Ph.D. THESIS

Functional Characterization of Phospholipid Transfer Protein at the Blood-Brain Barrier

submitted by

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DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other materials used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

The major part of investigation presented in this thesis has been summarized and published in the article entitled "**Phospholipid transfer protein is expressed in cerebrovascular endothelial cells and involved in HDL biogenesis and remodeling at the blood-brain barrier"** by Chirackal Manavalan, AP *et.al.* 2014 (1) in *The Journal of Biological Chemistry.*

Graz, 05.08.2014 Anil Paul CHIRACKAL MANAVALAN

Dedicated to my Pappa, Mummy & Ammu.....

The LORD is my strength and my shield; my heart trusts in him, and I am helped. My heart leaps for joy and I will give thanks to him in song.... Psalm 28:7

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"It is good to have an end to journey toward, but it is the journey that matters in the end."

Ernest Hemingway.

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ABBREVIATIONS

24*(S)*OH-C	24*(S)*-hydroxycholesterol
Aβ	amyloid beta
ABCA 1	ATP binding cassette transporter A1
ABCG1	ATP binding cassette transporter G1
ACAT 1	acyl-CoA cholesterol acyltransferase 1
ACTB	β-actin
AD	Alzheimer's disease
APP	amyloid precursor protein
Apo	apolipoprotein
BACE1	β-secretase β-site APP cleavage enzyme 1
BBB	blood-brain barrier
BCA	bicinchoninic acid
CAA	cerebral amyloid angiopathy
CETP	cholesterol ester transfer protein
CSF	cerebrospinal fluid
DPPC	L-α-dipalmitoyl phosphatidylcholine
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
FFA	free fatty acids
FXR	farnesoid X receptor
HDL	high-density lipoproteins
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-CoA reductase
HL	hepatic lipase
HPEC	human placental endothelial cells
HPRT1	hypoxanthine phosphoribosyltransferase 1
ISF	interstitial fluid
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LPL	lipoprotein lipase

LPS	lipopolysaccharide
LRP	LDL receptor related protein
LXRs	liver X receptors
LXRE	LXR-response elements
mBCEC	murine brain capillary endothelial cells
NDGGE	nondenaturing gradient gel electrophoresis
NFκB	nuclear factor kappa-B
NO	nitric oxide
pBCEC	porcine brain capillary endothelial cells
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PL	phospholipids
PLTP	phospholipid transfer protein
PON1	paraoxonase-1
PPAR	peroxisome proliferator-activated receptor
RAGE	receptor of advanced glycation end products
RCT	reverse cholesterol transport
RT-PCR	quantitative real-time polymerase chain reaction
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR-BI	scavenger receptor, class B, type I
SREBP-1c	sterol regulatory element binding protein 1c
TEER	transendothelial electrical resistance
TG	triglycerides
VEGF	vascular endothelial growth factor
VLDL	very-low-density lipoprotein
VD	vascular dementia
vWF	von Willebrand factor

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KURZFASSUNG

Phospholipid transfer protein (PLTP) gilt als Schlüsselprotein in der Biogenese und Remodellierung von Plasma HDL. In der Literatur wurden HDL zahlreiche neuro-protektive Eigenschaften zugeschrieben. Wie unsere Arbeitsgruppe bereits zuvor berichtet hat, führt die Aktivierung des Liver-X Rezeptors (LXR) in einem *in vitro* Modell der Blut-Hirn-Schranke (BHS), bestehend aus primären Schweinehirnendothelzellen (pBCEC), zu erhöhtem Cholesterinefflux und zur Entstehung von HDL ähnlichen Partikeln. Diese Arbeit beschreibt die Synthese von PLTP, dessen Regulierung und Schlüsselfunktion im HDL Metabolismus an der BHS. In einem polarisierten *in vitro* Modell, welches die BHS simuliert, sekretierten pBCEC verstärkt aktives PLTP in das basolaterale (‚Gehirn parenchymale') Kompartiment. Bei LXR Aktivierung mit 24(S)-Hydroxycholesterin (ein zerebraler Cholesterin-Metabolit) und TO901317 (ein synthetische LXR Agonist) wurde die PLTP Expression und Phospholipid Transferaktivität bis zu maximal 2.5-fach erhöht. In C57/BL6 Mäusen wurde eine erhöhte PLTP Aktivität in BCEC bei Verabreichung von TO901317 festgestellt. Bei Zugabe von supraphysiologischen Konzentrationen von Glukose/Insulin in pBCEC unter simuliertem diabetischem Zustand detektierten wir einen bis zu 2,2-fachen Anstieg an PLTP Proteinlevel und eine Reduktion in PLTP mRNA (40%) und Aktivität (35%). Vorinkubation mit HDL_3 und humanem Plasma PLTP resultierte in der Entstehung von kleineren und größeren HDL Partikeln mit einer bis zu 3-fachen erhöhten Kapazität für Cholesterin Efflux in pBCEC. Pre-β-HDL, welches mittels 2D-Immunoelektrophorese detektiert wurde, wurde von HDL_3 in pBCEC Überständen generiert und zeigte eine 1,9-fache Erhöhung durch LXR- Aktivierung. Zusätzlich fanden wir, dass Silencing von PLTP (bis zu 75%) mittels siRNA zu einer Reduktion von apoA-I abhängigen (67%) und HDL_3 (30%) abhängigen Cholesterinefflux in pBCEC führt. Des Weiteren wurde eine 1,8-fache Erhöhung von BACE1 Proteingehalt durch PLTP Silencing festgestellt. Behandlung mit HDL_3 und aktiven PLTP führte zu einer signifikanten Reduktion (63%) von Amyloid-Beta (Aβ) Oligomerisierung in pBCEC. Zusammenfassend und basierend auf diesen Resultaten, schlagen wir eine aktive Rolle von PLTP im Lipidtransfer, Cholesterinefflux, HDL Biogenese und am Remodellierung und Aβ Metabolismus vor.

ABSTRACT

Phospholipid transfer protein (PLTP) is a key protein involved in biogenesis and remodeling of plasma HDL. Several neuro-protective properties have been ascribed to HDL. We reported earlier that liver-X receptor (LXR) activation promotes cellular cholesterol efflux and formation of HDL-like particles in an established *in vitro* model of the blood-brain barrier (BBB) consisting of primary, porcine brain capillary endothelial cells (pBCEC). Here we report PLTP synthesis, regulation, and its key role in HDL metabolism at the BBB. We demonstrate that PLTP is highly expressed and secreted by pBCEC. In a polarized *in vitro* model mimicking the BBB, pBCEC secreted phospholipid-transfer active PLTP preferentially to the basolateral ('brain parenchymal') compartment. PLTP expression levels and phospholipid transfer activity were enhanced (up to 2.5-fold) by LXR activation using 24*(S)*-hydroxycholesterol (a cerebral cholesterol metabolite) or TO901317 (a synthetic LXR agonist). TO901317 administration elevated PLTP activity in BCEC from C57/BL6 mice. Under simulated diabetic conditions *in vitro* via treatment with supraphysiological concentration of glucose/insulin in pBCEC, we detected a significant increase (up to 2.2-fold) in PLTP protein levels but a reduced (up to 40% and 35%, respectively) PLTP mRNA levels and activity. Pre-incubation of HDL_3 with human plasma-derived active PLTP resulted in the formation of smaller and larger HDL particles and enhanced the capacity of the generated HDL particles to remove cholesterol from pBCEC by up to 3-fold. Pre-β-HDL, detected by two-dimensional crossed immunoelectrophoresis, was generated from HDL_3 in pBCEC-derived supernatants, and their generation was markedly enhanced (1.9-fold) upon LXR activation. Furthermore, RNA interference-mediated PLTP silencing (up to 75%) reduced both apoA-I dependent (67%) and HDL_3 dependent (30%) cholesterol efflux from pBCEC. Decreased (up to 63%) amyloid beta (Aβ) oligomerisation is observed in pBCEC treated with HDL_3 and active PLTP, and also detected an increased (1.8-fold) levels of beta-secretase (BACE1) mRNA in PLTP-silenced cells. Based on these findings, we propose that PLTP is actively involved in lipid transfer, cholesterol efflux, HDL biogenesis and remodeling, and Aβ metabolism at the BBB.

INTRODUCTION

1. INTRODUCTION

1.1. Plasma lipoprotein metabolism

Lipoprotein is a complex biochemical assembly of particles that are composed of both proteins and lipids. The five major classes of human plasma lipoproteins secreted by the intestine and liver in the order of increasing density are chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high-density lipoproteins (HDL). Plasma lipoprotein metabolism is accountable for the generation of lipoproteins and transport of lipids within the body. Since lipids are insoluble in plasma, circulating lipids are assembled as lipoprotein particles and transported to various tissues (2). Lipoproteins consist of four major classes of lipids (triacylglycerols, esterified and unesterified cholesterols and phospholipids) and proteins. The protein part of lipoprotein called apolipoproteins (apo) assists in lipid binding, target lipids to receptors and also act as enzyme cofactors. Lipoprotein receptors like low-density lipoprotein receptor (LDLR), low-density lipoprotein receptor-related protein (LRP), scavenger receptor class B type I (SR-BI) and ATP-binding cassette (ABC) transporters present on the surface of plasma membranes synchronize the uptake, processing and removal of lipids in the lipoproteins (3-7).

Based on the origin of lipoproteins, lipoprotein metabolism can be divided into either exogenous (dietary) or endogenous (liver) pathway. In the exogenous pathway, enterocytes in the small intestine absorb the dietary lipids which contain triglycerides (TG), phospholipids and cholesterol, and assembled apoB-48 containing TG-rich chylomicrons are then secreted by the enterocytes into the blood stream via the lymphatic system. The TG-rich nascent chylomicrons attain apolipoproteins including apoC-II, apoC-III and apoE from the circulation and also from HDL particles to become mature particles. Lipoprotein lipase (LPL), expressed on the endothelial cells lining the blood vessels gets activated via apoC-II and hydrolyzes the triglycerides in the

chylomicrons into glycerol and free fatty acids (FFA). Glycerol and FFA can then be absorbed by muscle, heart and adipose tissue, for energy and storage. The TG-poor chylomicrons called chylomicron remnants are taken up by liver via interaction with apoE and chylomicron remnant receptors, and are subsequently hydrolyzed for VLDL synthesis or stored for later use (8-11). During the endogenous pathway along with the lipids originating from the chylomicron remnants, hepatocytes also synthesize triglycerides and cholesterol to form VLDL particles. These apoB-100 containing nascent VLDL particles released into the bloodstream acquire apoC-II, apoC-III and apoE to become mature VLDL. Like chylomicrons, VLDL particles are then hydrolyzed by LPL and the resulting glycerol and FFA are principally absorbed from the blood by muscle, heart and adipose tissue, for energy and storage. The TG-poor VLDL particles called VLDL remnants are taken up by liver via interaction with apoE and remnant receptors, and can be subsequently hydrolyzed by hepatic lipase (HL) to form cholesterol-rich LDL particles. LDL can circulate and is absorbed by peripheral cells via interaction with apoB-100/apoE and LDLR, and gets hydrolyzed to release lipids, mainly cholesterol (12-15).

1.1.1. High-density lipoproteins metabolism and functions

Plasma HDL is an exceptionally heterogeneous group of lipoproteins. The main apolipoproteins present in human HDL are apoA-I, apoA-II, apoC-I, apoC-II, apoC-III, apoM, apoJ and apoE (16-19). Hepatocytes and intestinal cells secrete HDL particles in a lipid-free and/or lipid-poor apoA-I form. These small nascent HDL precursors incorporate cholesterol and phospholipid with the aid of ATP-binding cassette transporter A1 (ABCA1) transporter and phospholipid transfer protein (PLTP) to form discoidal pre-beta HDL particles. These are the initial events in the biogenesis of HDL. Discoidal HDLs are also formed when lipid-free and/or lipid-poor apoA-I accepts phospholipids and unesterified cholesterol released as surface fragments from lipolysed (by LPL) TG-rich lipoproteins (chylomicrons and VLDL). Further lipidation of HDL particle takes place with the help of ABCG1 and scavenger receptor, class B, type I (SR-BI). Finally, HDL particles mature with the help of lecithin-cholesterol acyltransferase

(LCAT) to become more spherical and functional HDL. Circulating HDL particles are continually being remodeled and interconverted by plasma factors including PLTP, cholesteryl ester transfer protein (CETP), HL and endothelial lipase (EL) to form several HDL subpopulations (20-24).

Based on density, HDL can be classified into HDL_2 and HDL_3, which can be further sub-fractionated by nondenaturing gradient gel electrophoresis into HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c} (25, 26). During agarose gel electrophoresis, HDL can also be resolved on the basis of electrophoretic mobility into pre-β- (most discoidal HDL, lipid-free apoA-I, and lipid-poor apoA-I), α- (spherical HDL) and γ- (spherical apoE-containing HDL) migrating particles (26-29). Different subpopulations of HDL exhibit distinct functions. HDL possesses several anti-atherogenic roles and is best known for its ability to perform reverse cholesterol transport (RCT), the process by which HDL acquires excess cholesterol from peripheral tissues and transports it back to the liver for excretion through bile. Besides RCT and lipid transport, circulating HDL exerts anti-oxidative, anti-inflammatory, anti-thrombotic and cytoprotective properties (21, 22). Eventhough the understanding of the metabolism and functions of plasma lipoproteins has been increased significantly in the recent years, not much is known about the lipoprotein metabolism in the central nervous system (CNS). More importantly, the presence of the blood-brain barrier (BBB) separates the plasma and brain lipoprotein metabolism into two largely independent compartments.

1.2. Blood-brain barrier and brain capillary endothelial cells

Paul Ehrlich and his student Edwin Goldman provided the first experimental evidence for the existence of "some sort of structure" that compartmentalizes brain from the rest of the body (30). But it was Lewandowsky, who first coined the term blood-brain barrier (BBB) while studying the penetration of potassium ferrocyanide into the brain (30). Owing to decades of research, now we know that the BBB is a complex and dynamic interface between blood and central nervous system (CNS) which limits and regulates

molecular exchange at the interface. Brain capillary endothelial cells (BCEC) that line the cerebrovasculature are the core anatomical constituent of the BBB (Fig. 1). In contrast to peripheral capillary endothelial cells, BCEC of the BBB are devoid of fenestrations and have a high number of mitochondria (31). BCEC act as physical and metabolic barrier by joining together the adjacent cells with highly specialized tight and adherens junction proteins (claudins, occludins, VE-cadherin, β-catenin). The BBB acts as a biological sieve which allows the entry of energy metabolites such as glucose and essential nutrients (like amino acids, vitamins) into the brain and assists in elimination of waste products out of the brain (32-34). The neurovascular unit comprises of BCEC, pericytes, vascular smooth muscle cells, glial cells and neurons (Fig. 1B). The functions of the CNS are mainly executed by neurons and glial cells. Out of the three major types of glial cells, oligodendrocytes and astrocytes are mainly involved in lipid and lipoprotein metabolism, while microglial cells are active in immune response reactions of the CNS (34). According to recent reports ~50% of cells in the human brain are non-neuronal (35). A growing body of literature now supports the idea that beyond its physical barrier function, BBB is also actively involved in CNS metabolic activities. The influx and efflux of biological molecules are tightly regulated at the blood-brain interface by BCEC. These specialized endothelial cells (EC) express a wide range of proteins, receptors and transporters at both their apical (blood) and basolateral (brain) side to maintain the structural integrity of BBB and for the bidirectional movement of bio-molecules. The major group of receptors and transporters expressed by BCEC are closely linked to brain lipoprotein metabolism and amyloid beta [Aβ, a major component of senile plaques in Alzheimer's disease (AD)] homeostasis which include LDLR, LRP1, megalin/LRP2, receptor of advanced glycation end products (RAGE), SR-BI, ABCA1, ABCB1, ABCG1, ABCG2 and ABCG4 (36-44).

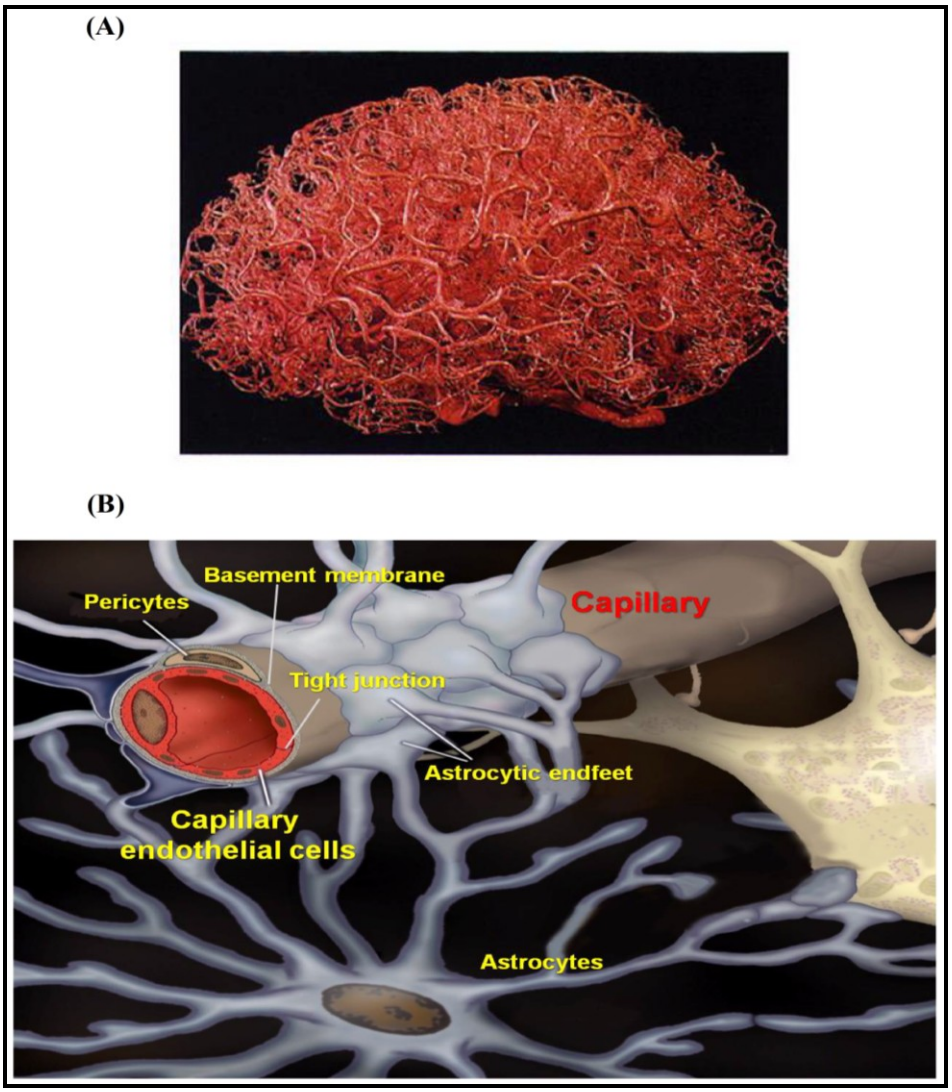


Figure 1: Blood-brain barrier. (A) Cerebrovascular network in a normal adult human brain after injecting a plastic emulsion which dissolves all the brain parenchymal tissues. The total length of capillaries is ~650 km with surface area of 20 m^2 [taken with permission from Zlokovic and Apuzzo, (45)]. (B) Main components of blood-brain barrier [modified from Carvey *et. al*., (46)].

