1$ and SR-BI were performed on 10 – 12 weeks old female mice fed a regular laboratory diet. For atherosclerosis studies, following induction of $S1P_1$ overexpression, 6-week-old female mice received high-fat atherogenic diet (w/w: 1.25% cholesterol; 16% fat; 0,5% sodium cholate; Altromin, Lage, Germany; corresponding to Research Diets D12109) for 30 weeks [25, 26].We used female mice because of substantial scientific evidence that atherosclerosis development is more prominent in female mice than in male mice including *Apoe* knock-out mice [27–29] (3 refs). This is even more relevant in *Apoe* haploinsufficient mice that inherently less susceptible to atherosclerosis than complete *Apoe* knock-out mice.

Animal experiments were carried out in compliance with the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and was approved by authorities in charge of animal protection (LANUV, permission 84–02.04.2011.A351, 84–02.04.2015.A505 and 84–02.04.2016.A545).

Assessment of vascular permeability for lipoproteins and Evan's Blue

Female $S1P_1$-iESKI or CTRL mice were intravenously administered with Evans blue (600μg/animal), or DyLight™-HDL or DyLight™-LDL (DyL-HDL and DyL-LDL, respectively; 350 μg/animal, prepared as described below) 15 minutes before LPS (25 μg/animal) was injected intraperitoneally. SEW2871 was diluted in PBS (to 0.1mg/ml in 1% DMSO) and administered as a single i.p. bolus injection (15μg/g body weight) 150 minutes prior to albumin or lipoprotein injection[12] Mice were sacrificed after 3h and their peritoneal cavities were washed with 10mL of ice-cold heparinized PBS. After spinning down cells, the supernatants were analyzed for Evans Blue with photometry (620nm, FluoStar Optima, BMG LabTech, Ortenberg, Germany) and for DyL-HDL or DyL-LDL with fluorescence spectrometry (560nm/590nm, FluoStar Optima).

En face immunostaining and atherosclerotic lesion analysis

At sacrifice, inferior vena cava was opened, and mice were perfused by intracardiac injection of ice-cold sterile saline followed by neutral buffered formalin (NBF, HT501128, Sigma Aldrich). Hearts and aortae were dissected and processed as follows: Hearts were further fixed in NBF for 30 minutes, incubated overnight at +4°C and successively embedded into Tissue-Tek O.C.T. compound. Arteries were incubated for 18h with primary antibodies diluted in blocking solution at 4°C under constant agitation (After three washes (10

minutes each) in washing solution (PBS, 0.1% Triton X-100), tissues were incubated with fluorescent secondary antibodies (Alexa Fluor dyes, Molecular Probes) for 1.5h, washed, counterstained and mounted with Fluoroshield DAPI mounting medium (Sigma-Aldrich), and then imaged using a Leica TCS SP2 confocal microscope. Atherosclerosis was evaluated in the aortic roots as described[30, 31]. Aortas were quickly cleaned of adventitial tissue, opened longitudinally and incubated for 1.5h at room temperature in the blocking solution (PBS, 2% BSA, 0.1% Triton X-100).

Cell culture

Primary Bovine aortic endothelial cells (BAECs) were cultured in Dulbecco's modified eagle's medium (DMEM) with 5% fetal bovine serum at 37°C in 5% CO_2, 95% air incubator. Human aortic endothelial cells (HAECs) were cultured in endothelial cell basal medium (LONZA Clonetics CC-3156 or ATCC PCS-100–030) with 5% fetal bovine serum (Sigma-Aldrich), 100U/mL of penicillin and 100μg/mL streptomycin (Sigma-Aldrich), supplemented with singleQuots (LONZA Clonetics CC-4176 or ATCC PCS-100–041, containing human Fibroblast Growth Factor, hVEGF, human insulin like growth factor 1, human epithelial growth factiorF, hydrocortisone, ascorbic acid, heparin) at 37°C in a humidified 5% CO_2, 95% air incubator as described previously [17, 32].

Lipoprotein Isolation and labeling

HDL (1.063<d<1.21kg/L) was isolated from fresh human normolipidemic plasma of blood donors or mouse plasma by sequential ultracentrifugation as described previously [18, 33]. ApoM-containing and apoM-free HDL of human plasma were separated by immunoaffinity chromatography as described previously [7]. HDL were radioiodinated with $Na^{125}I$ by the McFarlane monochloride procedure modified for lipoproteins [18]. Specific activities ranged between 300 and 900cpm/ng of protein. For assessment of vascular permeability of lipoproteins in mice, LDL and HDL were labeled with DyLight™ 550 fluorescent dye (DyL, Thermo Fischer)) according to the manufacturer's instruction. Briefly, HDL or LDL (2.0mg/mL, pH adjusted to 8.0 with 50mmol/L sodium borate) were mixed with DyLight™ 550 reagent (1:1, v/v), incubated for 60 minutes, and purified using spin columns provided by the manufacturer.

Small Interfering RNA Transfection

Endothelial cells were reverse transfected with small interfering RNA (Silencer Select, Thermo Fisher Scientific) targeted to $S1P_1$ (SR-BI) and non-silencing control (Main Resource Table) at a final concentration of 5nmol/L using Lipofectamine RNAiMAX transfection reagent (Invitrogen,) in an antibiotic-free medium. All experiments were performed 72h post-transfection and efficiency of transfection was confirmed with at least two siRNAs against each gene using quantitative RT-PCR and Western blotting.

Quantitative real time PCR

Total RNA was isolated using TRI reagent (Sigma T9424) according to the manufacturer's instruction. Genomic DNA was removed by digestion using recombinant DNase I (Roche) and RNase inhibitor (Ribolock, Thermo Scientific). Reverse transcription was performed

using M-MLVRT (Invitrogen, 200U/μL) according to the standard protocol provided by the manufacturer. Quantitative PCR was done with Lightcycler FastStart DNA Master SYBR Green I (Roche) using gene specific primers (for sequences, see Major Resource Table).

Lipoprotein Binding, Cell association and Transport

The methods for the quantification of binding, association, and transport of radiolabeled HDL by endothelial cells have been previously described [13,15–18,21,22]. All assays were performed in DMEM (Sigma) containing 25mmol/L HEPES and 0.2% BSA instead of serum. Cells were pretreated for 30 minutes at 37 °C with either $S1P_1$ agonist SEW2871 (Cat No:2284, Tocris, 20nM) or $S1P_1$ inhibitor W146 (Cat No:3602, Tocris, 20nM). The drugs were prepared as described by the vendor. After 30 minutes of treatment with either $S1P_1$ agonist SEW2871 or $S1P_1$ inhibitor W146, the cells were incubated with 10μg/mL of ^{125}I-HDL without (total) or with 40 times excess of non-labeled HDL (unspecific) for 1hour at 4°C for cellular binding and at 37°C for association and transport experiments. Specific cellular binding/ association/ transport was calculated by subtracting the values obtained in the presence of excess unlabeled HDL (unspecific) from those obtained in the absence of unlabeled HDL (total).