1.3. Brain cholesterol and lipoprotein metabolism

The presence of the BBB restrains the exchange of circulating plasma lipoproteins with the brain. Brain is the most lipid (and cholesterol) rich organ in the body and about a quarter of total body cholesterol resides in the brain. Cholesterol is indispensable for the structure and function of the CNS. A continuous supply of cholesterol is required for neurons and astrocytes for membrane synthesis and during myelinogenesis. Cholesterol plays a crucial role in the maintenance of synaptic plasticity and also regulates the release of neurotransmitter (47). In addition, cholesterol is an important precursor of several bio-molecules (like oxysterols, vitamin D and steroid hormones) (48). About 75% of cholesterol in the adult brain is present in myelin and since neurons cannot efficiently produce all the required cholesterol, neurons have to rely on external sources such as glial cells (49). Since cholesterol is such an essential biomolecule for the brain, any discrepancies in brain cholesterol levels are highly detrimental and may relate to many neurodegenerative diseases. And hence, the level of cholesterol is tightly monitored in the brain mainly by liver X receptors (LXR). Excess cholesterol leaves the brain in a hydroxylated form called 24*(S)*-hydroxycholesterol, a reaction which is catalyzed by cholesterol 24-hydroxylase (50, 51). Unlike cholesterol, 24*(S)*-hydroxycholesterol can cross the BBB and enter the blood stream whereby it is excreted into the bile through liver (52, 53).

In mammalian cells cholesterol is synthesized from acetyl-CoA, which involves over 20 enzymatic steps and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) being the rate-controlling enzyme. In peripheral tissues cholesterol is derived mainly from two sources, namely endogenous production and exogenous lipoproteins delivered from circulating plasma. However, in the case of CNS the presence of an intact BBB prevents the passage of lipoproteins from the circulation and hence almost all the brain cholesterol must be synthesized locally (48, 54). Oligodendrocytes govern the bulk of cholesterol synthesis in the brain followed by astrocytes and neurons (48). In the CNS, cholesterol is assembled and transported in form of lipoprotein particles. The three major sources of CNS lipoproteins are from astrocytes, cerebrospinal fluid (CSF) and BCEC

(55, 56). In contrast to the peripheral circulation which contains VLDL, LDL and HDL, the brain lipoprotein metabolism is centered exclusively on HDL-like particles, which resemble in size and density of plasma HDL (1, 57-60). The major HDL species categorized by density, size and apolipoprotein content in the CNS is outlined in Table 1.

Table 1: HDL species reported in plasma and brain.

	Plasma	**Brain**		
HDL type	**HDL** (61)	**BCEC-Lp** (40) and unpublished data from our group	**Astrocyte-Lp** (55, 60)	**CSF-Lp** (57)
Density (g/ml)	1.063 – 1.21	1.10 – 1.25	1.00 – 1.12	1.063 – 1.21
Size (nm)	5.4 - 14	n.d.	9 - 17	11 - 20
Shape	discoidal, spherical	n.d.	discoidal	mainly spherical
Apolipoproteins	apoA-I, apoA-II, apoC-l, apoC-II, apoD, apoE, apoJ, apoM	apoA-I, apoJ apoM,	apoE, apoJ	apoE, apoA-I, apoA-II, apoJ, apoD, apoC-I,

BCEC, brain capillary endothelial cells; Lp, lipoprotein; CSF, cerebrospinal fluid; n.d., not determined

LXR are considered as the master regulators of CNS lipid (cholesterol) homeostasis (40, 62, 63) and in brain their expression has been detected in glial cells, neurons and BCEC. LXR are members of nuclear transcription factors that orchestrate cholesterol, fatty acid and glucose homeostasis. LXRα and LXRβ (isoforms) form heterodimers with retinoid X receptors (RXR) and bind (LXR/RXR complex) to the promoter region of target genes containing LXR-response elements (LXRE) thereby modulating expression of target genes (64). The expression of LXRα is highest in liver, adipose tissue, intestine, kidney, adrenal glands and spleen, while LXRβ is found

ubiquitously including the CNS (65, 66). Both LXR isoforms exhibit minor differences in both DNA- and ligand-binding domains (65, 66). Oxysterols (like 24(*S*)-hydroxycholesterol) and also cholesterol intermediates (like desmosterol) are the natural ligands of LXR (65, 67). T0901317 and GW3965 are two non-steroidal synthetic LXR ligands that can cross the BBB and have been used in several studies (68, 69). Activation of LXR regulates the expression of target genes involved in lipid (cholesterol) metabolism including ABCA1, ABCG1, apoE, and sterol regulatory element binding protein 1c (SREBP-1c) (49, 70, 71). LXR activation enhances BBB integrity and is considered to be neuroprotective, owing to its ability to induce both reverse cholesterol transport, and degradation and clearance of toxic Aβ from the brain (49, 72).

Since apoE and apoA-I are the most abundant apolipoproteins in the brain, cholesterol and other lipid molecules are transported to the neurons or within the brain in the form of apoE or apoA-I containing HDL-like lipoproteins particles. Even though the exact mechanism of lipoprotein formation in the CNS is not fully elucidated, it is generally accepted that further addition of cholesterol and phospholipids to the newly formed apoE or apoA-I-containing lipoproteins particles is mediated by specific subtypes of ABC transporters and lipid transfer proteins (58). Lipidation of nascent lipoproteins is a crucial factor which decides its further function in the brain. The presence of LCAT activity in the brain parenchyma indicates that LCAT might play an important role in the maturation of newly formed HDL-like particles (73, 74). Similar to peripheral circulation, CNS also express many lipoprotein receptors and ABC transporters required for the lipoprotein transport system. The major lipoprotein receptors and lipid-transporters present in brain are LDLR, VLDLR, apoE receptor 2 (apoER2), LRP1, LRP1b, megalin/LRP2, SR-BI, ABCA1, ABCA2, ABCA7, ABCG1, ABCG4 and Niemann-Pick type C1 (NPC1) protein (56, 75-79). Finally after maturation the apoE or apoA-I containing HDL-like lipoproteins particles bind to LDL receptor family members expressed on neurons or astrocytes and are internalized. ApoE (and apoA-I) containing lipoproteins also induce axonal branching and outgrowth, prevent neuronal death, and engage in nerve repair (80-83). Taken together, apolipoproteins play a critical role in the transport and metabolism of lipids in the brain. Moreover, recent reports recognized that

beyond its role in lipid transport, apolipoproteins are critical for the normal functioning of brain.

1.3.1. ApoE

ApoE (34 kDa), the most abundant apolipoprotein in the CNS is synthesized and secreted mainly by astrocytes and also to some extent by microglia and neurons, and under the control of LXR (84). ApoE plays a central role in cholesterol, phospholipid and Aβ metabolism in the brain (85, 86). Nascent discoidal apoE-containing particles are gradually lipidated (by addition of cholesterol and phospholipid) and ultimately become mature spherical HDL-like particles by the collective action of ABCA1, ABCG1, LCAT and lipid transfer proteins. Matured apoE-containing particles then deliver cholesterol to neurons mainly by interaction through LDLR expressed on neurons and the released cholesterol can be used to support synaptogenesis and also for other purposes (87). ApoE levels are dramatically increased followed by nerve injury, in order to deliver more lipids to the regenerating neurons (87). The apoE lipidation state is the deciding factor for its function in the brain, as poorly lipidated apoE particle are susceptible to proteolysis and also considered as amyloidogenic. Several *in vitro* and *in vivo* data suggest that properly lipidated apoE can directly bind to Aβ in an isoform-specific manner and is actively involved in Aβ degradation (by microglia and astrocytes) and/or clearance across the BBB, and also in maintaining the BBB integrity (88). Functional ABCA1 is highly necessary for maintaining the lipidation of brain apoE particles by cholesterol and phospholipid. Hirsch-Reinshagen *et al*., (89) reported that in two different AD murine models, ABCA1 deficiency leads to a dramatic reduction in soluble apoE levels and lipidation in the CNS. Two other laboratories independently demonstrated that in AD murine models, targeted disruption of ABCA1 increases Aβ deposition in brain parenchyma and vasculature, and also found a significant reduction in soluble brain apoE levels and its lipidation state (90, 91).

Three major isoforms of apoE exists in humans - apoE2, apoE3 and apoE4, and apoE4 is considered as a key known genetic risk factor for the development of both AD and cerebral amyloid angiopathy (CAA), while apoE2 is neuroprotective (86, 92). Clinical studies indicate that apoE4 possession account for 65–80% of all AD cases and apoE4 is considered as less efficient in Aβ clearance (93, 94). Recent data suggests that apoE4 can cause direct toxic effects on the cerebrovasculature, independent of Aβ via the activation of proinflammatory cyclophilin-A (95). ApoE is suggested to be engaged in many other functions, including apoptosis, oxidative stress, neuroinflammation, cell signaling and synaptic plasticity.

1.3.2. ApoA-I

ApoA-I (28-KDa) is the main protein constituent of plasma HDL and the cofactor for LCAT. It is one of the most abundant apolipoprotein found in CSF and is present in relatively high amount (~4.0 µg/ml) in the CSF (57, 96, 97). BCEC is the main source of brain apoA-I in mammalian cells (40, 98-100) and is totally undetected in glial cells and neurons. It is also believed that plasma derived apoA-I can cross the BBB and contribute to the CNS pool, SR-BI expressed by BCECs is suggested to facilitate this selective uptake of HDL associated apoA-I at the BBB (43, 96, 101). Even though, apoA-I is the second most abundant apolipoprotein in the brain after apoE, the potential role of apoA-I in brain is not completely clear. However, emerging evidence supports the contribution of apoA-I towards cerebral health. Saczynski *et al*., (102) have reported that individuals with high serum apoA-I levels show a significantly lower risk of dementia. Proteomics-based diagnostic studies have revealed a decrease in apoA-I levels in CSF and brain tissue of AD patients (103-105). Nevertheless, Harr *et al*., (106) and Song *et al*.,(107) did not observe any difference in the CSF apoA-I levels in AD patients, while another study has reported an increase of apoA-1 in CSF (108). Intriguingly, several genetic studies have reported an association of apoA-I gene polymorphism with cognitive impairment, cholesterol homeostasis and risk of AD (109-111). Two recent *in vivo* studies have undoubtedly identified apoA-I as an important and beneficial determinant in the pathogenesis of AD (112, 113). It is interesting to note that exposure to LXR agonist in

8-month old AD mice significantly increased brain apoA-I protein levels independent of ABCA1 (114). In a murine model of AD, Lefterov *et al*., (112) reported that apoA-I deficiency aggravates cognitive deficits together with increased Aβ deposits in the cerebrovasculature. Meanwhile, over-expression of apoA-I enhanced cognitive function and attenuated cerebrovascular amyloid deposition in AD mice (113). ApoA-I co-localized to senile plaques in the brain of AD subjects has been also reported (106, 115, 116). It has been shown *in vitro* that both, lipidated and non-lipidated apoA-I (of mouse or human origin) can bind directly to Aβ and preclude its aggregation, thereby preventing Aβ-induced neurotoxicity (112, 117-119). All these data clearly suggest the need of more investigation to identify the exact role of apoA-I in normal environment and also in AD pathophysiology.

Using an *in vitro* model of BBB, we have reported that apoA-I is secreted to both the apical and basolateral sides of the BBB but more preferentially to the basolateral side (40). We have previously shown that primary porcine brain capillary endothelial cells (pBCEC) are involved in the formation of HDL-like particles at the "brain parenchymal side" of the BBB (40, 41). This process involves ABCA1- mediated lipidation of apoA-I, that is induced by LXR activation (40, 41). In the peripheral tissues the protective role of HDL is mainly due to its ability to perform reverse cholesterol transport (RCT), thus removing the excess cellular cholesterol, and apoA-I and ABCA1 are the key players in RCT. Like in the circulation, there is a possibility of similar reverse sterol transport system operating also in the CNS. Based on our previous studies we have proposed that a bidirectional delicately balanced sterol mobilization system is operating at the BBB with the aid of BCEC. At one side of this transport system it facilitates the removal of neurotoxic 24-(*S)*-hydroxycholesterol out of the brain to the peripheral circulation (SR-BI/HDL-dependent pathway), while at the opposite side ABCA1/apoA-I-dependent pathway enhances the recycling and secretion/transport of cholesterol to the brain (40, 41, 43).

1.4. Amyloid precursor protein (APP) metabolism in the brain

Mounting genetic and biochemical data support the hypothesis that overproduction, impaired clearance, and accumulation of Aβ peptide in various regions of the brain are the causative agent of AD (120). More than 25 million people are affected globally by AD, the most common (50-70%) cause of dementia that progressively impairs basic cognitive functions (121). Intracellular neurofibrillary tangles formed by hyper phosphorylated tau protein and extracellular amyloid plaques consisting of Aβ peptides are the two essential pathological hallmarks of AD (121, 122). The deposition of Aβ can also occurs within cerebral vessels causing cerebral amyloid angiopathy (CAA), which leads to BBB leakage, dysregulation of cerebral blood flow and intracerebral microhemorrhages (123). CAA is a common pathological feature seen in the majority of AD cases and also occurs in vascular dementia (VD), the second most common cause of dementia after AD. The cerebrovascular damage caused by both CAA and AD can exacerbate cognitive decline, implying a thus far underestimated role of the BBB in AD pathology (124).

Cerebral Aβ peptide is derived from sequential processing of a 135 kDa transmembrane protein (localized to the plasma membrane, endoplasmic reticulum and trans-Golgi network), namely amyloid precursor protein (APP) by two competing pathways (125, 126). In the non-amyloidogenic pathway APP undergoes cleavage by α-secretase within the Aβ domain releasing N-terminal soluble APPα (sAPPα), followed by a subsequent cleavage by γ-secretase complex at the remaining membrane associated C-terminal fragment to generate nontoxic p3 peptide as well as the APP intracellular domain (AICD). In the amyloidogenic pathway APP is cleaved first by β-secretase β-site APP cleavage enzyme 1 (BACE1) to release N-terminal soluble APPβ (sAPPβ) and subsequently cleaved at the transmembrane domain of C99 ($APP_{672-770}$) by γ-secretase complex to generate toxic Aβ peptides with varying length and also the AICD (79, 127). $Aβ_{40}$ is the most prevalent form of toxic Aβ species; while $Aβ_{42}$ accounts for ~10% of total Aβ is very fibrillogenic (128). The accumulation of toxic Aβ is the initial hit that triggers a series of events that finally leads to neuronal dysfunction and death. The exact

mechanisms underlying the regulation of these two pathways, physiological role of APP/Aβ and also the trigger that convert the nontoxic soluble monomeric Aβ to toxic insoluble oligomeric Aβ are remains largely unclear.

1.4.1. Role of brain capillary endothelial cells in Aβ homeostasis

Clearance of Aβ peptides from the brain through the BBB is very crucial for the normal functioning of brain and hence BCEC are essentially involved in Aβ homeostasis. Aβ is produced mainly in neurons and glial cells, and recently our group has detected the presence of Aβ also in cultured BCEC of the BBB (129, 130). Neurotoxic Aβ is cleared from the brain by two major pathways. Firstly, through the endopeptidase-mediated proteolytic degradation of Aβ by insulin-degrading enzyme (extracellular Aβ) and neprilysin (intracellular Aβ) (131). Second and the major clearance route is through, LRP1-mediated endocytosis of Aβ across brain endothelium of the BBB via interstitial fluid (ISF)/CSF route into the circulation (and for final elimination in to liver) (44). We and others have reported that BCEC are able to synthesize, process and degrade Aβ and, also express the receptors to eliminate Aβ (44, 130, 132-134).

Reduced expression of LRP1 is associated with enhanced vascular accumulation of Aβ in brains of Alzheimer's patients (135, 136). Moreover, Owen *et al*., (137) reported that LRP1 is in an oxidized state in AD brains which may contribute to Aβ retention in brain, as oxidized LRP1 cannot bind and transport Aβ. A soluble form of LRP1 (sLRP1) is also reported in human plasma, which can bind up to 90% of circulating Aβ and prevent its entry to CNS (138). In AD patients, the levels of circulating sLRP1 are reduced and its binding capacity to Aβ is also compromised due to the increased oxidative damage of sLRP1 (138). LRP1 also binds to other ligands implicated in the pathogenesis of AD such as apoE, APP, α2-macroglobulin and matrix metallopeptidase 9 (76). Megalin/LRP2, ABCB1 (also known as P-glycoprotein) and ABCG2 (also known as breast cancer resistance protein) expressed in BCEC also participate in endocytosis and efflux of Aβ across the BBB (139-142). On the other hand,

a continuous influx of plasma-derived Aβ into the CNS is occurring across the BBB, which is mediated by receptor of advanced glycation end products (RAGE) and can lead to abnormal levels of Aβ in the CNS (44). RAGE expression is increased in brain endothelium of patients with AD and in AD murine models, while inhibition of RAGE protects against the accumulation of cerebral Aβ in an AD murine model (143, 144). So the BCEC of the BBB control the levels of neurotoxic Aβ in the CNS by regulating the efflux and influx of Aβ mainly through LRP1 and RAGE, respectively. But, how these receptors/transporters (LRP1, Megalin/LRP2, ABCB1 and ABCG2) of BCEC select which Aβ species to be egressed from brain and also what triggers the binding to Aβ is not well documented.

1.4.2. Cholesterol in Aβ metabolism

Escalating evidence suggests that dysregulation of cholesterol homeostasis is the major reason for the development of AD. Cellular cholesterol and phospholipid (such as phosphatidylinositol, phosphatidic acid and sphingomyelin) levels have been implicated in controlling the trafficking and processing of APP. Amyloidogenic and non-amyloidogenic events are believed to occur in different cellular compartments. APP processed within the cholesterol-enriched membrane microdomains known as lipid rafts is predominantly amyloidogenic and in contrast, APP processed outside lipid rafts appears to be non-amyloidogenic (145, 146). A recent structural study based on nuclear magnetic resonance spectroscopy of APP revealed that the β-secretase-generated transmembrane domain of C99 has a flexible binding site for cholesterol and the binding of cholesterol to this motif promotes amyloidogenesis (147). It is widely accepted, at least in part, that lowering the level of intracellular free cholesterol is neuroprotective (148). Depletion of cellular cholesterol either by statin (inhibitors of HMG-CoA reductase) or by methyl-β-cyclodextrin (MβCD; extracts and removes cholesterol from membranes) treatments inhibits the generation of toxic Aβ in neuronal cells (149). We have also reported that in cultured primary BCEC, when *de novo* cholesterol biosynthesis was inhibited (by simvastatin) the Aβ deposition was significantly decreased while the level of beneficial sAAPα was elevated (130). Refolo *et al*., (150) have identified that a

high-cholesterol diet increases Aβ accumulation and accelerates the amyloidogenic processing of APP, and the same research group (151) has also reported that a cholesterol reducing compound attenuates Aβ levels in AD murine models. Along with APP, BACE1, α-secretase and γ-secretase complex are all transmembrane proteins, and cellular cholesterol can directly modulate secretase activities there by controlling the processing of APP. Even though APP, BACE1 and γ-secretase complex are localized both in lipid raft and outside lipid raft regions, the reduction in cellular cholesterol will weaken the association of BACE1 with lipid rafts which in turn attenuates APP processing by the amyloidogenic pathway (145, 152, 153).

Moreover, recent studies indicate that not only the amount but also the distribution and turnover of intracellular cholesterol correlate to AD pathology. In the CNS excess free cholesterol is esterified and stored as cholesterol esters by acyl-CoA: cholesterol acyl-transferase 1 (ACAT1), which is also expressed in BCEC. Growing evidence proposes that ACAT1 activity will also regulate amyloidogenesis and pharmacological inhibition of ACAT1 might reduce Aβ generation (154, 155). *In vivo* studies have provided substantial evidence to confirm that ACAT1 inhibitors or ACAT1 deficiency could significantly improve AD pathology, but the exact mechanism is not fully elucidated. Although, numerous *in vivo* studies have reported the association of intracellular cholesterol lowering to AD pathogenesis, clinical trials with statins have generated mixed and disappointing results (156, 157). A combination of statins and ACAT inhibitors can be considered as a promising disease-modifying strategy for AD.

1.5. Importance of the cerebrovasculature in maintaining brain health

The brain is a highly vascularized organ that receives up to 20% of cardiac output, with a total length of ~650 km of vasculature and an available surface area of ~20 m^2 for molecular transport/exchange between blood and brain interstitial fluid (ISF; Fig. 1A) (158). Over several years the main research focus of AD was centered on neurons and glia or was "neuro-centric" ignoring the influence of vascular system, but emerging findings underlie the significance of the cerebrovascular system in the pathogenesis of AD. Substantial epidemiological evidence confirmed that atherosclerosis, stroke, hypertension, hyperlipidemia, obesity, and type 2 diabetes mellitus are the major vascular risk factors of AD (159, 160). There is an increasing risk for developing AD or vascular dementia in individuals with cardiovascular diseases and atherosclerosis (161). Indeed, advances of state-of-the-art research techniques and novel approaches do support the idea of neuronal-vascular axis in the development and/or progression of AD. Nevertheless, till now the researchers cannot reach to a consensus about whether BBB impairment is a cause or consequence in AD. BBB dysfunction is a multifaceted event which includes BBB rupture, anomaly in transporters and receptors expressed by ECs, and altered expression and secretions of other proteins by ECs and neighboring cell types. But the general notion that cerebrovascular dysfunction as one of the 'initial hit' is getting more attention, and the subsequent cascade of events that includes Aβ deposition and its reduced clearance will then accelerate vascular damage and neuronal dysfunction and ultimately lead to neurodegeneration and cognitive decline (158). This concept signifies the thus far underappreciated role of brain capillary endothelium in the progression of AD.

Several human studies and murine models of AD documented that cerebrovascular aberration takes place before the progression of cognitive impairment (162). Neurovascular perturbations probably due to cardiovascular/metabolic diseases or impaired Aβ clearance promote cerebral hypoperfusion/hypoxia, BBB leakage, neuroinflammation and angiogenic neurovascular remodeling. Indeed, brain microvessels isolated from AD subjects release cytokines, chemokines, reactive oxygen

species and proteases that can directly affect neuronal viability and even in turn damage the microvessels itself (163). A recent genome-wide expression study using AD human brain revealed that over 2000 genes are differentially altered in AD microvessels and most of the altered genes are associated with immune and inflammatory responses, and signal transduction pathways (164).

1.6. Phospholipid transfer protein

Phospholipid transfer protein (PLTP) belongs to the lipopolysaccharide (LPS)-binding/lipid transfer gene family and facilitates the exchange of phospholipids, cholesterol, LPS and vitamin E among various lipoproteins as well as between lipoproteins and cells (23, 165). PLTP displays 23-27 % amino acid homology with cholesteryl ester transfer protein (CETP), which is also a member of the LPS-binding/lipid transfer gene family. The PLTP gene is localized on chromosome 20q12-13.1 with an approximate length of 13.3 kilo bases (kb) and the mature secreted PLTP protein has a predicted molecular weight of 54.7 kDa. Mature PLTP protein seen in human plasma contains 476 amino acid residues and has 93% and 83% homology with porcine and mouse PLTP, respectively (166). The apparent molecular weight of PLTP is 80 kDa due to extensive N-glycosylation (six potential sites). PLTP mRNA (a single transcript of 1.8 kb) is expressed in different cell types and tissues, including macrophages, liver (the major site of expression), small intestine, ovary, placenta, lung and CNS (167). Apart from plasma, PLTP is also present in human tear fluid, semen and CSF. Not much is known on the regulation of PLTP activity. Reportedly, PLTP activity increased following incubation in the presence of apolipoproteins such as apoA-I and apoE (168, 169). The gene expression of PLTP can be regulated by members of the nuclear receptor family of transcription factors like LXRs, farnesoid X receptor, and peroxisome proliferator-activated receptor (170-172).

1.6.1. Plasma PLTP complexes

PLTP exists in two distinct forms in human plasma - an "active" (high-active) and "inactive" (low-active) form based on its ability/inability to transfer phosphatidylcholine from liposomes to HDL particles (173-175). In plasma these two forms are found in complexes of different size, the active PLTP is localized in plasma macromolecules with molecular mass of 160-210 kDa and Stokes diameter of 7.6-12.0 nm, which is similar to HDL particles size range. Inactive PLTP is present in larger macromolecules with molecular mass of 340-520 kDa and Stokes diameter of 12.0-17.0 nm (176). The physiological role of inactive PLTP is presently unknown and since its lipid binding capacity is still intact, inactive PLTP may play other important roles in circulation. There is also evidence that under proper stimulation inactive PLTP can be transformed to the active form (168). Although, the inactive form of PLTP is present in human plasma there is no evidence of inactive PLTP in human tear fluid and CSF (177, 178). Proteomics studies detected the association of twenty-four proteins in plasma PLTP complexes and out of this six proteins are apolipoproteins, and apoJ, PLTP, fibrinogen, plasminogen and apoA-I being the most abundant proteins (179). The existing information regarding the association of a particular apolipoprotein with a specific form (active or inactive) of PLTP remains controversial. Cheung *et. al*, (180) reported that active plasma PLTP is associated with apoA-I but not apoE-containing lipoproteins, while Karkkainen *et. al*., (174) co-purified inactive (low-active) PLTP with apoA-I and active PLTP with apoE.

1.6.2. Role of PLTP in lipoprotein metabolism

Plasma PLTP mediates the transfer and exchange of phospholipids (PL) between TG-rich lipoproteins and HDL particles during lipolysis (181). PLTP is capable of transferring all common PL species and hence it is a non-specific PL transfer protein. PLTP is a main plasma factor that modulates the size and composition of HDL particles. PLTP-mediated PL activity is essential for HDL enlargement and this process is markedly enhanced by TG-enriched HDL particles (182, 183). Human plasma PLTP plays a significant role in the conversion of circulating HDL into populations of larger

(10.9 nm, HDL_2) and smaller (7.8 nm, pre-β) HDL particles, a process known as HDL remodeling (184). This process involves the fusion of HDL particles which depends on the interaction of PLTP with apoA-I on HDL, followed by the formation of an unstable fusion intermediate which would eventually dissociate to form lipid-poor preβ-HDL and enlarged HDL particle (185-187).

PLTP deficiency in mice causes a marked decrease (60-70%) in the HDL-associated PL and apoA-I and a complete loss of ability to transfer *in vivo* radiolabeled-phosphatidylcholine (PC) from apoB-containing lipoprotein particles to HDL (188). Addition of a human CETP transgene to PLTP KO-mice does not compensate for the missing PL-transfer activity *in vivo*, indicating that there is no redundancy in function of PLTP and CETP and, PLTP is exclusively accountable for the plasma PL-transfer activity (189). Based on PLTP-deficient *in vivo* studies we can conclude that PLTP deficiency significantly decreases plasma levels of HDL associated PL, cholesterol, and apoA-1 (188, 190-192). High overexpression of PLTP with adenovirus (10- to 40-fold increase in PL-transfer activity) *in vivo*, resulted in a striking increase and decrease in preβ-HDL and α-HDL cholesterol levels, respectively (193, 194). A similar phenotype was also observed during moderate overexpression of PLTP by transgene (2.5- to 4.5-fold increase in PL-transfer activity), which resulted in a 30-40% reduction in plasma HDL cholesterol and a 2-3% increase in preβ-HDL levels (195, 196). Overall, these animal studies suggest that a balanced PLTP activity is critical in maintaining the normal HDL levels in plasma.

In addition to PL, PLTP also transfers cellular cholesterol and hence plays a potential role in cholesterol efflux. However the role of PLTP in RCT is controversial, both pro- and anti-atherogenic roles of PLTP have been reported (197). Macrophage mediated cholesterol efflux and RCT are considered as anti-atherogenic. Oram *et. al*., (198) reported that PLTP enhances cholesterol efflux from macrophages through a ABCA1-dependent pathway. *In vivo* studies confirmed that PLTP deficiency impairs ABCA1-dependent cholesterol efflux from macrophage foam cells (199). However, elevation of systemic human PLTP in transgenic mice decreases HDL cholesterol efflux

and RCT from mouse peritoneal macrophages, indicating that high levels of systemic PLTP may contribute to the development of atherosclerosis (200). Similarly, Moerland *et al*., (201) observed that HDL isolated from human PLTP/apoA-I double-transgenic mice was less efficient in removing cholesterol from macrophages compared to HDL isolated from human apoA-I transgenic mice. Consistent with these studies in mice, Masson *et al*., (202) recently reported that human PLTP expression in rabbits significantly increased the cholesterol content of plasma apoB-containing lipoproteins, and when these animals were exposed to high fat/cholesterol diet, increased atherosclerosis was observed. More studies are definitely needed to draw a clear conclusion concerning PLTP's pro- or anti-atherogenic roles. Based on the existing data the generally accepted notion is that PLTP exhibits a site-specific difference in its function *i.e.* local PLTP production (such as by macrophages) could be anti-atherogenic, while elevation of systemic PLTP could contribute to atherosclerosis development.

1.6.3. Other functions of PLTP

Alpha tocopherol (αTocH)/vitamin E, the potent antioxidant associated with HDL plays a significant role in the prevention of lipoprotein oxidation and also in maintaining normal endothelial function (203). PLTP facilitates the transfer of αTocH between lipoproteins and cell membranes (203, 204). PLTP also plays important functions in immunity and inflammatory pathways, as indicated by the association of plasma PLTP complexes with acute phase response, innate immunity and complement system proteins (179). In macrophages, PLTP activates the ABCA1/signal transducer and activator of transcription 3 (STAT3) anti-inflammatory signaling, and also reduces the nuclear levels and activation of nuclear factor kappa-B (NFκB) (205). These anti-inflammatory properties of PLTP in macrophages are independent of its lipid transfer ability (205).

1.6.4. Metabolic disorders associated with PLTP

Human PLTP deficiency has not been reported and the reports on PLTP single nucleotide polymorphism (SNPs) are contradictory. Tahvanainen *et al*., (206) reported six intragenic PLTP polymorphisms in the Finnish population and observed no significant association between these polymorphisms and serum PLTP activity. However, Vergeer *et al*., (207) reported the association of two PLTP SNPs with lower PLTP activity, higher HDL levels and decreased risk for coronary heart disease (CHD). Another research group reported that, SNPs located near the PLTP gene are connected with higher PLTP activity, higher HDL and lower TG levels (208). It has been reported that plasma PLTP activity increases with age, and also plasma PLTP activity correlated positively with body mass index (BMI), serum cholesterol and TG levels and correlated negatively with levels of apoA-I in HDL particles containing only apoA-I (206). Several epidemiological studies have reported that PLTP activity is elevated in both type I and type 2 diabetic conditions, and also in obesity (209-211). However an increased PLTP activity and mass in type 2 diabetic subjects is often associated with high plasma TG levels and obesity, while the plasma PLTP activity is not elevated in patients with normal BMI (211-214). But in the case of type 1 diabetes, the increased PLTP activity is independent of BMI and TG levels (209). Patients with CHD have shown increased serum PLTP activity (215), while Schgoer *et al*., (216) reported that low PLTP activity is a risk factor for peripheral atherosclerosis. However, the underlying mechanisms behind these variations in PLTP activity under pathophysiological conditions are largely unknown and warrant further investigation.

1.6.5. Role of PLTP in the central nervous system

In the CNS, PLTP is expressed mainly in neurons, choroid plexus and glial cells, and its phospholipid transfer active form is present both in CSF and in brain tissue (178, 217). PLTP secreted by these cells contributes to CSF PLTP levels and activity and CSF represents 15% of plasma PLTP activity. Unlike plasma, all the CSF PLTP is active in

lipid transfer and PLTP may play multiple roles in the CNS. PLTP is differentially expressed in different regions of the brain, indicating site specific functions of PLTP in various brain regions (217). PLTP might be involved in the maintenance of functional and structural integrity of myelin (217). In CSF, PLTP is mainly seen in association with apoE-containing lipoproteins and, moreover PLTP was found to be involved in regulating apoE expression and secretion in primary human astrocytes (178). PLTP was further reported to be involved in the reduction of abnormal Tau phosphorylation in human neuronal cells (218). At the intracellular level, in human neurons PLTP is an important regulator of signal transduction pathway through multiple pathways, including phosphatidyl inositol-3 kinase (PI3K)/protein kinase B (Akt or PKB) and insulin receptor (IR)/insulin-like growth factor 1 receptor (IGFR) (218). In addition, nuclear trans(location) of active PLTP has been also reported (218, 219).

PLTP deficiency significantly reduces brain vitamin E content and accumulates brain cholesterol derivates, and has been also associated with increased anxiety in mice (220). In addition, an increase (up to 33% and 450%, respectively) in the mean index of oxidative stress and amount of lipofusin (end product of lipid peroxidation) was detected in PLTP-deficient mouse brain (220).Two recent *in vivo* studies have demonstrated the association of PLTP in Aβ metabolism and the authors concluded that genetic inactivation of PLTP increases Aβ-induced memory deficits in mice (221, 222). Desrumaux *et al*., (221) reported that PLTP exerts its role in Aβ metabolism through the ability to transport vitamin E to the brain. A significant increase in Aβ levels and oxidative stress makers are observed in PLTP-KO animals. Furthermore, the restoration of brain vitamin E levels by dietary supplementation showed improved memory deficits and reduced cerebral oxidative stress and toxicity (221). In another study, Wang *et al*., (222) reported that PLTP deficiency enhanced the levels of $A\beta_{42}$ in the cerebral cortex and hippocampus regions of mice, due to increased expression of APP and BACE1. In addition, the levels of apoE were significantly decreased in both cortex and the hippocampus regions of PLTP KO animals (222). Moreover, it has been shown recently that PLTP deletion impairs the BBB integrity by elevating cerebrovascular oxidative stress (223). BBB permeability was increased in PLTP-deficient animals due to the reduced expression of tight junction proteins including occludin, zona occludens-1 (ZO-

1) and claudin-5 (223). Chronic dietary supplementation of vitamin E improved the BBB integrity and increased the expression of tight junction proteins in PLTP KO mice (223). Taken together, these *in vivo* studies confirm the crucial role of PLTP in the CNS, but only explored the vitamin E-axis of PLTP function, while the potential role of PLTP in relation to brain lipoprotein metabolism is largely unexplored.

PLTP levels or activity are differentially regulated in numerous human diseases, including neurodegenerative and neuroinflammatory diseases. Epidemiological studies reported altered PLTP levels and activity in brain tissue and CSF of patients suffering from AD (178, 217). PLTP mRNA levels were significantly lower in brains of patients with Down syndrome and CSF PLTP activity was considerably reduced in patients with multiple sclerosis (MS) (178, 224, 225). In addition, a significant correlation between CSF PLTP activity and total CSF cholesterol was observed in control groups but not in patients with MS, suggesting a disease-linked difference in the association between CSF cholesterol and PLTP activity (224). All these emerging informations collectively suggest the crucial roles of PLTP in both physiological and pathophysiological processes in the brain, and also warrant further investigation.

AIMS OF THE Ph.D. THESIS

2. AIMS OF THE Ph.D. THESIS

Escalating evidence suggests that disparities in lipid metabolism, mainly associated with impaired cholesterol and lipoprotein homeostasis, play a role in the pathogenesis of several neurodegenerative diseases such as AD (48, 50). Cholesterol and phospholipid transport within the CNS is mediated by HDL-like particles (55). The BBB (in particular BCEC) express several lipoprotein receptors, lipid transporters, proteins, and apolipoproteins important for both cholesterol turnover and HDL metabolism. We have previously proposed that primary pBCEC are involved in the biogenesis of HDL-like particles at the "brain parenchymal side" of the BBB (40, 41). PLTP is a glycoprotein involved in lipid and lipoprotein metabolism, and is also highly expressed in the CNS (226), however the potential role of PLTP in relation with brain lipoprotein metabolism is largely unknown.

In this study, we addressed the following aims:

I. To investigate the possible roles of PLTP at the interface between the brain and the peripheral circulation. In particular, we investigated its expression, regulation, and potential functions in HDL metabolism, using an established *in vitro* model of the BBB.

II. To identify the regulation of PLTP in the brain and more specifically in murine brain capillary endothelial cells (mBCEC) *in vivo*.