Inulin permeability

After culturing for 72h in transwells, HAECs were treated with SEW2871 or W146 for 30 minutes before 2 mCi/mL of ^{3}H-inulin were added into the apical compartment. After 1h of incubation, the filtrated radioactivity was harvested from the basolateral compartment [18].

Western Blotting

Endothelial cells were lysed in RIPA buffer (10mmol/L Tris pH 7.4, 150mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, with protease and phosphatase inhibitors (complete EDTA (Roche)). Amount of protein in the lysed samples is quantified using Pierce BCA protein assay kit (Thermo Fisher Scientific) 30μg of protein were separated on SDS-PAGE and trans-blotted onto PVDF membrane (GE Healthcare). Membranes were blocked in appropriate blocking buffer recommended for the antibody (PBS-T supplemented with 5% milk or BSA) and incubated either for 1hour or overnight on shaker at 4 °C with primary antibodies at a dilution of 1:1000 in the same blocking buffer. Membranes were incubated for 1h with HRP-conjugated secondary antibody (Dako) in blocking buffer (antibody concentrations are provided in the Major Resource Table). Membranes were further incubated with chemiluminescence substrate for 1 minute (Pierce ECL plus, Thermo Fisher Scientific) and imaged using Fusion Fx (Vilber). As indicated, TATA binding protein was used as loading control with primary antibody at 1:5000 and secondary antibody at 1:10000 dilutions. The silencing efficiencies of $S1P_1$ and SR-BI were evaluated and compared to TATA binding protein (TBP) (for sources and concentrations of antibodies, see Major Resource Table).

Cell surface expression analysis

Biotinylation of intact cells was performed using 20mg/mL EZ-Link sulfo-NHS-S-S-Biotin (Thermo Fisher Scientific) in the cold for 1 hour with mild shaking and quenched with ice-

cold 50mM Tris pH 7.4. Cells were lysed in RIPA buffer (total cell lysate) and 200–500μg of lysates were incubated with 20μL of BSA-blocked streptavidin beads suspension (GE Healthcare) for 16 hours at 4°C and pelleted by centrifugation; the pellet represents surface proteins. Proteins were dissociated from the pellet by boiling with SDS loading buffer and analyzed by SDS-PAGE and immunoblotted with SR-BI antibody, LDL-receptor and TATA binding proteinor Na^+/K^+- ATPase which were used as loading control (for sources and concentrations of antibodies, see Major Resource Table).

Statistical Analysis

The data sets were analyzed using the GraphPad Prism 5 software. Comparison between two groups was performed using unpaired Mann Whitney U-tests. The data was obtained from at least three independent experiments, performed in triplicates or quadruplets. Values are expressed as mean ± SEM. $P<0.05$ was regarded as significant.

Results

The content in apoM modulates the uptake and transendothelial transport of HDL by bovine aortic endothelial cells (BAECs) –

HDL isolated from plasma of *Apom* knock-out mice showed 21% reduced binding, 30% reduced association, and 48% reduced apical-to-basolateral transport by bovine aortic endothelial cells (BAECs) as compared to HDL of wild type mice (Figures 1A, 1B, and 1C). Likewise, human apoM-depleted HDL, which lacks S1P [5,7], showed reduced association with BAECs as compared to total HDL (Figures 1D, 1E, and 1F). Conversely, HDL isolated from mice overexpressing human apoM were more effectively associating with BAECs (Figure 2; $P = 0.0052$).

In human aortic endothelial cells (HAECs), $S1P_1$ regulates cellular binding, association and transendothelial transport of HDL –

We next investigated how the cognate G protein coupled S1P receptor $S1P_1$ regulates the binding, uptake, and transport of HDL by cultivated HAECs. They express $S1P_1$ as analyzed at the protein level (Supplemental Figure IIa). Treatment of HAECs for 30 minutes prior to the assays with 20nM of the $S1P_1$ agonist SEW2871 increased the specific cellular binding of ^{125}I-HDL at 4°C by 95% (Figure 3A), the cellular association of ^{125}I-HDL at 37°C by 39% (Figure 3B), and the transendothelial transport of ^{125}I-HDL from apical to basolateral compartments by 37% (Figure 3C). Conversely, treatment with 20nM of the $S1P_1$ inhibitor W146 (20nM) decreased the specific binding, association, and transport of ^{125}I-HDL by 61% (Figure 3D), 46% (Figure 3E), and 59% (Figure 3F), respectively. Inhibition of $S1P_1$ interfered neither with the permeability of ^{3}H-inulin (Supplemental Figure IIIa) nor the confluence of HAECs as assessed by immunostaining of tight-junction protein 1 (Supplemental Figure IIIb). Like the pharmacological inhibition, silencing of $S1P_1$, decreased the cellular binding, association, and transport of ^{125}I-HDL by 49%, 53% and 42%, respectively (Supplemental Figures IIb to IId).

$S1P_1$ regulate cellular binding, association and transendothelial transport of HDL via SR-BI

Because SR-BI mediates the binding, uptake and transport of HDL by both BAECs and HAECs [17,22], we next investigated by RNA interference whether it is involved in the $S1P_1$-stimulated trans-endothelial transport of HDL. The knockdown of SR-BI was efficient at protein level (Figure 4A). Specific binding, association, and transport of HDL by HAECs was significantly decreased by 40%, 36%, and 43% after silencing of SR-BI and remained unrestored in the presence of the $S1P_1$ agonist (Figures 4B, 4C, and 4D, respectively). Cell surface biotinylation experiments showed that activation of $S1P_1$ receptor increases the cell surface abundance of SR-BI but not of Na/K ATPase which was included as the loading control (Figure 5

Endothelial overexpression of $S1P_1$ in mice increases SR-BI in the aortic endothelium and endothelial permeability for HDL but decreases endothelial permeability for LDL and Evan's Blue