III. To investigate the potential role of PLTP in Aβ metabolism at the interface between the brain and the peripheral circulation, using an established *in vitro* model of the BBB.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and materials used for the study

Cell culture flasks, plates and other plastic ware were purchased from Greiner Bio-One (Kremsmünster, Austria). Transwell multiwell plates (poly-ester membrane inserts, 0.4 μM pore size) were obtained from Corning/Szabo-Scandic (Vienna, Austria). Medium M199, Minimal essential medium (MEM), porcine serum and dispase were obtained from Invitrogen (Vienna, Austria) and bovine calf-skin collagen G from Biochrom (Berlin, Germany). Culture media additives, trypsin-EDTA and DMEM/Ham´s F 12 medium were purchased from PAA (Pasching, Austria) and collagenase/dispase from Roche (Vienna, Austria). Protease inhibitor cocktail, percoll, L-α-Phosphatidylcholine (egg PC), butylated hydroxytoluene (BHT), hydrocortisone and heparin were from Sigma Aldrich (Vienna, Austria). [^{3}H]L-α-dipalmitoyl phosphatidylcholine (specific activity-1.550 TBq/mmol), [1, 2-^{3}H(N)]-cholesterol (specific activity-1.772 TBq/mmol; [^{3}H]-cholesterol) and Ultima Gold scintillation cocktail were purchased from New England Nuclear (Vienna, Austria). 24(*S)*-hydroxycholesterol was purchased from Medical Isotopes (Pelham, Canada) and TO901317 from Cayman Chemicals (MI, USA). RNeasy Mini kit and small interfering RNA (siRNA) targeting for human PLTP were from Qiagen (Vienna, Austria). siRNA transfection reagents were obtained from Lonza (MD, USA) and negative siRNA control from Dharmacon (MA, USA). PD-10 desalting columns and polyvinylidene fluoride (PVDF; 0.45 μm) transfer membranes were purchased from GE Health care (Vienna, Austria). Centrifugal filter units (Amicon Ultra-15) for concentrating culture media from Merck Millipore (Vienna, Austria). Antibodies were purchased from Abcam (PLTP, IgG; Cambridge, UK), Invitrogen (APP; Vienna, Austria), Millipore (A11 amyloid oligomer; Vienna, Austria) and Sigma Aldrich (β-actin; Vienna, Austria). PCR reagents were obtained from Bio-Rad (Vienna, Austria) and primers from Invitrogen (Vienna, Austria). Anti-human apoA-I antibodies were raised in

rabbits by immunizing the animals subcutaneously with 100 µg of apoA-I purified from human plasma HDL. All other solvents and chemicals were of reagent grade quality and purchased either from Sigma Aldrich (Vienna, Austria) or Merck (Vienna, Austria).

3.1.2. Reagents and buffers used for the study

Composition of self-made reagents, cell culture media and buffers are listed below.

Phosphate buffered saline (PBS; 1X; pH 7.4; 1 L)

NaCl	8.0 g
KCl	0.20 g
KH_2PO_4	0.25 g
$Na_2HPO_4.2H_2O$	1.42 g
Glucose	2.0 g
Check the pH and fill up to 1 L with double-distilled water (ddH_2O). Sterile-filter.	

Preparation medium

M199 medium (1X)	500 ml
Penicillin/Streptomycin (stock conc. 10000 U/ml)	5 ml
20 mM Gentamycin	5 ml
200 mM Glutamine	2.5 ml

Plating medium A

M199 medium (1X)	500 ml
Penicillin/Streptomycin (stock conc. 10000 U/ml)	5 ml
20 mM Gentamycin	5 ml
200 mM Glutamine	2.5 ml
Porcine serum (1X)	50 ml

Plating medium B

M199 medium (1X)	500 ml
Penicillin/Streptomycin (stock conc. 10000 U/ml)	5 ml
200 mM Glutamine	2.5 ml
Porcine serum (1X)	50 ml

Serum-free medium

M199 medium (1X)	500 ml
Penicillin/Streptomycin (stock conc. 10000 U/ml)	5 ml
200 mM Glutamine	2.5 ml

Hydrocortisone stock solution (50 µg/ml)

Dissolve 1 mg of hydrocortisone in 20 ml Ham´s F12 medium and store the aliquots at -20^0C.

DMEM/Ham´s F12 medium

DMEM/Ham´s F12 medium	500 ml
Penicillin/Streptomycin (stock conc. 10000 U/ml)	5 ml
200 mM Glutamine	1.8 ml

MCDB 131 medium

MCDB 131 medium (1X)	500 ml
Fetal calf serum (FCS; 1X)	10 ml
Penicillin/Streptomycin (stock conc. 10000 U/ml)	5 ml
200 mM Glutamine	2.5 ml

Collagenase/Dispase solution

Collagenase/Dispase	100 mg
Plating medium A	10 ml
Mix and sterile filter (0.2 μm). Store the aliquots at -20^{0}C.	

Percoll® solution (pH 8.5 -9.5; 1.03 g/mL density; 50 ml)

PBS (1X)	40 ml
Percoll®	9 ml
MEM (10X)	1 ml

Percoll® solution (pH 8.5 -9.5; 1.07 g/ml density; 50 ml)

PBS (1X)	20 ml
Percoll®	27 ml
MEM (10X)	3 ml

Dextran solution (1.2 L; 1.0612 g/L density)

Dextran	200 g
NaHCO3	2.4 g
Minimum essential medium (MEM; 10X)	109.1 ml
Add 1.2 L ddH_2O and stir overnight at 4^0C. Adjust the density on the next day, mark the liquid level and autoclave the solution. After autoclaving fill up to the mark using sterile ddH_20 .	

24*(S)*-hydroxycholesterol stock solution (20 mM)

Dissolve 5 mg of 24*(S)*-hydroxycholesterol in 625 µl of 100% ethanol and store the aliquots at -20^0C.

TO903017 stock solution (100 mM)

Dissolve 50 mg of TO903017 in 1039 µl of 100% ethanol to obtain a 100 mM stock. Dilute 1:10 with ethanol to get a 10 mM working stock solution and store the aliquots at -20^0C.

Human insulin stock solution (10 mM)

Dissolve 5.8 mg insulin in 100 µl of 0.01 N HCl and dilute (1:1000) the stock to obtain the working stock solution (10 µM). Stock solution is stable for up to 4 weeks at 4^0C.

D-glucose stock solution (1 M)

Dissolve 1.8 g of glucose in 10 ml of PBS (1X) and sterile-filter. Store at 4^0C.

Protein lysis buffer (10 ml; pH 7.5)

1 M Tris-HCl	500 µl
1 M EDTA	100 µl
Triton-X 100	100 µl
Protease inhibitor cocktail (25X)	400 µl
Mix all ingredients and fill up to 10 ml with ddH_20. Store the aliquots at -20^0C.	

TAE buffer (50X; pH 8.5; 1 L)

Tris base	242 g
EDTA	18.6 g
Glacial acetic acid	57.1 ml
Adjust the pH and make up to 1L with ddH_20.	

Western blot (WB) running buffer (10X; pH 8.3; 1 L)

Tris base	30.3 g
Glycine	144 g
SDS	10 g
Make up to 1L with ddH_20.	

WB transfer buffer (1X; 1 L)

WB running buffer (10X)	100 ml
Methanol	200 ml
Make up to 1L with ddH_20.	

WB wash buffer (1X; 1 L)

1 M Tris	20 ml
5 M NaCl	50 ml
Triton-X 100	2 ml
Tween® 20	0.5 ml
Make up to 1L with ddH_20.	

Blocking solution (5%; 100 ml)

Dissolve 5 g of blotting-grade blocker (nonfat dry milk powder) in 95 ml 1X PBS and 0.2 ml Tween® 20.

Nondenaturing gradient gel electrophoresis running buffer (1X; pH 8.3, 1 L)

Tris base	10.9 g
Boric acid	4.95 g
0.5 M EDTA	6 ml
Make up to lL with ddH_20.	

Nondenaturing gradient gel electrophoresis transfer buffer (10X; pH 8.3, 1 L)

Tris base	30.3 g
Glycine	144 g
Make up to 1L with ddH_20.	

Coomassie brilliant blue (CBB) staining solution (100 ml)

CBB G-250	0.1 g
Methanol	50 ml
Glacial acetic acid	10 ml
Make up to 100 ml with ddH_20	

Destaining solution (100 ml)

Methanol	50 ml
Glacial acetic acid	10 ml
Make up to 100 ml with ddH_20.	

Fixing solution for electrophoresis (10%; 100 ml)

Dissolve 10 g of 5-Sulfosalicylic acid dehydrate in 90 ml of ddH_20.

KBr solution (1.063 g/ml density; 500 ml)

Dissolve 47.13 g of KBr in 500 ml of ddH_20. Sterile-filter and store at 4^0C.

Substrate buffer for PLTP activity assay (20X; pH 7.4; 100 ml)

Tris base	2.423 g
NaCl	17.530 g
$Na_2EDTA.2H_20$	0.744 g
Mix all ingredients and adjust the pH. Fill up to 100 ml with ddH_20. Sterile-filter and store at 4^0C.	

Stop solution for PLTP activity assay (1X; 100 ml)

NaCl	3.133 g
MnCl2	7.183 g
Heparin (195.9 U/mg)	0.09 g
Mix all ingredients and fill up to 100 ml with ddH_20. Prepare fresh for each assay.	

3.2. Methods

3.2.1. Immunohisto- and immunocyto-chemical staining

Studies were carried out on 5 µm coronal cryosections of porcine brain samples. For immunocytochemistry, pBCEC were cultured on chamber slides. Tissue sections or cells were fixed in acetone for 5 min and air dried 20 min prior to immunostaining using the UltraVision anti-polyvalent, LP HRP Detection System (Thermo Scientific, CA, USA) according to manufacturer's instructions. Sections were washed with 0.01M PBS (pH7.4) before primary antibodies were incubated for 30 min at room temperature. Antibody concentrations were 0.7 µg/ml for rabbit anti-human von Willebrand factor (vWF, Dako, CA, USA), 1 µg/ml for rabbit anti-human PLTP (H-273; Santa Cruz, CA, USA) and 1 µg/ml for normal rabbit immunoglobulin fraction (Dako ,CA,USA). After rinsing for 5 min with PBS, slides were incubated with primary antibody enhancer (10 min) followed by horseradish peroxidase polymer (15 min). The slides were washed and immunolabeling was visualized by incubating with 3-amino-9-ethylcarbazol (AEC; Thermo Scientific) for 5 min. Slides were counterstained with Mayer's hematoxylin (Merck) and mounted with Kaiser's glycerol gelatin (Merck).

3.2.2. Isolation and culture of primary porcine brain capillary endothelial cells (pBCEC)

pBCEC were isolated from freshly slaughtered pigs (about 6 months old) obtained from the local slaughterhouse as described by Franke *et al.* (227). After removal of the secretory areas and visible large vessels of the porcine brain, pBCEC were isolated from the remaining cerebral cortex by sequential enzymatic digestion and centrifugation steps as described (227). Briefly, brain tissue homogenate was digested with dispase (50 mg/brain) in a final volume of 100 ml of 'preparation medium' (M199 medium containing 1% penicillin/ streptomycin, 1% gentamicin and 1mM L-glutamine) per brain for 1 h with continuous stirring in a water bath at 37^0C. The homogenate was suspended

in 10% w/v dextran solution and centrifuged at 6800 g for 10 min at 4^0C and the pellets were resuspended in 'preparation medium'. After filtered through a nylon mesh (180 µm pore size), the capillary-fragment suspension was carefully placed onto the top of the percoll gradient (20 ml of 1.03 g/ml, 15 ml of 1.07 g/ml) solution and centrifuged at 1300 g for 10 min at room temperature in a swing-out bucket rotor. Brain capillary endothelial cells being enriched at the interphase were carefully recovered and washed with an excess of 'preparation medium' to remove percoll. pBCEC were then plated onto collagen-coated (60 µg/ml) 75 cm^2 culture flasks with 'plating medium A' (M199 medium containing 1% penicillin/ streptomycin, 1% gentamicin, 1mM L-glutamine and 10% porcine serum). Cells were washed twice with PBS after 24 h to remove cell debris and non-adherent cells, and cultured in fresh 'plating medium B' (M199 medium containing 1% penicillin/ streptomycin,1mM L-glutamine and 10% porcine serum) until confluent. After 3 days the cells were trypsinised and plated onto collagen-coated (60 µg/ml or 120 µg/ml) multiwell culture plates, flasks, or transwell filter plates and grown until confluent. For treatments, pBCEC monolayers were incubated in the absence or presence of the indicated concentrations of glucose, insulin or their combination for 24 h or with LXR agonists (24*(S)*-hydroxycholesterol or TO901317) for 24 h in serum free medium. All cell culture incubations were performed at 37^0C in a humidified 5% CO_2 incubator.

3.2.3. Isolation of murine brain capillary endothelial cells (mBCEC)

Mice were sacrificed by cervical dislocation and brains were immediately transferred in to ice-cold PBS. After removal of the secretory areas, the brain cortices were homogenised in 1 ml of 'preparation medium' using a Dounce tissue grinder (4-6 strokes), and another 1ml of 'preparation medium' was added . The homogenate was digested with dispase (0.02 g/ml MCDB 131 medium) for 1 h with continuous stirring in a water bath at 37^0C. The homogenate was suspended in 5 ml of dextran solution (density 1.0612 g/ml) and centrifuged at 1000 g for 10 min at 4^0C and the pellets were washed and resuspended in 2ml of 'preparation medium'. The suspension was carefully placed onto the top of the percoll gradient (4 ml of 1.03 g/ml, 3 ml of 1.07 g/ml) solution

and centrifuged at 1300 g for 10 min at room temperature in a swing-out bucket rotor. Brain capillary endothelial cells floating at the interphase were carefully recovered and washed with an excess of 'preparation medium' to remove percoll. The pelleted mBCEC were stored at -80°C until analyzed.

3.2.4. Transwell studies

To establish polarized pBCEC cultures, cells were plated onto collagen-coated (120 µg/ml) transwell (6- or 12-well) culture dishes at a density of 40 000 cells/cm^2. Cells were grown for 2 to 3 days depending on the transendothelial electrical resistance (TEER; ≥ 50 ohms/cm^2). The tightness of the transwell culture was assessed by measuring the TEER using an EndOhm tissue resistance measurement chamber and EndOhm ohmmeter (World precision Instruments, Florida, USA). TEERs of collagen coated, cell-free filters were used as blanks. Tight junction formation was induced (overnight) by adding DMEM/Ham´s F12 medium containing 550 nM hydrocortisone, 1% penicillin/ streptomycin and 0.7 mM L-glutamine along with the indicated concentrations of LXR agonists. Establishment of intact tight junctions was indicated by rising TEERs in the *in vitro* BBB model system.

3.2.5. Immunoblot analysis

pBCEC supernatants were collected, centrifuged (10000 g, 10 min, 4^0C) and secreted proteins were precipitated with 3% (v/v) tricholoacetic acid (TCA). Protein pellets were washed twice with acetone and resuspended in 1x sample buffer (Bio-Rad). For total cell proteins, pBCEC were lysed in protein lysis buffer (50 mMTris, 10 mM EDTA, 1% Triton X-100, 0.5% v/v protease inhibitor cocktail, pH 7.4), sonicated and centrifuged (10000 g, 10 min, 4^0C). Cellular protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Vienna, Austria). Equal amounts of proteins (cellular and secreted) were loaded onto NuPAGE Novex 4-12 % Bis-Tris Midi gels (Invitrogen) and proteins were separated by SDS-PAGE in MOPS

running buffer (Invitrogen) under denaturing conditions (200 V for 55 min). For immunoblotting, proteins were electrophoretically transferred to 0.45 µm PVDF membranes (50 V for 1 h). After blocking with 5% non-fat dry milk (Bio-Rad) for 90 min at room temperature, the membranes were probed using rabbit polyclonal anti-PLTP antibody (Abcam; 1:2000), rabbit polyclonal anti-APP antibody (Invitrogen; 1:1000), rabbit polyclonal A11 anti-amyloid oligomers antibody (Millipore; 1:10000) or rabbit polyclonal anti-β-actin (Sigma Aldrich; 1:5000) overnight at 4^0C. Membranes were washed and subsequently incubated with streptavidin-HRP (horseradish peroxidase; Bio-Rad) labeled goat polyclonal antibody against rabbit IgG (Abcam; 1:5000) for 1 h at room temperature. Equal loading and transfer of proteins were confirmed by β-actin detection. Immunoreactive bands were detected on CL-XPosure films (Thermo Scientific) using enhanced chemiluminescence (Western C Immunstar, Bio-Rad). Bands were quantified densitometrically using ImageJ version 1.42 software (NIH).

3.2.6. RNA Isolation and mRNA quantification

Total RNA was isolated from pBCEC using RNeasy Mini kit according to the manufacturer's protocol and RNA integrity was assessed by 1% agarose gel electrophoresis. RNA concentration was determined spectrophotometrically and total RNA (1 µg) was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) on a C1000 Thermal Cycler (Bio-Rad). Quantitative gene expression analysis of *PLTP* and reference genes *HPRT1* (hypoxanthine phosphoribosyltransferase 1), *ACTB* (beta actin), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, *TBP* (TATA box binding protein), *RPL4* (ribosomal protein L4), and *HMBS* (hydroxymethylbilane synthase) were performed on a CFX 96 Real-Time System (Bio-Rad) using SYBR Green technology. In general, each reaction (10 µl) contained 1X iQ SYBR Green Supermix (Bio-Rad), 300 nM of each primer (Table 2) and 20 ng cDNA template, PCR cycling conditions consisted of 40 cycles at 95^0C for 20 s, 60^0C for 40 s and 72^0C for 40 s. All reactions were run in triplicates and melting curve analyses were routinely performed to monitor the specificity of the PCR product. The relative gene expression ratio was determined using a standard curve method (228).