Treatment of wild type mice with the $S1P_1$ agonist SEW2871versus saline significantly decreased the occurrence of Evan's Blue which binds to albumin by 19% but increased the occurrence of DyL-HDL by 37% in the peritoneal lavage (Supplemental Table I). To further investigate the impact of $S1P_1$ on transendothelial lipoprotein transport *in vivo*, we generated *Apoe* haploinsufficient mice which overexpress human $S1P_1$ ($S1P_1$-iECKI) receptors under the control of a tamoxifen-inducible VE-cadherin promotor in endothelial cells only. The successful knock-in was demonstrated by genotyping (Supplemental Figure Ib) as well as the indirect immunofluorescence microscopy of aortas (Figure 6 and Supplemental Figure IV). The anti-$S1P_1$ immunoreactivity of the endothelium was rather weak in aortas of CTRL mice (Figure 6A) but much enhanced in aortas of $S1P_1$-iECKI (Figure 6B). Likewise, the anti-SR-BI immunoreactivity was strongly enhanced in the aortic endothelium of S1P1-iECKI mice compared to CTRL mice (Figures 6c and 6d and Supplemental Figure V). While consuming a regular laboratory diet, CTRL and $S1P_1$-iECKI mice had similar plasma levels of cholesterol and triglycerides (Table 1). Upon gel filtration of plasma, no major difference in the distribution of cholesterol and triglycerides among lipoproteins was seen (not shown). Compared to CTRL mice, $S1P_1$ -iECKI mice showed 46% and 40% decreases of Evan's Blue, and DyL-LDL but a 77% increase of DyL-HDL in the peritoneal lavage (Table 1). After 30 weeks feeding of a 1.25% cholesterol containing Western diet, the $S1P_1$-iECKI mice had developed less pronounced hypercholesterolema as well as 30% less fatty lesions in their sinus aortae than Apoe haploinsufficient CTRL mice (Supplemental Table II).

Discussion

In mice, the knock-out of the S1P-binding protein apoM caused a strong decrease of S1P levels in plasma and HDL as well as increases in the permeability of lung capillaries and the blood brain barrier for albumin [5, 10, 12]. Likewise, the endothelium-specific knock-out of the *S1p1* receptor increased the endothelial permeability of albumin [11]. Contradicting a general inhibitory effect of S1P on transendothelial macromolecule transport, we here demonstrate that the apoM/S1P/$S1P_1$ interaction promotes rather than inhibits the transendothelial

transport of HDL, which may be important in facilitating specific HDL entry into extravascular tissues where the particles exert most of its protective functions [2].

For our in vitro experiments, we cultivated BAECs or HAECs either in monolayers for binding and association experiments or in transwells for the transport experiments. We have used these models previously for the identification and validation of several proteins as facilitating factors of the transendothelial transport of apoA-I or HDL, namely ABCA1 and ABCG1, SR-BI, LIPG and the ecto-ATPase/P2Y receptor axis [16–19]. Among them, SR-BI was also identified as a limiting factor of LDL transport by independent laboratories and methods [24, 34, 35].

To study the role of apoM and S1P in transendothelial HDL transport, we compared HDL of wild type mice and *Apom* knock-out mice for binding, uptake, and transport by BAECs. Christoffersen et al. have shown that the lack of apoM leads to the near disappearance of S1P from HDL[5–7]. The absence of apoM and S1P caused 25% to 50% decrease in the binding, association, and transport of radioiodinated HDL by BAECs (Figures 1A, 1B, and 1C). Similar significant decreases were seen for apoM-depleted HDL (Figures 1D, E and 1F), which also is completely devoid of S1P [5]. Since apoM predominates in smaller HDL3 particles [36], one may wonder whether different particle size rather than difference in apoM and S1P content caused the differences in uptake and transport. However HDL of mice transgenic for human apoM, which have 150% to 200% higher levels of S1P [33, 22], showed a significantly higher endothelial cell association than HDL of wild type control mice (figure 2) despite their increased particle size[22, 37]. Moreover, we showed previously that HDL3 and HDL2 are equally bound, internalized and transported through aortic endothelial cells and that HDL size rather decreases during the transport [17, 18]. Therefore, and because of the presence of apoM in only 5% of HDL particles[36], it is very unlikely that the increased transendothelial transport of apoM containing HDL compared to apoM deficient HDL is simply caused by smaller particle size.

Binding, uptake, and transport of HDL by HAECs were promoted by the $S1P_1$ agonist SEW2871 and impaired by the $S1P_1$ inhibitor W146. These drugs were previously used to show the involvement of $S1P_1$ in mediating inhibitory HDL effects on adhesion molecule expression [9]. The present study validates the specificity of the drugs as RNA interference mimicked the effects of W146 and abrogated the effects of SEW2871. In addition, interference with SR-BI abrogated the enhanced binding, uptake, and transport of HDL otherwise apparent in the presence of the $S1P_1$-agonist. Likewise, increased cell surface abundance of SR-BI underlies the enhanced transendothelial transport upon stimulation with VEGF-A [32]. Activation of either $S1P_1$ and VEGFR-2 induces Akt phosphorylation [38], which promotes SR-BI translocation to the plasma membrane and binding, uptake and transport of HDL by HAECs [32]. A recent untargeted proteomics approach found that the expression of *S1p1* in murine pulmonary lung endothelial cells alters the apical plasma membrane abundance of several proteins [39]. One of them was CD36, which is very homologous to SR-BI. The mechanism by which $S1P_1$ or VEGFR-2 regulate the cell surface abundance of SR-BI is not known. One candidate is the regulation of the retromer that mediates the recycling of endocytosed lipoprotein receptors including SR-BI to the cell surface[40]. Moreover, it is important to consider that $S1P_1$ might regulate SR-BI by different

mechanisms, depending on the time kinetics: Translocation of preformed SR-BI may explain the quick effects of apoM/S1P as well as the pharmacological interventions with $S1P_1$ on HDL uptake and transport. Both the knock-down of S1P1 by RNA interference in vitro as well as the knock-in of $S1P_1$ in vivo, however, may also regulate the production of SR-BI. Indeed, the aortic endothelium of $S1P_1$-iECKI mice presented with increased anti-SR-BI immunoreactivity. Moreover, we previously reported decreased SR-BI mRNA and protein expression of SR-BI in HAECs after knock-down of $S1P_1$[41].

The in vitro findings regarding $S1P_1$ activation and its role in the transendothelial transport were principally recapitulated under in vivo conditions. In mice, both treatment with the $S1P_1$ agonist and the endothelium-specific overexpression of $S1P_1$ led to increased transport of fluorescent HDL from the blood stream into the peritoneal cavity. Interestingly the transport of Evan's Blue, which is bound by albumin and hence reflects albumin transport is decreased by $S1P_1$ overexpression. We also found transport of fluorescent LDL into the peritoneal cavity decreased in our model. Likewise, mice with a knock-out of sphingosine kinase 2, resulting in increased S1P levels in plasma, show decreased transport of both dextran beads and LDL into the peritoneal cavity [42]. Conversely, endothelium specific knock-out of endothelial $S1P_1$ showed enhanced exudation of Evan's Blue into the lung [11].

Taken together, the in vivo and in vitro data indicate that transendothelial HDL transport is promoted rather than inhibited by the S1P/$S1P_1$ interaction. Of note, we made these complementary findings in different endothelial cell systems. Our in vivo model like the ones used by others previously on apoM and $S1P_1$ reflects macromolecule transport through capillaries, which is happening either paracellularly or transcellularly through pores [43]. The in vitro experiments were done in confluent macrovascular endothelial cells from two different species, which is supposed to involve vesicular transport at least in addition to those described before. Of note, however, apoM/S1P was previously shown to limit the transendothelial transport of albumin through the blood brain barrier through either trafficking route [12]. Thus, apoM/S1P/$S1P_1$ interactions facilitate rather than restrict HDL transport from the blood stream into extravascular tissues. At first sight, the effects are even unexpectedly strong in view of the rather low concentration of apoM and S1P in HDL as the consequence of which only five percent of HDL particles are supposed to contain one molecule of apoM and S1P[30,33]. However, HDL promotes efflux of S1P in both apoM dependent and independent manner [6,7], so that the ex vivo measured S1P may underestimate the local concentration of S1P and thereby also the potential effect on endothelial transport. In addition, the promotion of SR-BI expression and cell surface abundance upon apoM/S1P/S1P receptor interaction will facilitate the binding, uptake, and transport of HDL particles in general, independently of their load of S1P and apoM.

By promoting the transendothelial transport of potentially anti-atherogenic HDL, apoM/S1P and $S1P_1$ may play an important role in the pathogenesis of atherosclerosis. At first sight in agreement, we found atherosclerosis reduced in $S1P_1$-iECKI mice. However, this difference may simply reflect less pronounced dyslipidemia. Moreover, it is important to keep in mind that S1P and $S1P_1$ exert many vasoprotective effects on the endothelium beyond regulating transendothelial transport, for example on the transmigration of leukocytes and nitric oxide production [44]. Therefore, further studies are needed to show the pathogenic relevance for

the regulation of transendothelial HDL transport through apoM/S1P and its cognate receptor $S1P_1$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ABC	ATP binding cassette transporter
Apo	apolipoprotein
BAECs	bovine aortic endothelial cells
CHD	coronary heart disease
CTRL	control mice, i.e apoE-haploinsufficient mice without endothelium specific knock-in of $S1P_1$
HAECs	human aortic endothethelial cells
HDL	high density lipoprotein
LDL	low density lipoprotein
LIPG	gene encoding endothelial lipase
S1P	sphingosine-1-phosphate
S1P1	sphingosine-1-phosphate receptor 1
$S1P_1$-iECKI	apoE-haploinsufficient mice with endothelium specific knock-in of $S1P_1$
SR-BI	scavenger receptor BI
VEGF	vascular endothelial growth factor

VEGFR	VEGF receptor

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Highlights

- The presence of apolipoprotein M increases binding, association, and transport of HDL by bovine aortic endothelial cells
- Binding, association, and transport of HDL by human aortic endothelial cells is promoted by agonists of the sphingosine-1-phosphate receptor type 1 ($S1P_1$) but decreased by inhibitors or knock-down of $S1P_1$
- The stimulatory effect of $S1P_1$ activation on transendothelial HDL transport depends on the expression of SR-BI
- Endothelium-specific overexpression of *S1p1* in Apoe haploinsufficient mice increases SR-BI expression in the aortal endothelium
- Endothelium-specific overexpression of *S1p1* in Apoe haploinsufficient mice decreases transport of albumin but increases transport of HDL from blood into the peritoneal cavity

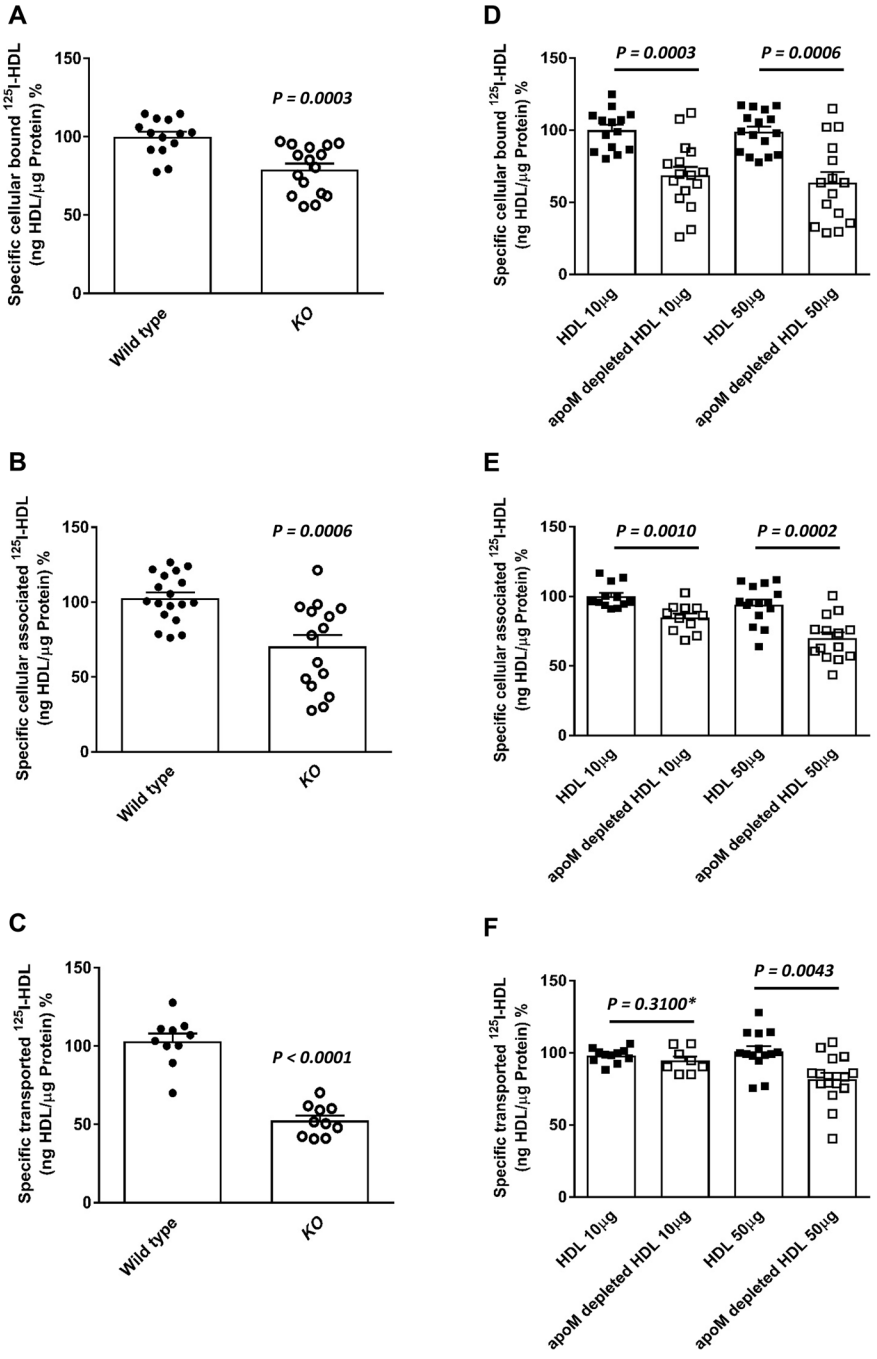

Figure 1: Presence of ApoM enhances binding binding, association and transendothelial transport of HDL in bovine aortic endothelial cells (BAECs).