Table 2: Primer sequences used for real-time PCR. (*ss- Sus scrofa, hs- Homosapiens*)

Gene	NCBI gene ID or	Primer sequence (5'- 3')	Amplicon length
ssPLTP	397527	F: CCCTCTTCCTAGTGCTGCTG R: CAGATCCGGAATGGTAATGG	146
hsPLTP	(26)	F: CAGGCGCACATGCAGAGTTC R: GCAGCTCTGTGACCTTCACC	183
ssHPRT1	15452	F: AGGACCTCTCGAAGTGTTGG R: CAGATGGCCACAGGACTAGA	247
hsHPRT1	(26)	F: GACCAGTCAACAGGGGACAT R: CTGCATTGTTTTGCCAGTGT	111
ssACTB	414396	F: GGCCAGGTCATCACCATT R: GGATGTCCACGTCACACTTC	140
hsACTB	(26)	F: TCCCTGGAGAAGAGCTACG R: GTAGTTTCGTGGATGCCACA	131
ssGAPDH	396823	F: GCCGCGTCCCTGAGACAC R: GGCGGGATCTCGCTCCT	262
hsGAPDH	2597	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	226
ssTBP	(27)	F: AACAGTTCAGTAGTTATGAGCCAGA R:AGATGTTCTCAAACGCTTCG	153
hsTBP	6908	F: TGCACAGGAGCCAAGAGTGAA R: CACATCACAGCTCCCCACCA	132
ssRPL4	(27)	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	122
hsRPL4	(28)	F: GCTCTGGCCAGGGTGCTTTTG R: ATGGCGTATCGTTTTTGGGTTGT	154
ssHMBS	(27)	F: AGGATGGGCAACTCTACCTG R: GATGGTGGCCTGCATAGTCT	83
hsHMBS	3145	F: TGCTCGCATACAGACGGAC R:GGTAAACAGGCTTTTCTCTCCAA	111
ssAPP	(53)	F: GTGAAGATGGATGCGGAGTT R: GTGATGACAATCACGGTTGC	152
ssBACE1	100511707	F: TGGACTGCCTCATGGTGTG R: GTGACCAAAGTGAACCACCG	155

3.2.7. Measurement of phospholipid transfer activity

Phospholipid transfer activity of PLTP was assessed based on the transfer of [^{3}H] L-α-dipalmitoyl phosphatidylcholine (DPPC) from liposomes to HDL_3 using an established radiometric assay (229). In brief, 129 µmol/µl egg PC, 1 nmol/µl butyrated hydroxytoluene and 1µCi/µl [^{3}H]-α-DPPC were dried under nitrogen and resuspended in 1 ml of substrate buffer (10 mM Tris-HCl, 150 mM NaCl and 1 mM EDTA, pH 7.4). To obtain clear liposomes, the above solution was sonicated and centrifuged (12000 g, 10 min, at room temperature). Radio-labeled liposomes (150 nmol) and plasma HDL_3 (200 µg) were incubated with aliquots of pBCEC supernatants (300 µl) or cellular lysates (100 µl; in substrate buffer) for 1 h at 37^{0}C. The reaction was stopped and liposomes were selectively precipitated by the addition of stop solution (536 mM NaCl, 363 mM $MnCl_2$ and 52 units of heparin). After incubation for 30 min on ice, the mixture was centrifuged (12000 g for 10 min) at room temperature and radioactivity transferred to HDL_3 in the supernatant (500 µl) was determined on a Tri-Carb 2100 TR Liquid Scintillation Counter (Packard Bioscience Co.) after mixing with 5 ml Ultima Gold scintillation cocktail. The specificity of the PLTP activity assay in pBCEC lysates and supernatants was validated by antibody inhibition and heat inactivation control experiments, as recently described (230). To control for inter-assay variability, an aliquot (1µl) of freshly thawed (-70^{0}C) human plasma was included in each assay. Cellular protein content was determined by BCA protein assay. PLTP activity was expressed in absolute activity as nmol PL transferred per mg of total cell protein per ml medium or cell lysates and per hour measured under control conditions.

3.2.8. Isolation of plasma HDL and apoA-I

HDL_3 (1.125-1.210 g/ml) was isolated fresh from normolipidemic human EDTA-plasma by density gradient ultracentrifugation (231). Briefly, density of plasma was adjusted with potassium bromide (KBr) to 1.24 g/ml and density-adjusted plasma was then gently layered underneath an aqueous solution of KBr (1.063 g/ml). The samples were centrifuged at 694,000 g for 4h (10^{0}C) using an Optima L-90K Ultracentrifuge

(Beckman Coulter, Vienna, Austria). HDL_3 was carefully recovered, stored at 4^0C and desalted before use with PD-10 columns containing Sephadex G-25 medium. Protein content of HDL_3 was determined using Quanti-IT protein assay kit (Invitrogen). Apo A-I was purified after delipidation of HDL by size exclusion chromatography on a Sephacryl S-200 column (3×150 cm) as described (232).

3.2.9. Purification of human plasma PLTP

PLTP was purified from human plasma as described as Marques-Vidal *et al.* (233). Briefly, the human plasma fraction (density>1.22 g/ml) was applied sequentially to five different high performance chromatographic columns and the active PLTP fraction was finally eluted at a Na-phosphate concentration of about 140 mM. Depending on the preparation the activity of purified PLTP ranged between 5.0 to 9.0 μmol PL transferred/ml/h. All of the preparations were free of CETP, hepatic lipase, LCAT and phospholipase activity.

3.2.10. HDL_3 conversion/modification assay

For HDL_3 conversion assay, fresh human HDL_3 (250 μg) was incubated with purified active human PLTP (250 nmol/h) at 37^0C for 24 h. Control incubations of HDL_3 at 4^0C and 37^0C in the absence or presence of purified PLTP were also performed. HDL particle size was analyzed (10 μg protein) by non-denaturing gradient gel electrophoresis and PLTP-modified HDL particles were used as acceptors for cellular cholesterol efflux studies, as described below.

3.2.11. *Nondenaturing gradient gel electrophoresis (NDGGE) and immunoblotting*

Nondenaturing 4-15% (Tris-HCl gel, Bio-Rad) polyacrylamide gradient gel electrophoresis was used to determine HDL subclass distribution as described (234) with minor modifications. After pre-running the gels for 20 min at 140 V, samples were applied and electrophoresed for 30 min at 70 V, followed by another 6 h run at 140 V. Nascent HDL particle formation in pBCEC conditioned media was determined by directly transferring the proteins after electrophoresis (4-20% Tris-HCl gel, Bio-Rad) to PVDF membranes (40 min at 100 V). All the above procedures were performed at 4^0C. Subsequent immuno-blotting with polyclonal rabbit anti-human apoA-I antibody (1:2500, a kind gift from Dr. Ernst Malle, Medical University of Graz, Austria) was performed as described in 'immunoblot analysis' section. Gels were stained with Coomassie Brillant Blue G250 after fixing in 10% sulfosalicylic acid for 30 min. A high molecular weight protein marker (NativeMark, Invitrogen) was used as the size standard (7.1, 8.2, 11.0, 13.4 and 18.0 nm).

3.2.12. Pre-β-HDL determination by two dimensional (2D) crossed immunoelectrophoresis

pBCEC monolayers grown in flasks (75 cm^2), were incubated with fresh human HDL_3 for 24 h at 37^0C. Cell-free control incubations of HDL_3 in serum-free medium were performed at 4^0C and 37^0C. On the next day, culture media were collected and concentrated using Amicon Ultra-15 Filter Devices. Pre-β-HDL formed in pBCEC conditioned media during incubation with HDL_3 was quantified by 2D crossed immunoelectrophoresis as described (196). In brief, in the first dimension, pre-β- and α-mobile lipoproteins present in the media (4 μg of protein) were resolved by 1% native agarose gel electrophoresis (4^0C, 2 h, 320 V). In the second dimension, separated lipoproteins were electrophoresed at right angles into a freshly applied layer of agarose gel (1%) containing 7.5% (vol/vol) rabbit anti-human apoA-I antiserum (4^0C for 20 h, 50 V). Gels were air dried after staining with Coomassie Brillant Blue R250. The relative

amount of pre-β-HDL was calculated as the percentage of the sum of α-HDL and pre-β-HDL peak areas.

3.2.13. Cellular cholesterol efflux assay

Cellular cholesterol efflux was determined as previously described (40). In brief, pBCEC monolayers on 6 or 12-well plates were cholesterol labeled with 0.5 μCi/ml [^{3}H]-cholesterol in M199 (containing 1% penicillin/ streptomycin, 1 mM L-glutamine and 10% porcine serum) for 24 h. Polarized pBCEC grown on 12-well transwell filter plates were labeled with 1.0 μCi/ml [^{3}H]-cholesterol added to the basolateral compartment (lower chamber, representing the brain parenchymal side) for 24 h. LXR agonist TO901317 was added to the apical compartment (upper compartment, representing the microvessel luminal side) during induction of tight junctions (16 h). Cells were washed and cellular cholesterol pools were equilibrated in serum free medium for 2 h (transwell experiments) or 16 h. Subsequently, cells were washed twice with PBS and cholesterol acceptors apoA-I (10 μg/ml), plasma HDL_3 (50 μg/ml) or plasma HDL_3 modified with purified human plasma PLTP (250 nmol/h, 37^0C, 24 h) were added in serum free medium. For transwell experiments, plasma HDL_3 (50 μg/ml) or plasma HDL_3 modified with purified human plasma PLTP (250 nmol/h, 37^0C, 24 h) were added either to the apical or basolateral compartment in serum free medium. Aliquots of media were collected at the indicated time intervals and radioactivity was determined with Ultima Gold scintillation cocktail on a Tri-Carb 2100 TR Liquid Scintillation Counter (Packard Bioscience Co.). Cells were washed twice with ice-cold PBS and lysed in 0.3 N NaOH. Remaining [^{3}H]-cholesterol radioactivity in the cell lysates was determined and total cellular protein concentration was quantified using the Qubit fluorometer (Quanti-IT protein assay kit, Invitrogen). Cholesterol efflux was calculated as the percentage of cpm /mg cell protein in the supernatants relative to the total counts in the supernatants plus cell lysates.

3.2.14. RNA interference mediated PLTP silencing in pBCEC

A pool of four small interfering (si) RNAs targeting the human PLTP sequence was obtained from Qiagen. At 50-60% confluency, pBCEC were transfected with human PLTP siRNAs at a final concentration of 25 nM using PrimeFect siRNA Transfection Reagent (3 µl), as described by Stefulj & Panzenboeck *et al*., (235). Non-targeting control (NTC) with minimal unintended off-target effects was used as negative control. For cholesterol efflux assays, pBCEC were labeled with 0.5 µCi/ml [^{3}H]-cholesterol for 40 h, and simultaneously transfected with siRNA or NTC. Subsequently, cholesterol pools were equilibrated for 2 h and cholesterol efflux was determined as described under section 'cellular cholesterol efflux assay'. Silencing efficiency (relative to non-targeting siRNA) was monitored at the mRNA, protein, and phospholipid transfer-activity level as described above.

3.2.15. Animal experiments

Animal experiments were performed in accordance with the standards established by the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments (Vienna, Austria). Male C57/BL6J mice 20 weeks of age (25-30g) were maintained under a 12 h light/12 h dark cycle in a temperature-controlled environment and had free access to chow diet (Ssniff®, Soest, Germany) and water. For 7 days mice were 4 h fasted each day and dosed orally with vehicle [0.5% (w/v) carboxymethyl cellulose (CMC)/H_2O; 10 µl/g mouse] or synthetic LXR agonist T0901317 (Cayman Chemicals, MI, USA) [50 mg/kg in 0.5% CMC]. After 7 days of treatment, blood was drawn via retro-orbital puncture and plasma was isolated for analysis. Mice were sacrificed by cervical dislocation. Brain and liver tissues were isolated, snap-frozen and stored at -80°C until analyzed. For PL-transfer activity assay, brain and liver samples were homogenized in 1ml of substrate buffer containing protease inhibitor cock tail at 4^{o}C using an Ultra-Turrax T25 homogenizer (IKA labortechnic, Linz, Austria). Mouse brain capillary endothelial cells (mBCEC) were isolated from a pool of hemispheres (n = 7) essentially as described in section 3.2.3. For PL-transfer

activity assay, mBCEC were homogenized in 1ml of substrate buffer containing protease inhibitor cocktail at 4^0C using Sonoplus HD 3080 (Bandelin, Berlin, Germany). After homogenization the mixture was centrifuged (1000 g, 5 min, 4^0C). PLTP activity (100 µl per assay) and total protein content were analyzed from the clear supernatant. PLTP activity was measured as described above and expressed as nmol PL transferred/h/mg protein.

3.2.16. Murine plasma PLTP and lipid analysis

Plasma (1 µl undiluted mouse EDTA-plasma) phospholipid transfer activity was measured by radiometric assay as described above and expressed as nmol PL transferred/ml/h. Plasma levels of total cholesterol, unesterified cholesterol, total phospholipids, and triglycerides were assayed using enzymatic kits according to the manufacturer's instructions (DiaSys Diagnostics, Holzheim, Germany).

3.2.17. Statistical analysis

Results are reported as means ± S.E. unless stated otherwise. All experiments were performed at least three or more times in triplicate. Statistical significances (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$) were determined by two-tailed Student's t-test or analysis fo variance (ANOVA) followed by Bonferroni´s post hoc test using Prism software (*Graphpad version 5*, CA, USA).

RESULTS

4. RESULTS

4.1. PLTP is synthesized in porcine cerebrovascular endothelial cells

Previous studies have identified the brain as one of the organs where PLTP is expressed (217). Immunohistochemistry has suggested that PLTP is present in neurons, astrocytes, and at the BBB (217). However, the contribution of BCEC to cerebral PLTP expression has not been evaluated. Hence, we first performed immunostaining on coronal sections of porcine midbrain using endothelial cell specific marker vWF (Fig. 2A) and PLTP antibody (Fig. 2B). Rabbit IgG was used as negative control (Fig. 2C). We detected distinct PLTP staining in cerebral vessels (Fig. 2A), more prominent than in brain parenchymal tissue, indicating that PLTP is highly expressed at the BBB. Immunocytochemical analysis of cultured primary pBCEC (Fig. 2 D-F) also showed distinct PLTP staining (Fig. 2E), confirming PLTP expression in the cerebral microvasculature.

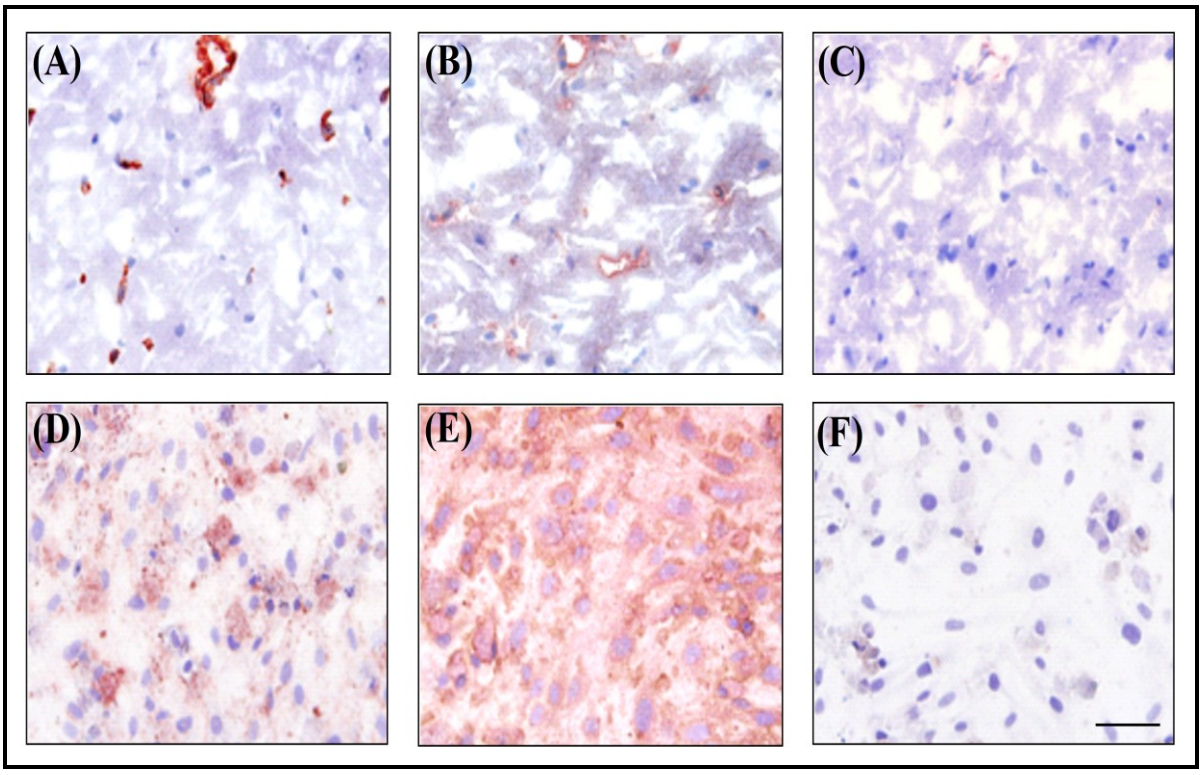


FIGURE 2: Cerebrovascular endothelial cells express PLTP. Immunocytochemical analysis of porcine brain endothelial cells is shown *in situ* on coronal sections of porcine midbrain (A-C) and *in vitro* in cultured pBCEC (D-F). Cerebral vessels (CV) and cultured endothelial cells express the endothelial marker von Willebrand factor (A, D) and PLTP (B, E). (C, F) non-immune rabbit IgG control. Cells were stained with Mayer´s hematoxylin to visualize cell nuclei. Scale bar: 50µm.

The presence of PLTP mRNA by real-time PCR in isolated cerebral vessels confirmed that PLTP is of endogenous origin (Fig. 3). Human liver expresses high levels of PLTP; hence we used human liver RNA for comparison. PLTP mRNA expression levels (normalized to the geometric mean of four reference genes) were 6.8-fold and 1.8-fold higher in isolated porcine cerebral vessels as compared to whole porcine brain and liver, respectively (Fig. 3). The enrichment of PLTP mRNA in isolated cerebral vessels as compared to total brain tissue strongly supports an important role of PLTP in BBB physiology.