BAECs were cultured for 72 hours before they were incubated for 1 hour with 10μg/mL of ^{125}I-HDL from wild type mice or *apom* knock-out mice (**A- C**) or radioioidinated total HDL or radioioidinated apoM-depleted HDL from humans at indicated amounts (**D-F**) in the absence (total) or in the presence of 40-fold excess of unlabeled HDL, to record nonspecific interactions. Specific binding, association and transport were calculated by subtracting nonspecific values from total values. Specific binding was measured by incubating cells

with ^{125}I-HDL (**A, D**) at 4 °C. To measure specific cell association, cells were incubated with ^{125}I-HDL (**B, E**) at 37 °C. For the measurement of transport, BAECs were cultured on inserts. The transport of ^{125}I-HDL (**C, F**) from the apical to basolateral compartment was measured at 37 °C. The results are presented as means ± SEM of three to six independent triplicate experiments (n=3). P was calculated by unpaired Mann Whitney U-test

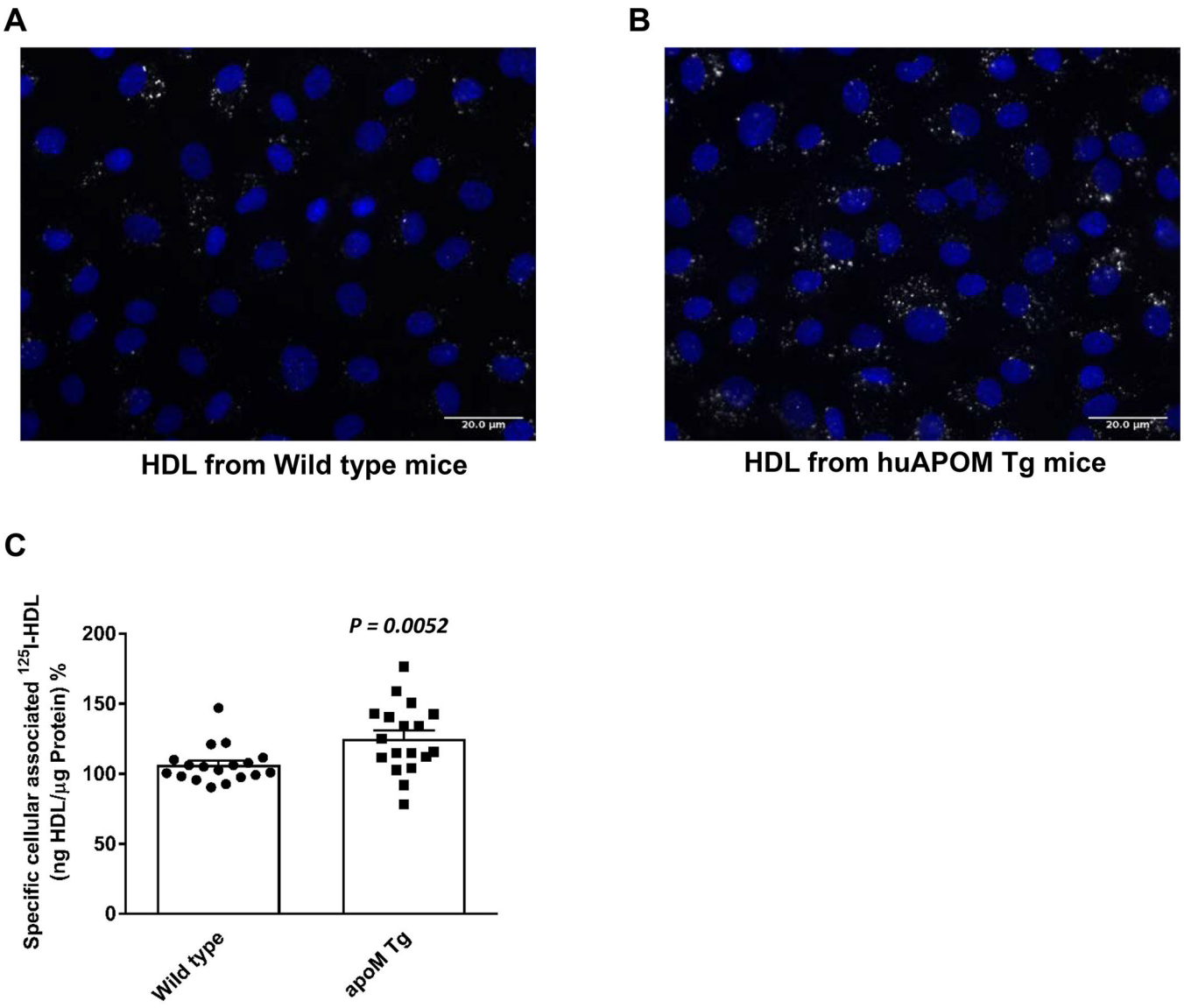

Figure 2: Enhanced uptake of HDL from human *APOM* transgenic mice by bovine aortic endothelial cells (BAECs).

For fluorescence microscopy (**A**), BAECs were cultured until confluence on cover slips and incubated for 60min at 37° with 50 µg/ml 594Atto-HDL, fixed with 3.75% formaldehyde and counter-stained with DAPI for nuclear staining. (**B**) BAECs were incubated with ^{125}I-HDL of *APOM* transgenic mice at 37 °C for 1 hour in the absence (total) or in the presence of 40-fold excess of unlabeled HDL. Specific association was calculated by subtracting unspecific values from total values. The results are presented as means ± SEM of six independent triplicate experiments (n=3). P was calculated by unpaired Mann Whitney U-test.

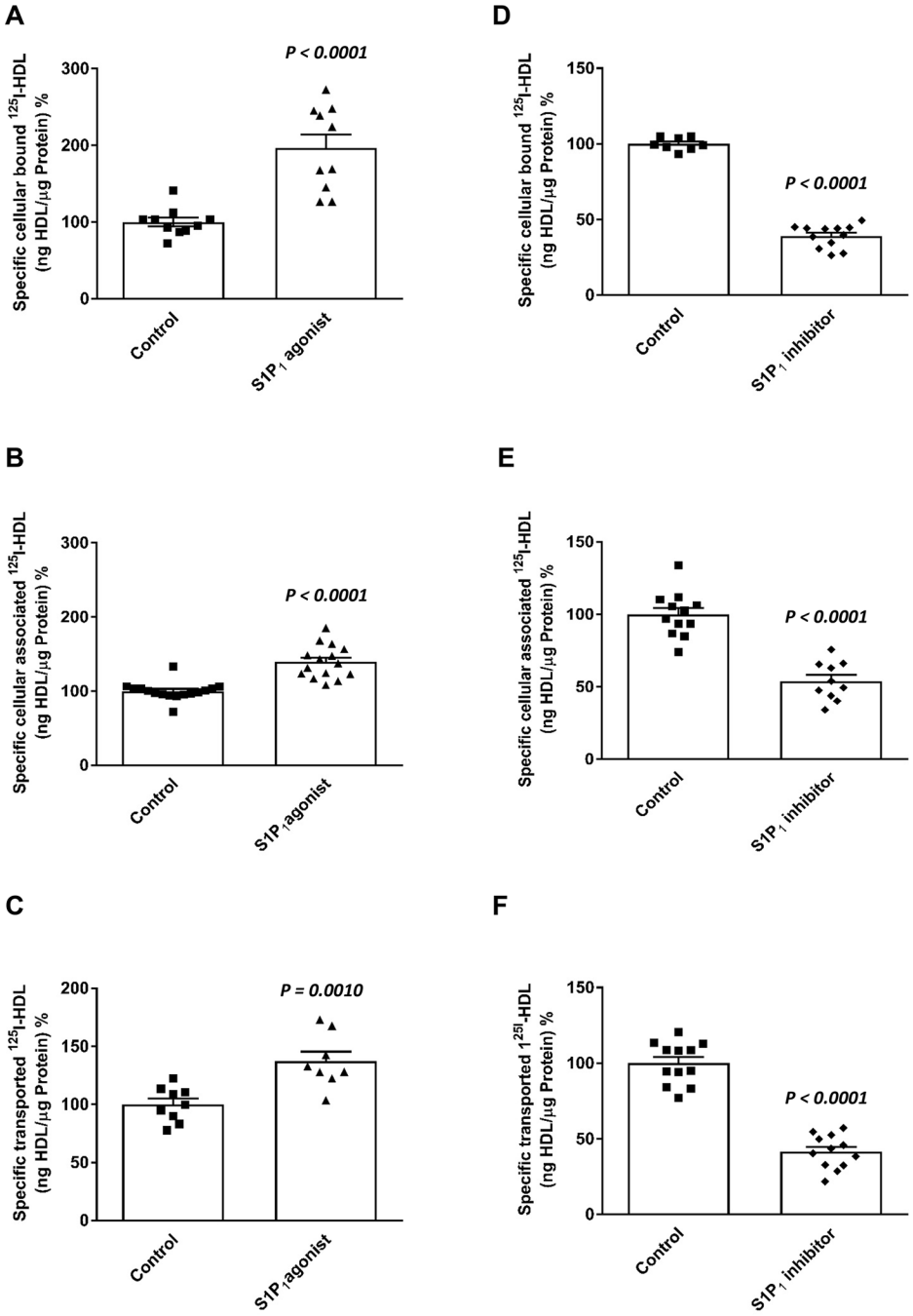

Figure 3: Agonists (a-c) and inhibitors (d-f) of $S1P_1$ regulate binding, association and transendothelial transport of HDL in human aortic endothelial cells (HAECs).

HAECs were cultured for 72h. Cells were then treated with $S1P_1$ agonist (SEW2871, 20nM; **A-C**) or $S1P_1$ inhibitor (W146, 20nM, **D-F**) for 30 minutes, at 37 °C as indicated. To study cellular binding, association and transport, HAECs were incubated with 10μg/mL of ^{125}I-HDL for 1 hour in the absence (total) or in the presence of 40-fold excess of unlabeled HDL, to record nonspecific interactions. Specific binding, association and transport were calculated by subtracting nonspecific values from total values. Specific

binding was measured by incubating cells with ^{125}I-HDL (**A, D**) at 4°C. To measure specific cell association, cells were incubated with ^{125}I-HDL (**B, E**) at 37°C. For the measurement of transport, HAECs were cultured on inserts. The transport of ^{125}I-HDL (**C, F**) from the apical to basolateral compartment was measured at 37°C. The results are presented as means ± SEM of three independent triplicate experiments (n=3). P was calculated by unpaired Mann Whitney U-test.