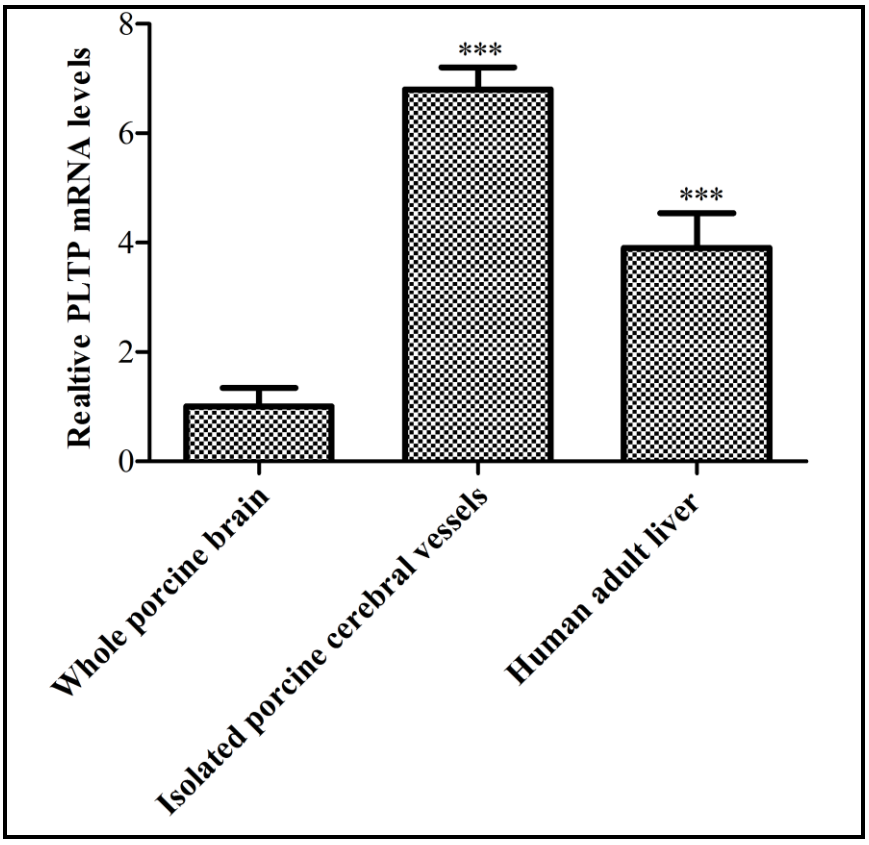
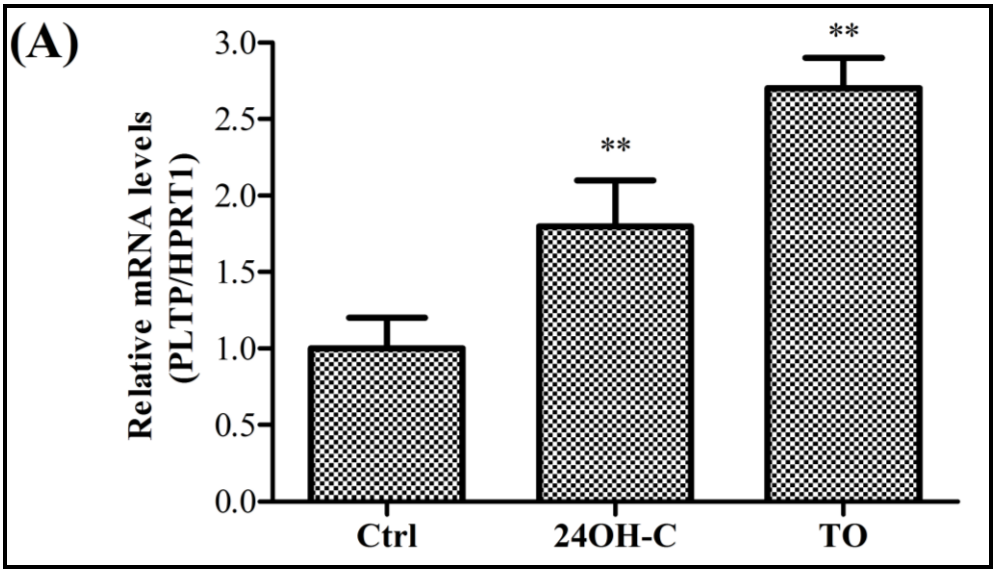


FIGURE 3: High expression of PLTP mRNA in cerebrovascular endothelial cells. Total RNA was isolated from porcine brain tissue, porcine cerebral vessels, and human liver (positive control), reverse transcribed, and real-time PCR was performed on a CFX 96 Real-time System (Bio-Rad) using SYBR Green technology. PLTP mRNA levels were normalized to four reference genes (*HPRT1*, *GAPDH*, *RPL4* and *HMBS*). Data shown are means ± SD of triplicate analyses (* p< 0.05, *** p< 0.001 *versus* whole porcine brain).

4.2. PLTP expression and levels are enhanced by LXR activation

It has been reported that LXR up-regulates expression of PLTP in HepG2 cells, macrophages, and murine liver and enhances PLTP activity in plasma (170, 171). Hence, we next investigated whether PLTP expression in pBCEC is modulated by LXR activation. The brain-specific natural LXR ligand, 24*(S)*-hydroxycholesterol (24OH-C) under estimated physiological concentration (10 μM) induced PLTP mRNA expression levels by 1.8-fold (Fig. 4A). Treatment with the synthetic LXR agonist TO901317 (5 μM) resulted in an even more pronounced, 2.7-fold up-regulation of PLTP mRNA expression levels (Fig.4A). PLTP protein was immunodetected both in cell lysates and in supernatants (Fig. 4B). LXR activation using both, synthetic or natural ligands increased PLTP mass in pBCEC by 1.4 -1.5-fold (Fig. 4B).



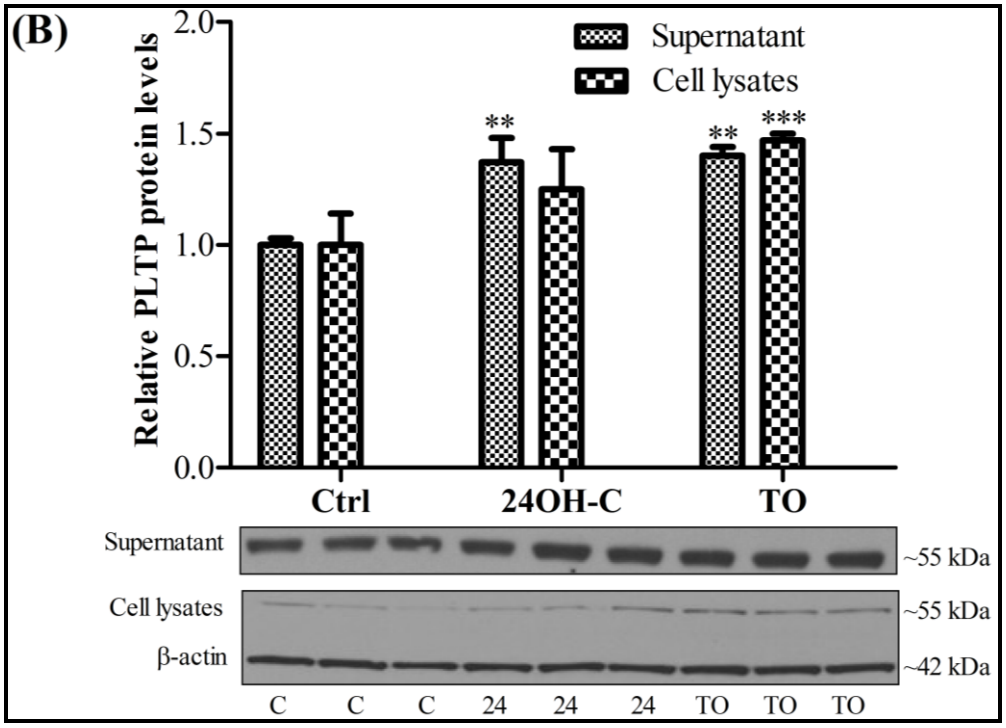


FIGURE 4: LXR activation up-regulates PLTP protein and mRNA expression. Capillary endothelial cells were isolated from porcine brains and cultured on 6-well plates. Confluent pBCEC were incubated in the presence or absence of synthetic (5 µM TO901317, TO) or natural (10 µM 24*(S)*-hydroxycholesterol, 24OH-C) LXR ligands for 24 h in serum-free medium. (A) Total RNA was isolated, reverse transcribed and real-time PCR was performed on a CFX 96 Real-time System (Bio-Rad) using SYBR Green technology. mRNA expression levels were normalized to HPRT1. Bars represent means ± SEM of 3 independent experiments, each performed in triplicates (** $p < 0.01$ *versus* controls). (B) Proteins were extracted from cells or TCA-precipitated from supernatants, separated by SDS-PAGE (4-12 %) and blotted onto PVDF membranes. Secreted and intracellular PLTP was immunodetected using rabbit polyclonal anti-PLTP antibody. Image shown is a representative immunoblot of three independent experiments. (C, control; 24, 24(S)-hydroxycholesterol; TO, TO901317). Band intensities were evaluated by densitometric scanning. Data shown are means ± SEM of three experiments performed in triplicates (** $p < 0.01$, *** $p < 0.001$ *versus* controls).

4.3. PLTP-mediated phospholipid transfer activity is augmented by LXR activation

We further investigated whether the PLTP secreted by pBCEC has ability to transfer phospholipid and thus performed phospholipid transfer assays based on the rate of transport of radiolabeled phosphatidyl choline (PC) from liposomes to human plasma HDL_3. Both, secreted and cellular PLTP efficiently transferred PC to HDL_3 (Fig. 5). Furthermore, LXR activation induced an increase in PLTP activity in both supernatants as well as in cell lysates, with somewhat less pronounced effects elicited by TO901317 (1.5 and 1.4-fold for supernatant and intracellular activity, respectively) as compared to the endogenous LXR ligand 24OH-C (2.0 and 1.6-fold, for supernatant and intracellular activity, respectively; Fig. 5).

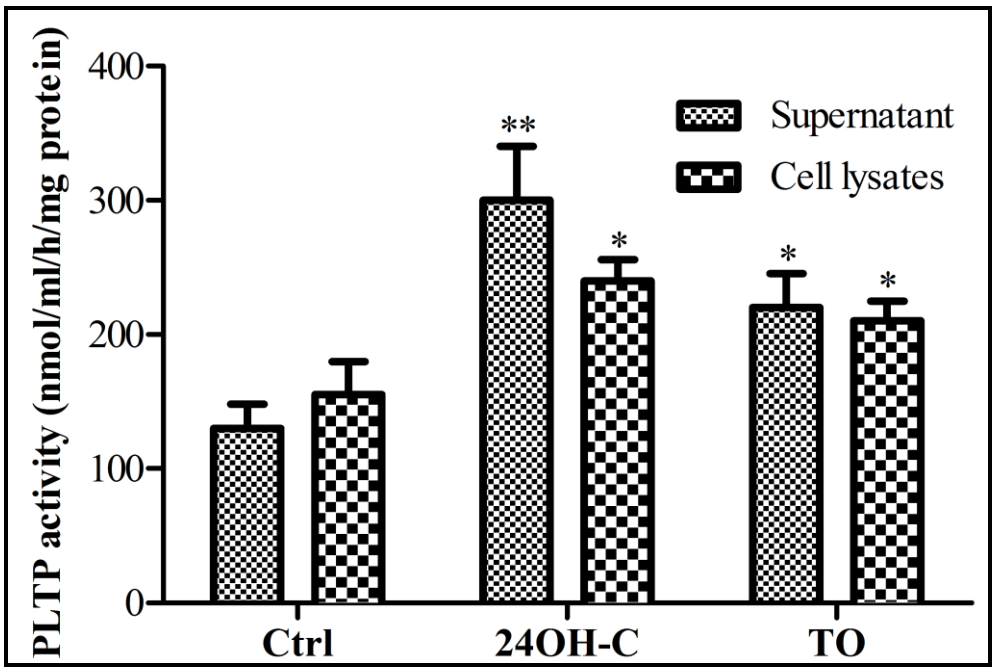


FIGURE 5: LXR activation enhances PLTP-mediated phospholipid transfer-activity. Capillary endothelial cells were isolated from porcine brains and cultured on 6-well plates. Confluent pBCEC were incubated in the presence or absence of synthetic (5 µM TO901317, TO) or natural (10 µM 24(S)-hydroxycholesterol, 24OH-C) LXR ligands for 24 h in serum-free medium. PLTP activity in the supernatants and cell lysates were determined based on the rate of transfer of [^{3}H]-phosphatidylcholine (PC) from liposomes to human HDL_3. Values were normalized to total cellular protein contents and are

expressed as means ± SD of one experiment representative of at least 4 independent experiments performed in triplicates (* $p< 0.05$, ** $p< 0.01$ *versus* controls).

4.4. Polarized pBCEC secrete PLTP preferentially to the brain side of the in vitro BBB model

To characterize polarized expression/release of PLTP and to investigate how LXR agonists affect the polarized secretion pattern/activity of PLTP, pBCEC cells were grown on transwell filter plates and tight junction formation was induced and assessed by measuring transendothelial electrical resistance. At 24 h significant amounts of PLTP were detected that was secreted to both, apical (mimicking the side facing the peripheral circulation) and basolateral (mimicking the side facing the brain parenchymal tissue) compartments (Fig. 6A). Notably, the amount of PLTP detected in the basolateral compartment was 1.8-fold higher as compared to the apical compartment (Fig. 6A). Furthermore, in line with results obtained with pBCEC monolayers, PLTP secretion was under control of LXR activation in both apical and basolateral compartments. PLTP secretion into the apical compartment was increased in response to 24OH-C (1.5-fold) and TO901317 (1.3-fold; Fig. 6A). PLTP protein levels secreted to the basolateral compartment within 24 h were 1.5-fold higher upon 24OH-C and 1.2-fold higher upon TO901317 treatment (as compared to basolateral levels detected under control conditions; Fig. 6A).

We next investigated the phospholipid transfer activity of PLTP secreted to apical and basolateral compartments. In line with higher PLTP levels immunodetected in the basolateral compartments, the PL transfer activity was also enhanced (1.7-fold) in the basolateral supernatants (Fig. 6B). Furthermore, LXR activation induced an increase in PLTP activity in both apical as well as in basolateral compartments. The endogenous LXR ligand, 24OH-C elicited more pronounced effects on phospholipid transfer activity detected in both, apical (1.6-fold relative control) and basolateral (1.7-fold relative to

control) compartments as compared to TO901317 (1.4-fold PLTP activity in apical, 1.2-fold in basolateral supernatants) (Fig. 6B).

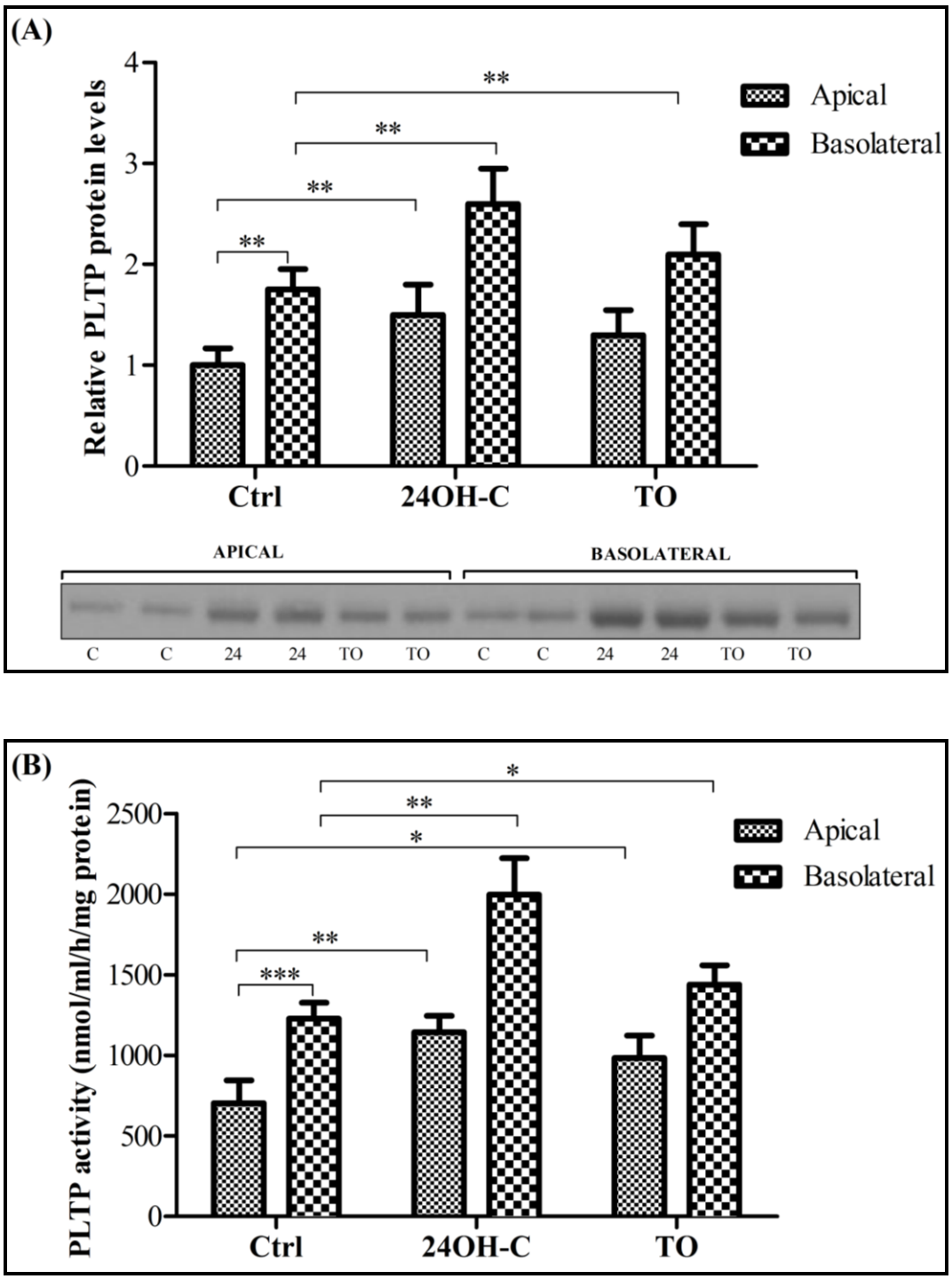


FIGURE 6: Polarized release of active PLTP from pBCEC into apical ('blood compartment') and basolateral ('brain parenchymal compartment') direction is enhanced by LXR activation. pBCEC

were cultured on 6-well transwell filter plates and incubated for 24 h in the presence or absence of synthetic (5 µM TO901317) or natural (10 µM 24OH-C) LXR ligands in serum-free medium. (A) Apical (1.5 ml/well) and basolateral (2.6 ml/well) media were collected and proteins were TCA-precipitated, aliquots corresponding to 1 ml of supernatant and normalized to total cellular protein, were separated by SDS-PAGE 4-12 %) and blotted onto PVDF membranes. Secreted PLTP (~55 kDa) was immunodetected using rabbit polyclonal anti-PLTP antibody. Image shown is a representative immunoblot of four independent experiments. (C, control; 24, 24(S)-hydroxycholesterol; TO, TO901317). Band intensities were evaluated by densitometric scanning. Data shown are means ± SD of one experiment representative of 4 independent experiments, each performed in triplicates (** $p< 0.01$ *versus* controls). (B) PLTP activity in the supernatants was determined based on the transfer rate of [^{3}H]-phosphatidylcholine (PC) from liposomes to human HDL_3. Values were normalized to total cellular protein contents and are expressed as PL transfer activity (per well) related to apical release under control conditions. Means ± SD of one experiment representative of at least 3 independent experiments performed in triplicate (* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ *versus* controls.