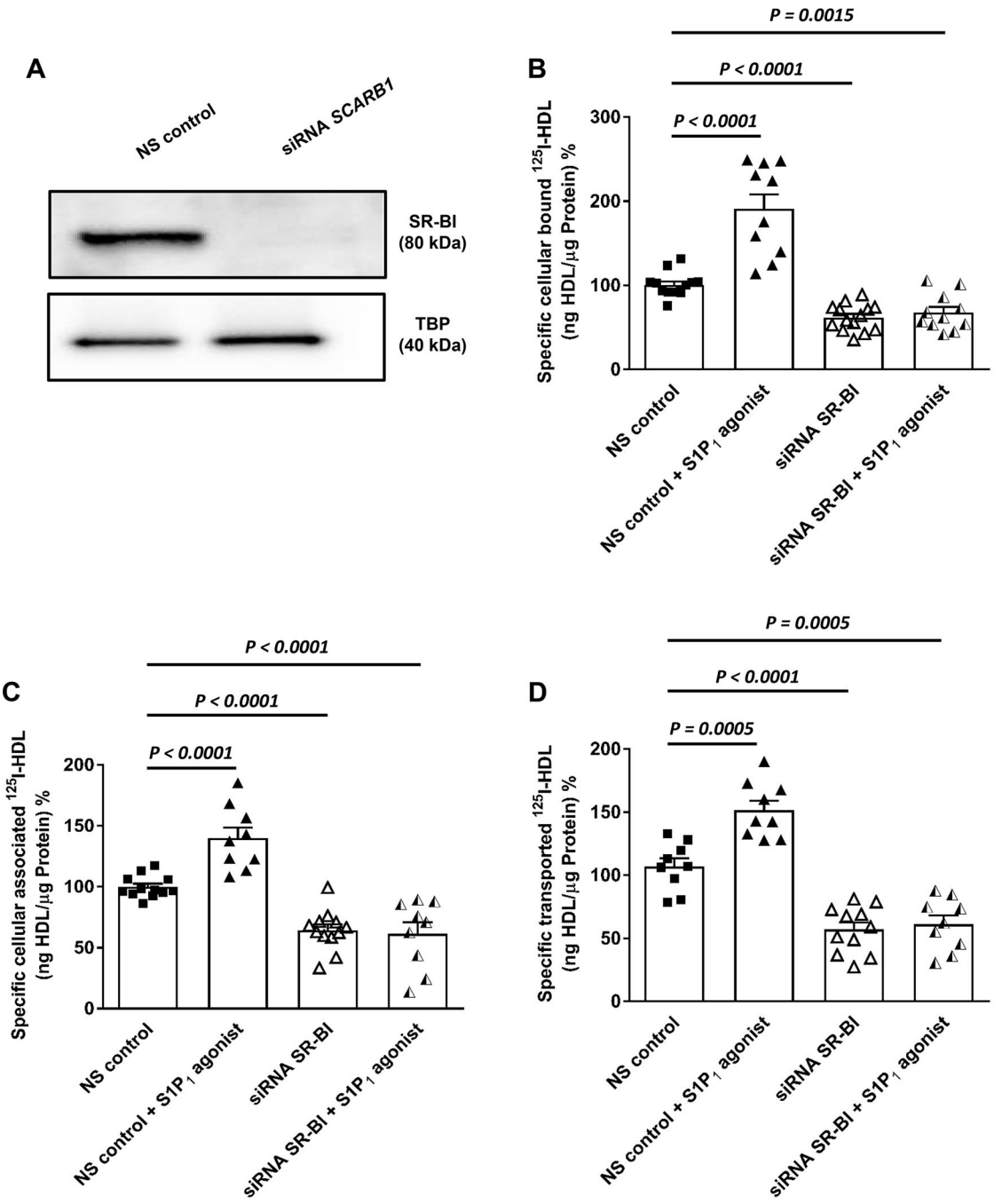

Figure 4: SR-BI is involved in the $S1P_1$ regulated binding, association and transport of HDL by human aortic endothelial cells (HAECs).

HAECs were transfected with a specific siRNA against *SCARB1* or with non-silencing control siRNA (NS control). Assays were performed 72 hours post-transfection. (**A**) representative Western blot showing the efficacy of the silencing relative to the non-silencing siRNA (NS control) and TATA-binding protein (TBP) used as the loading control. Cells were then treated with $S1P_1$ agonist (SEW2871, 20nM) for 30 minutes at 37 °C. (**B**) cellular binding of ^{125}I-HDL was measured at 4 °C after pre-treating cells with the $S1P_1$ agonist.

(**C**) cellular association of ^{125}I-HDL was measured at 37 °C. (**D**) for the measurement of transport of ^{125}I-HDL, HAECs were cultured on inserts. The transport of ^{125}I-HDL was measured after pre-treatment with the $S1P_1$ agonist from the apical to basolateral compartment was measured at 37 °C. The results are presented as means ± SEM of three independent triplicate experiments (n=3). P was calculated by unpaired Mann Whitney U-test).

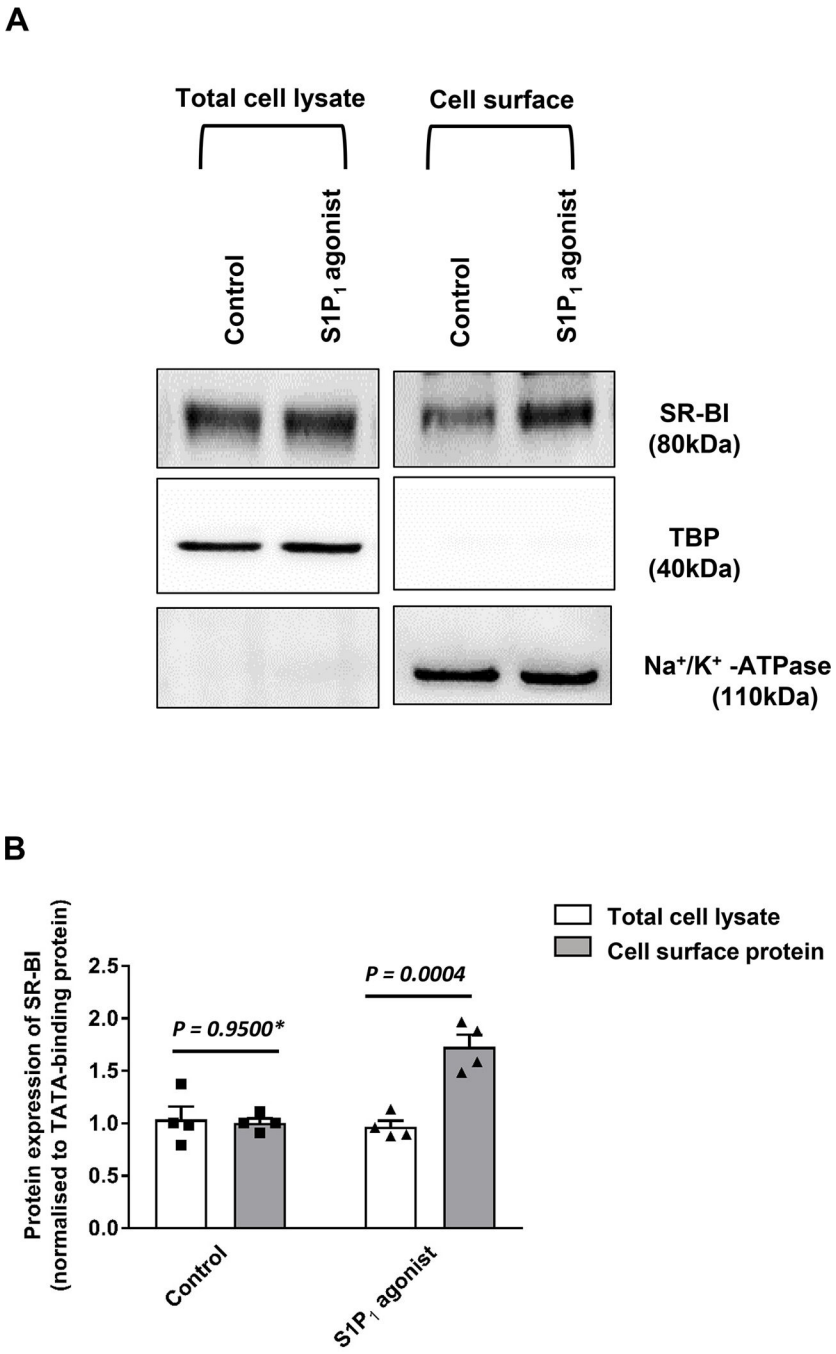

Figure 5: Effects of $S1P_1$ activation on the cell surface abundance of SR-BI.