4.5. Simulated diabetic conditions reduce both PLTP activity and expression

Altered plasma PLTP activity levels have been reported in obesity and insulin resistance associated diabetes mellitus (209-211). To determine if diabetic conditions affect PLTP expression and activity in pBCEC, we mimicked diabetic conditions *in vitro*, i.e., pBCEC were treated in the presence of supraphysiological concentration of glucose (25 mM) or insulin (10 nM) or a combination of both for 24 h. PLTP activity was measured in cell supernatants, insulin decreased the activity of PLTP by 35% and glucose by 25% (Fig. 7). The combination of insulin and glucose treatment also reduced PLTP activity by 20% (Fig. 7).

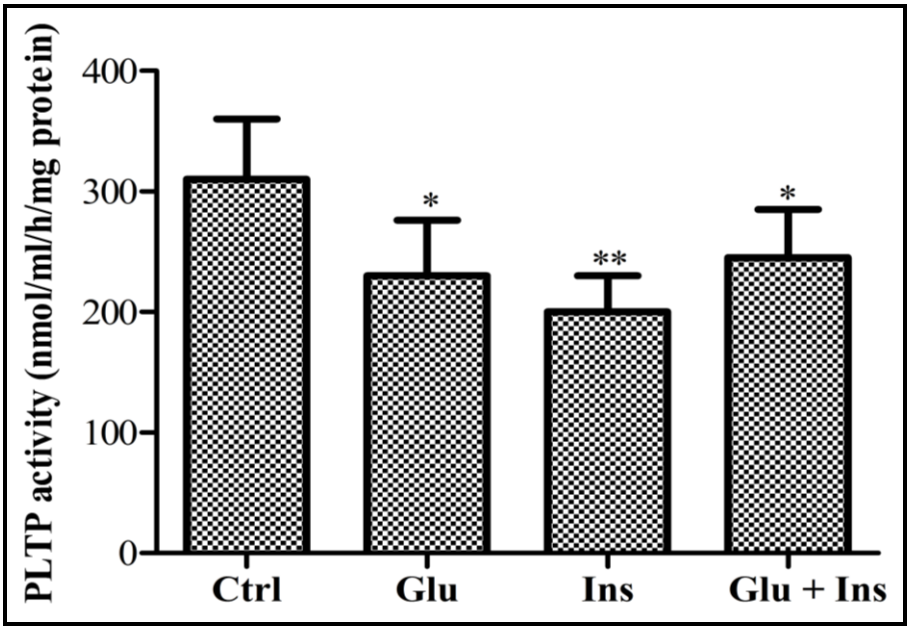


FIGURE 7: Simulated diabetic conditions reduce PLTP activity in supernatants of pBCEC. Cells were cultured on 6-well plates and incubated in absence (Ctrl) or presence of glucose (Glu; 25 mM) or insulin (Ins; 10 nM) or their combination (Glu; 25 mM + Ins; 10 nM) for 24 h. PLTP activity of media was determined based on the transfer of [^{3}H]-phosphatidylcholine from liposomes to human HDL_3. Values were normalized to total cellular protein contents and are expressed as means ± SD of one experiment representative of at least 4 independent experiments performed in triplicates (* $p< 0.05$, ** $p< 0.01$ *versus* controls).

In addition, the decrease in PLTP activity observed was associated with a decrease in PLTP mRNA levels. I.e., real-time PCR showed a 40% decrease in PLTP mRNA with insulin treatment and a 30% decrease with high glucose (Fig.8). Interestingly, the combination of high glucose and insulin resulted in only a 10% decrease in PLTP mRNA levels (Fig. 8).

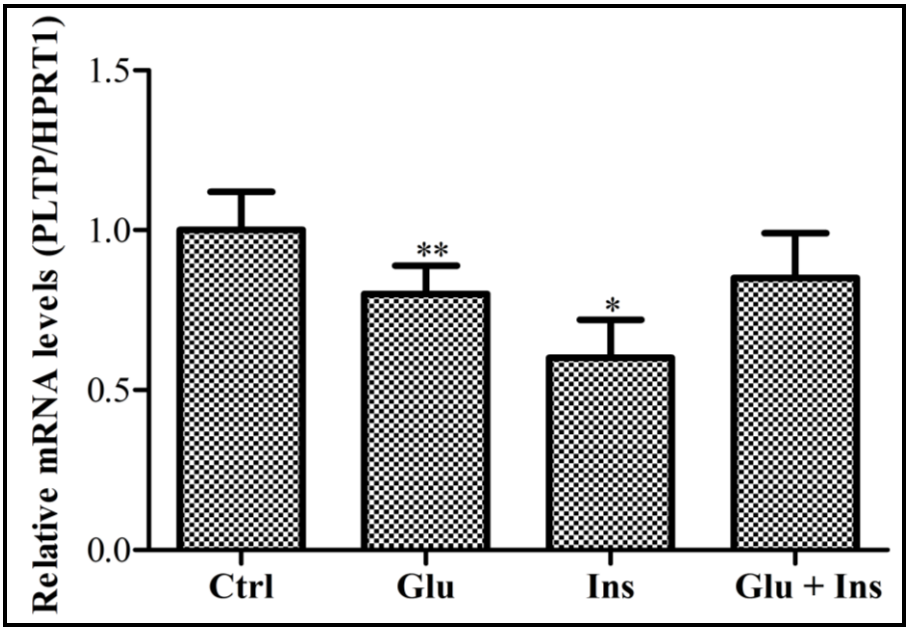


FIGURE 8: PLTP mRNA expression is reduced under simulated diabetic conditions in pBCEC. Cells were cultured on 6-well plates and incubated in absence (Ctrl) or presence of glucose (Glu; 25 mM) or insulin (Ins; 10 nM) or their combination (Glu; 25 mM + Ins; 10 nM) for 24 h. Total RNA was isolated, reversed transcribed and real-time PCR was performed on a CFX 96 real-time System (Biorad) using SYBR Green technology. Relative mRNA expression levels normalized to HPRT1 were determined. Bars shown are means ± SEM of three independent experiments performed in triplicates (* $p< 0.05$, ** $p< 0.01$ *versus* controls).

However, PLTP protein levels in the cells and pBCEC supernatants did not correlate with the PLTP activity and expression levels. Thus, immunoblotting showed an increase in both cellular (1.5-fold) and secreted (2.0-fold) PLTP after the addition of high glucose and also with the combination of glucose and insulin (1.9 and 2.2 fold respectively; Fig. 9). There was also a non-significant trend towards increased PLTP levels with high insulin (Fig. 9).

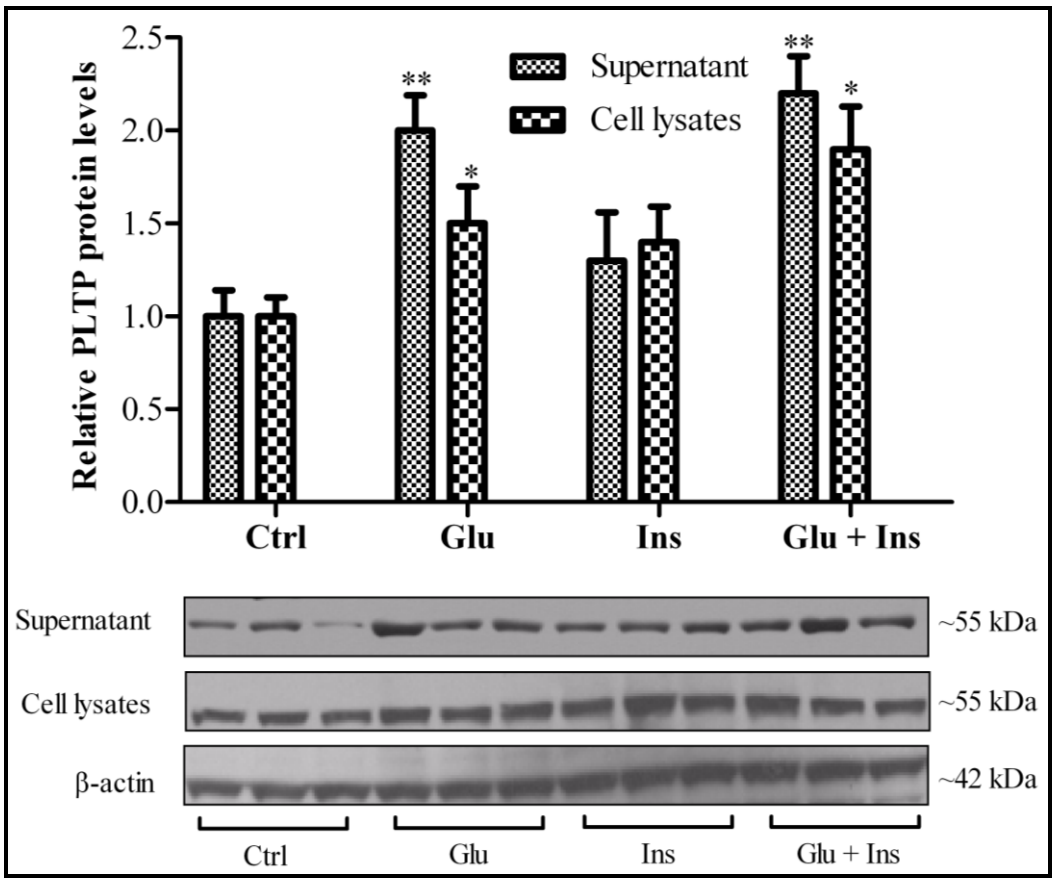
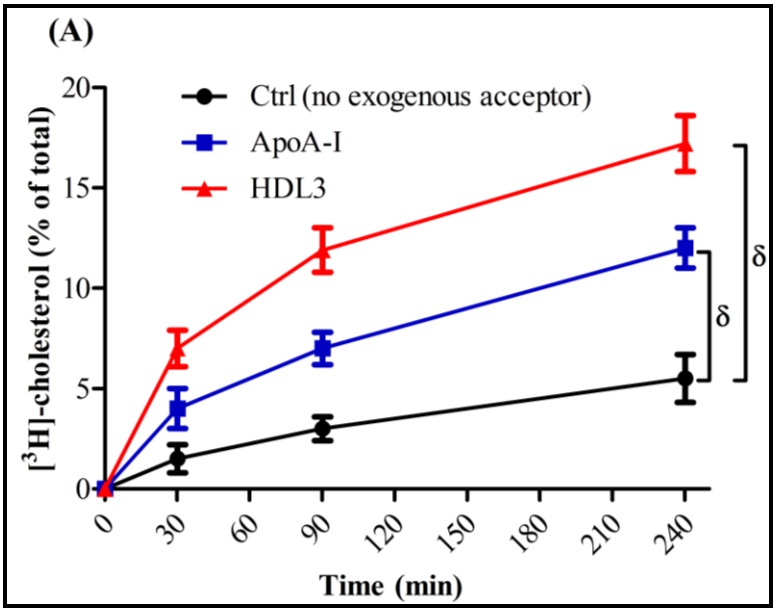


FIGURE 9: Effect of high glucose and insulin on PLTP protein levels. pBCEC were cultured on 6-well plates and incubated in absence (Ctrl) or presence of glucose (Glu; 25 mM) or insulin (Ins; 10 nM) or their combination (Glu; 25 mM + Ins; 10 nM) for 24 h. Proteins were extracted from cells or TCA-precipitated from supernatants, separated by SDS-PAGE (4-12 %) and blotted onto PVDF membranes. Secreted and intracellular PLTP were immunodetected using rabbit polyclonal anti-PLTP antibody. Image shown is a representative immunoblot of three independent experiments. Band intensities were evaluated by densitometric scanning. Data shown are means ± SEM of three experiments performed in triplicates (* $p< 0.05$, ** $p< 0.01$ *versus* controls).

4.6. Efficient cholesterol release from pBCEC to plasma HDL_3 and lipid-free apoA-I

Mammalian cells can remove excess intracellular cholesterol to maintain the sterol homeostasis and, apoA-I and HDL_3 are ideal cholesterol acceptors in the cells (21, 236). In order to evaluate the cholesterol efflux in pBCEC, cells were radiolabeled with cholesterol and [^{3}H]-cholesterol release to exogenous plasma HDL_3 or lipid-free apoA-I was measured (Fig. 10). About 12% of total [^{3}H]-cholesterol release from pBCEC is mediated by HDL_3, while apoA-1 accounts for 6.5% of total [^{3}H]-cholesterol efflux (Fig. 10B). It may be important to the note that the basal cholesterol efflux (*i.e.* in the absence of exogenous apoA-I or HDL_3) from pBCEC was 5% (Fig. 10A). Since pBCEC also secrete apoA-I, it is reasonable to assume that endogenous apoA-I and other unknown acceptors are responsible for the cholesterol efflux under basal conditions (40).

HDL_3-mediated cholesterol efflux was 4.7- to 3.1-fold (at 30 and 240 min, respectively) and apoA-I-mediated cholesterol efflux was 2.7- to 2.2-fold (at 30 and 240 min, respectively) higher than the basal (Ctrl) cholesterol efflux (Fig 10 A). Furthermore, compared to apoA-I, the HDL_3-mediated cholesterol release was increased from 1.8- (at 240 min) to 2.2- fold (at 30 min) in pBCEC (Fig. 10 B).



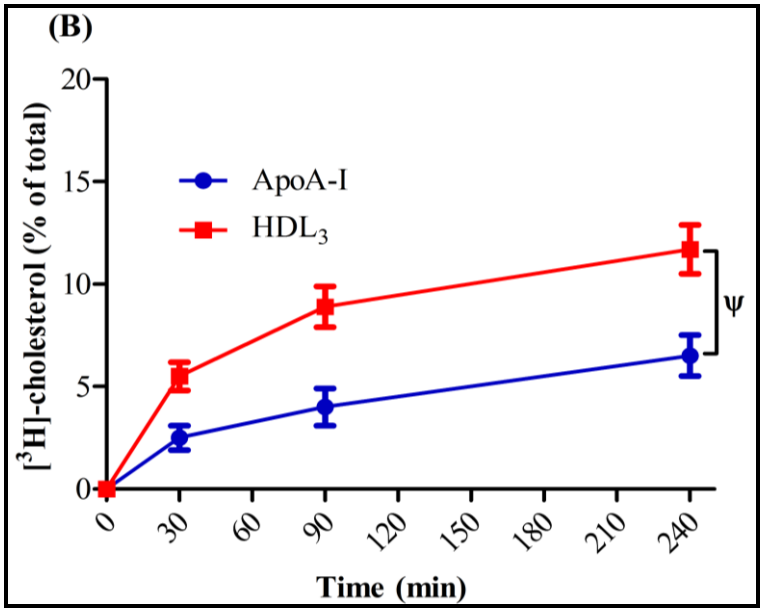


FIGURE 10: Time-dependent release of cellular cholesterol from pBCEC to plasma HDL_3 and plasma lipid-free apoA-I. pBCEC were cultured on 12-well multi-plates labeled with [^{3}H]-cholesterol (0.5 µCi/ml) for 24 h. Cellular cholesterol pools were equilibrated for 2 h. Aliquots of medium were collected and time-dependent cellular [^{3}H]-cholesterol release in the absence (Ctrl, no exogenous acceptors) or presence of HDL_3 (50 µg/ml) or apoA-I (10 µg/ml) was determined at the indicated time points. Radioactivity remained in the cell lysate was also measured. Cholesterol efflux was calculated as the percentage of cpm /mg cell protein in the supernatants relative to the total counts in the supernatants plus cell lysates. A, percentage total [^{3}H]-cholesterol efflux with basal cholesterol efflux (no exogenous acceptor). B, percentage total [^{3}H]-cholesterol efflux after subtracting the basal cholesterol efflux. Data shown are means ± SE of 3 independent experiments performed in triplicates. δ, $p< 0.001$ Ctrl (basal cholesterol efflux) *versus* HDL_3 or apoA-I-mediated cholesterol efflux. ψ, $p< 0.001$ apoA-I *versus* HDL_3-mediated cholesterol efflux (analysis of variance).

4.7. Exogenous PLTP remodels human plasma HDL_3

PLTP remodels HDL_3, generating larger HDL particles with concomitant production of small apoA-I containing preβ-HDL particles (184, 185). We preincubated human plasma HDL_3 with purified, active plasma PLTP (250 nmol/h) and checked the HDL-particles formation by NDGGE. In accordance with previous reports (184, 185), particle size determination by NDGGE (Fig. 11) confirmed that HDL_3 incubation with PLTP resulted in the formation of two major distinct particle populations, one with larger (11 ± 0.5 nm) and the other with small mean diameter (7.4 ± 0.5 nm).