HAECs were cultured for 72 hours. Cells were then treated with 20nM SEW2871 for 30 minutes at 37 °C. Cell surface expression of SR-BI in HAECs was measured using Western blot analysis in total cell lysates (left) and on the cell surface (right). The western blots were probed with anti-SR-BI (82kDa), anti-TBP (40kDa, used as a control for intracellular protein expression) and Na^+/K^+-ATPase (110 kDa, used as a loading control for cell surface protein expression). **(A)** shows a representative western blot. **(B)** shows the summary of four

independent experiments quantified by densitometry. P was calculated by unpaired Mann Whitney U-test.

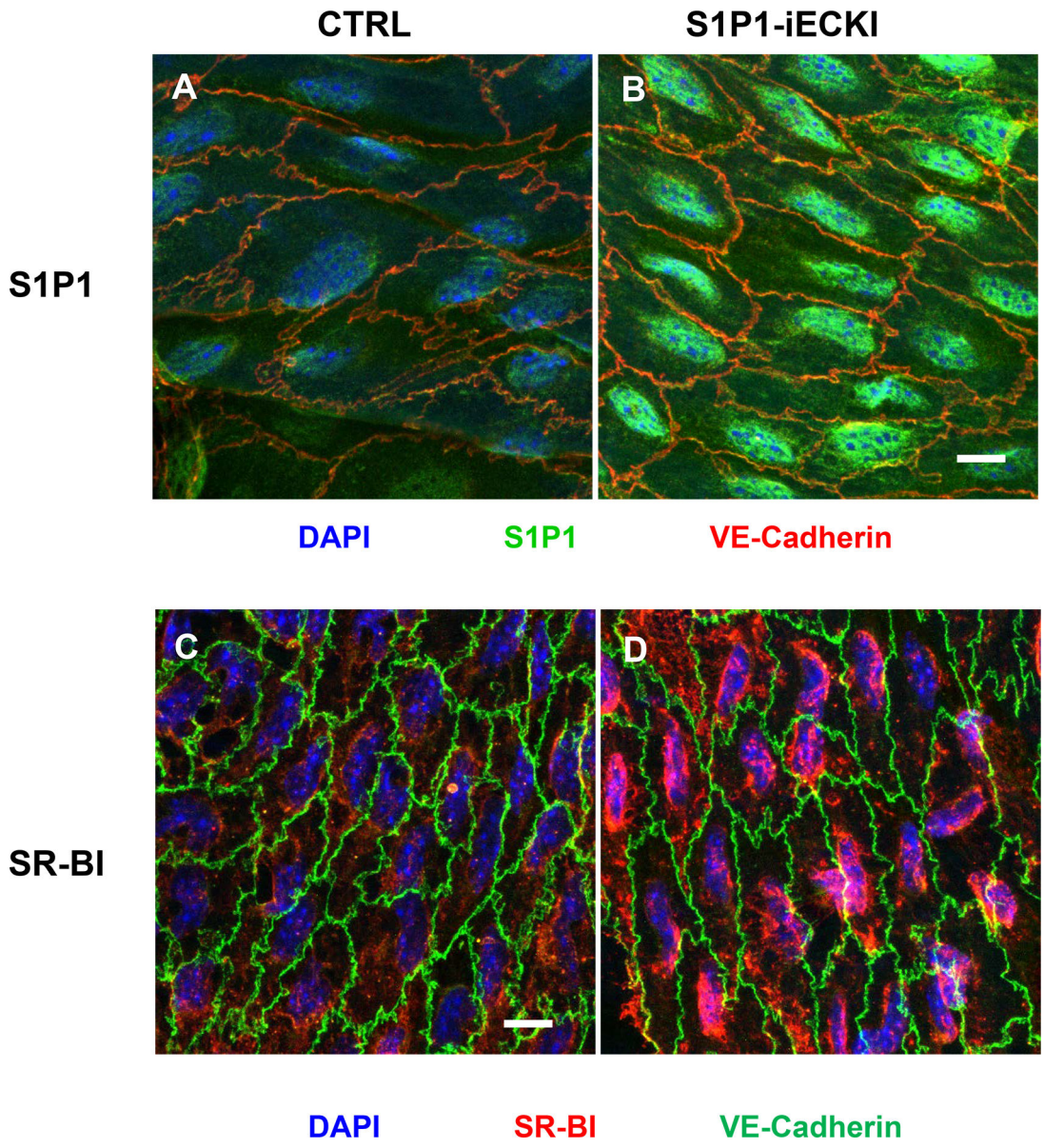

Figure 6: Demonstration of $S1P_1$ (A, B) or SR-BI (C, D) in the endothelium of aortas from Apoe haploinsufficient mice without (CTRL: A, C) or with overexpression of $S1P_1$ ($S1P_1$-iECKI; B, D).

Figure shows en-face prepared aortic immunostainings. Aortas were quickly cleaned of adventitial tissue, opened longitudinally and incubated with primary and secondary antibodies conjugated with green or red fluorescent dyes, as indicated. Nuclei were counterstained with DAPI. Images were captured by confocal microscope and z-axis projections of 14 scanned planes are shown. Scale bar = 10μm. Original micrographs are shown as supplemental figures IV and V.

Table 1:

Endothelium-specific overexpression of $S1P_1$ does not alter lipids but differentially regulates endothelial permeability for HDL and Evan's Blue in Apoe haploinsufficient mice

i.v. injection of Evan's Blue or DyLight-HDL and i. p. stimulation with LPS. Collection of peritoneal fluid after 3 hours. (N = 4 per group; results are presented as means ± SEM

	CTRL	$S1P_1$ -iECKI	P*
Cholesterol (mmol/L)	1.15 ± 0.16	1.05 ± 0.10	0.317
Triglycerides (mmol/L)	0.78 ± 0.11	0.65 ± 0.05	0.258
Evans' Blue in the peritoneal fluid (arbU)	0.13 ± 0.01	0.07 ± 0.02	0.029
DyL-LDL in the peritoneal fluid (arbU)	89.0 ± 1.6	53.0 ± 6.1	0.028
DyL-HDL in the peritoneal fluid (arbU)	18.5 ± 2.6	32.7 ± 2.8	0.019

*:statistical significance calculated by Mann-Whitney U-Test)