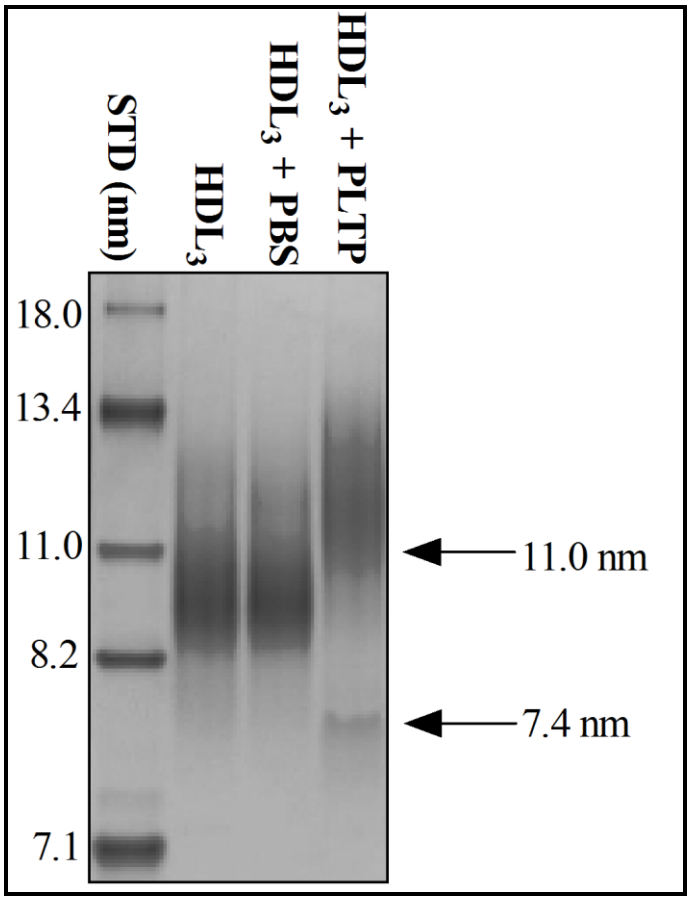
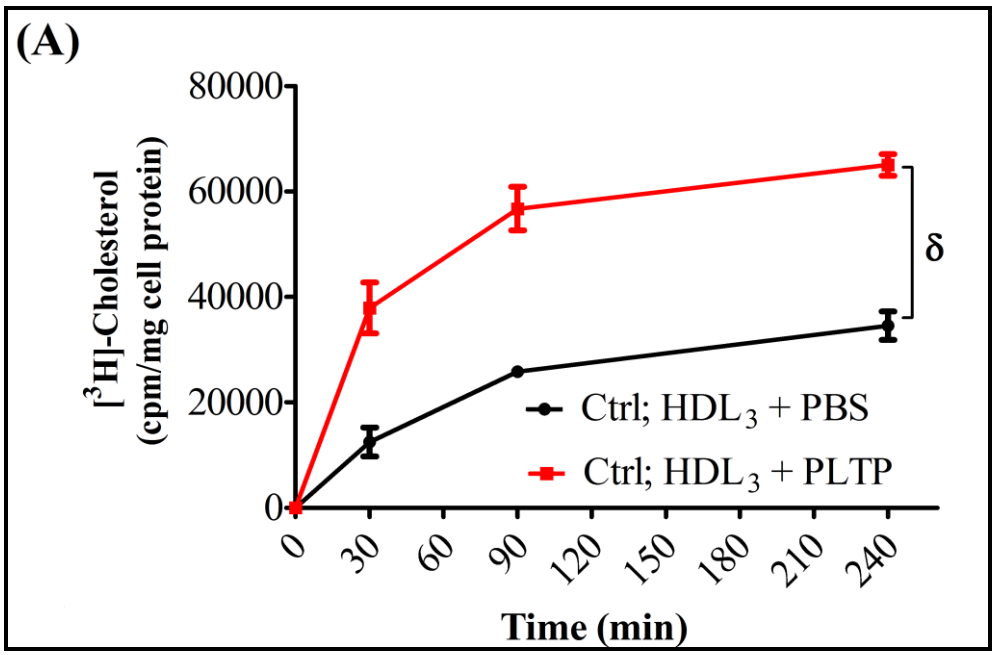


FIGURE 11: Incubation of human plasma HDL_3 with purified PLTP changes HDL particle size. HDL_3 (250 µg) was preincubated in the absence (PBS) or presence of plasma PLTP (250 nmol/h) at 37^0 C for 24 h. Subsequently, 10 µg of HDL protein was used for NDGGE analysis and stained with Coomassie Brillant Blue G250. PLTP-mediated particle remodeling is indicated by the arrows. Gel shown is representative of three independent experiments (STD, protein standards of known diameter).

4.8. PLTP-modified HDL_3 elicits enhanced cholesterol efflux from pBCEC

To investigate whether PLTP may alter the capacity of HDL particles to remove cellular cholesterol from pBCEC, we preincubated human plasma HDL_3 with purified, active plasma PLTP (250 nmol/h) and used these HDL particles as acceptors. NDGGE confirmed that HDL_3 was modified by PLTP (Fig. 11). Strikingly, PLTP-modification of HDL_3 enhanced time-dependent cholesterol release from pBCEC, as compared to unmodified HDL_3 particles, under basal (control) conditions (from 1.9- to 3.0-fold at 240 and 30 min, respectively; Fig. 12A) as well as in the presence of 24OH-C (from 1.5- to 1.8-fold at 240 and 30 min, respectively; Fig. 12B) or TO901317 (from 1.3- to 1.5-fold at 240 and 30 min, respectively; Fig. 11C). Cholesterol efflux to modified HDL_3 particles was enhanced by 1.2- and 1.5-fold (at 30 min), respectively, upon 24OHC and TO901317 treatment relative to control conditions, indicating additive effects of PLTP-treatment and LXR activation (Figs. 12 A-C).



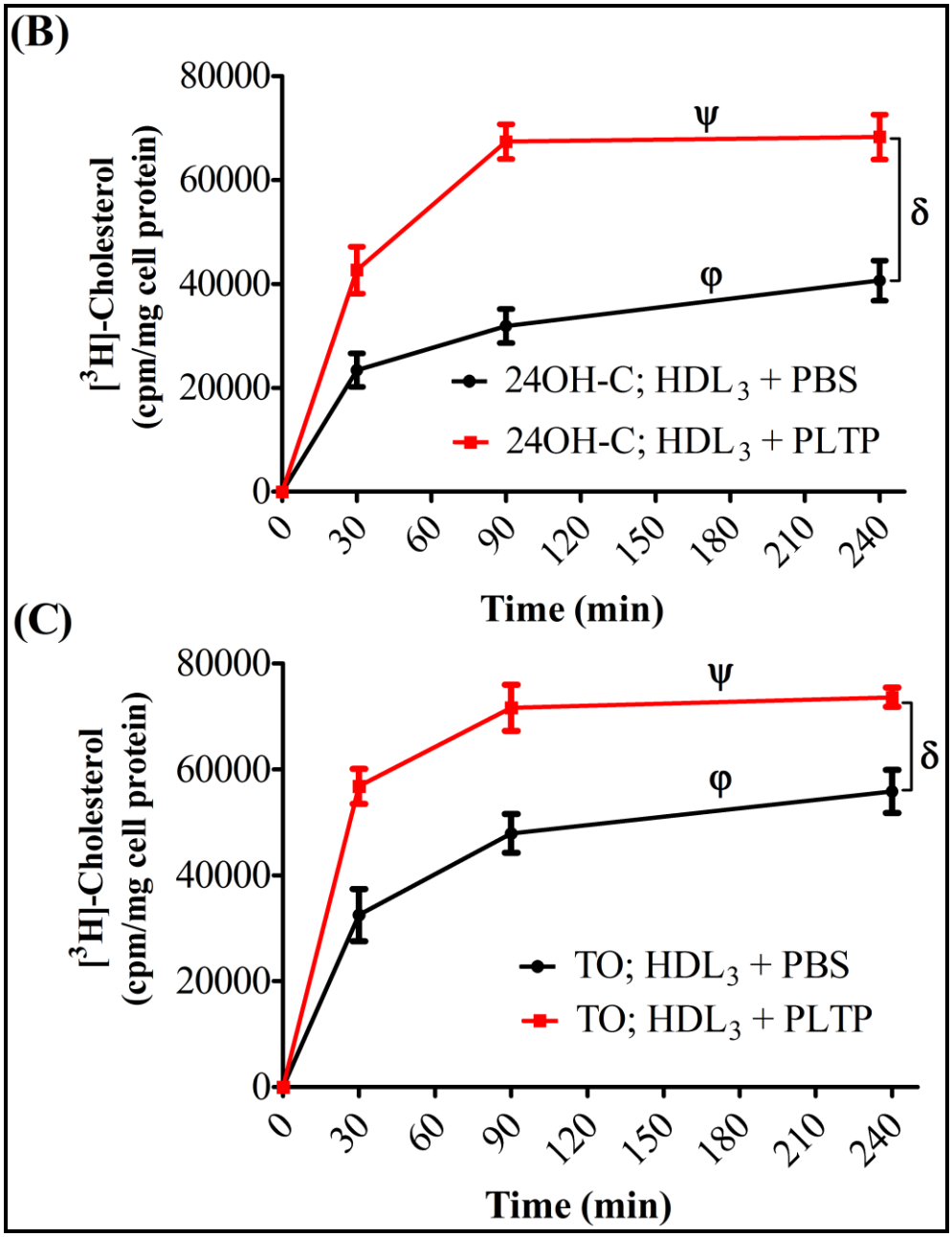


FIGURE 12: PLTP-mediated remodeling increases the cholesterol removal capacity of HDL_3. pBCEC were cultured on 12-well multi-plates labeled with [^{3}H]-cholesterol (0.5 µCi/ml) for 24 h in the absence (A) or presence of 24OH-C (10 µM; B) or TO901317 (5 µM; C). HDL_3 (250 µg) was preincubated in the absence (PBS) or presence of plasma PLTP (250 nmol/h) at 37^0 C for 24 h. Cellular cholesterol pools were equilibrated for 16 h. Aliquots of medium were collected and time-dependent cellular [^{3}H]-cholesterol efflux to untreated (+ PBS) or PLTP-modified (+PLTP) HDL_3 (50 µg/ml) was determined at the indicated time points. Radioactivity remained in the cell lysate was also measured. Data shown are means ± SE of 3 independent experiments performed in triplicates. δ, $p< 0.0001$ HDL_3+ PLTP *versus* HDL_3 + PBS; ψ, $p< 0.0001$ TO/24OH-C *versus* basal conditions in the presence of HDL_3 + PLTP; φ, $p< 0.0001$ TO/24OH-C *versus* basal conditions in the presence of HDL_3 + PBS (analysis of variance).

4.9. PLTP-modified HDL_3 enhances cholesterol release to both apical and basolateral compartments from polarized pBCEC

In order to further elucidate the effect of PLTP-mediated HDL_3 modification on cholesterol mobilization in polarized pBCEC cultured on transwell filters, we preincubated human plasma HDL_3 with purified, active plasma PLTP (250 nmol/h) and added these HDL particles to either apical or basolateral compartment as acceptors. NDGGE confirmed that HDL_3 was modified by PLTP (Fig. 11).

Interestingly, as compared to unmodified particles, PLTP-modified HDL_3 removed cholesterol from polarized pBCEC more efficiently to the basolateral compartment of transwell chambers. Thus, cholesterol removal under basal (control) conditions was increased from 2.0- to 1.6-fold at 30 and 240 min, respectively (Fig. 13A). Noticeably, under basal conditions PLTP-modification of HDL_3 did not have any significant effect on cellular cholesterol removal to the apical compartment (Fig. 13B).

However, in the presence of LXR agonist TO901317, PLTP modification of HDL_3 enhanced time-dependent cholesterol release from polarized pBCEC to both the basolateral (by 1.3- and 1.8-fold at 30 and 240 min, respectively; Fig. 13D) as well as to the apical (by 1.7- and 2.0-fold at 30 and 240 min, respectively; Fig. 13C) compartments.

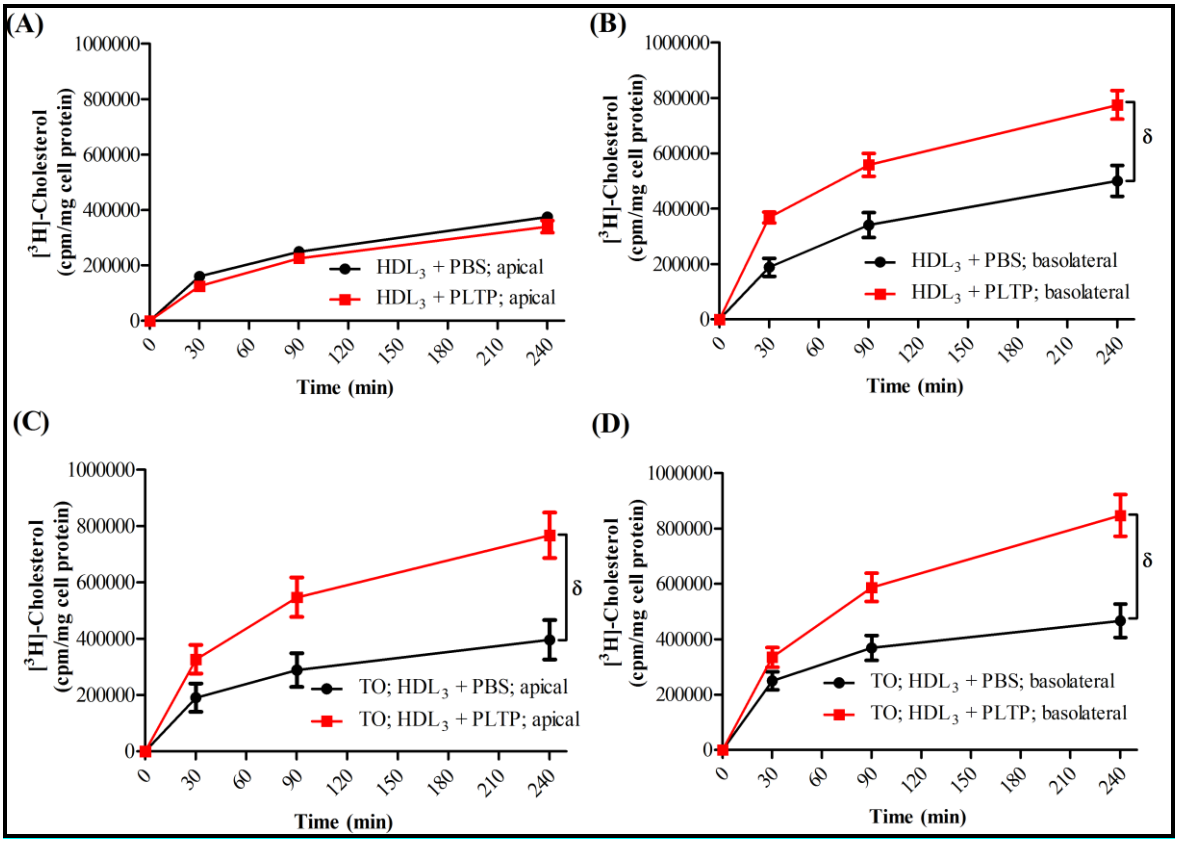


FIGURE 13: Under basal condition, PLTP-mediated remodeling increases the cholesterol efflux capacity of HDL_3 only to the basolateral compartment from polarized pBCEC, while to both compartments upon LXR activation. pBCEC were cultured on 12-well transwell filter plates, labeled with [^{3}H]-cholesterol (1.0 µCi/ml) for 24 h in the absence (A and B) or presence of TO901317 (5 µM; C and D). HDL_3 (250 µg) was preincubated in the absence (PBS) or presence of plasma PLTP (250 nmol/h) at 37^{0}C for 24 h. Cellular cholesterol pools were equilibrated for 16 h and time-dependent cellular [^{3}H]-cholesterol efflux to untreated (+ PBS) or PLTP-modified (+PLTP) HDL_3 (50 µg/ml) was determined at the indicated time points. Aliquots of medium from apical (A and C) and basolateral (B and D) compartments were collected and time-dependent cellular [^{3}H]-cholesterol efflux to untreated (+ PBS) or PLTP-modified (+PLTP) HDL_3 (50 µg/ml) was determined at the indicated time points. Data shown are means ± SE of 3 independent experiments performed in triplicates. δ $p < 0.0001$ HDL_3+ PLTP *versus* HDL_3 + PBS (analysis of variance).

4.10. pBCEC induce remodeling of HDL_3 and pre-β-HDL formation is augmented upon LXR activation

Pre-β-HDL has been suggested to function among the most efficient HDL acceptors for cellular cholesterol (236). Previous studies have indicated that PLTP enhances the formation of pre-β-HDL particles from reconstituted HDL (187). To investigate whether PLTP secreted by pBCEC is capable of executing similar functions, we incubated the cells with human HDL_3, and pre-β-HDL particles were quantified by 2D crossed immunoelectrophoresis after 24 h. We have immuno-detected substantial amounts of pre-β-HDL in pBCEC-derived supernatants (Fig. 14). Moreover, the formation of pre-β-HDL particles was markedly increased (1.9-fold) in TO901317 treated pBCEC as compared to the untreated cells (Fig. 14 A, B and E) which is in line with (2.2-fold) increased PLTP activity detected in the supernatants (Fig. 14F). We were unable to immuno-detect any pre-β-HDL particles in cell-free control incubations of HDL_3 (Fig. 14 C and D).

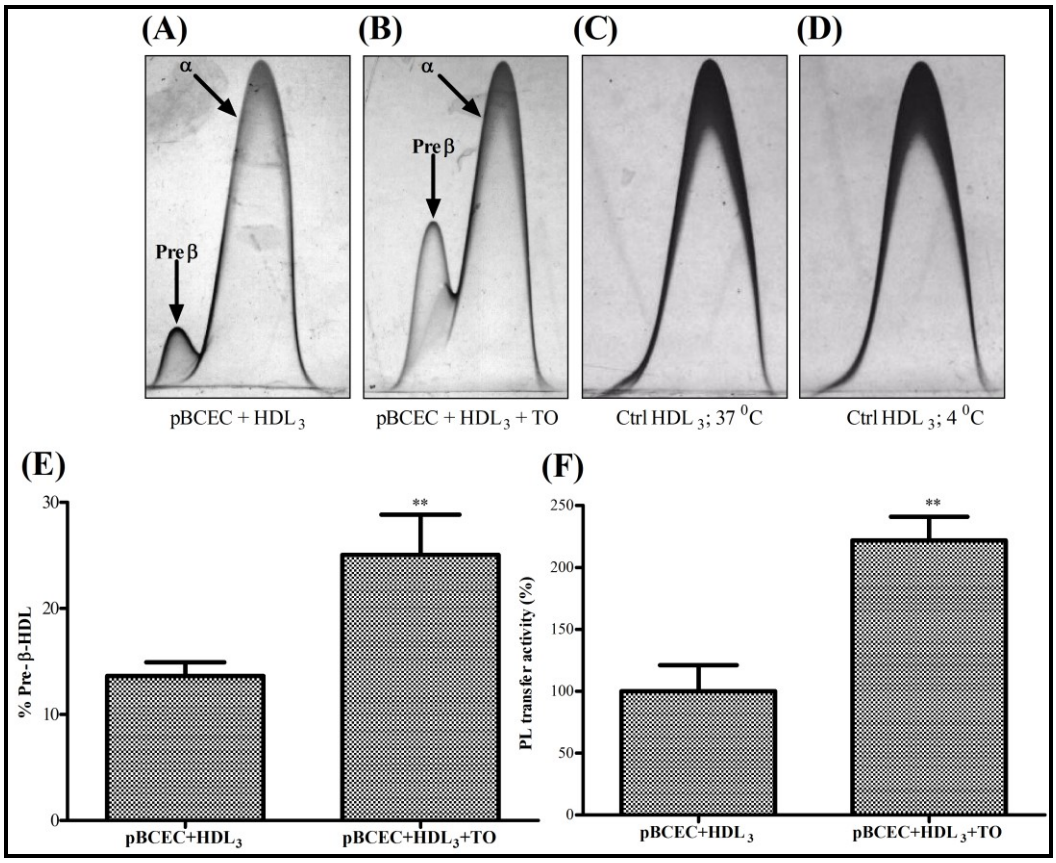


FIGURE 14: Pre-β-HDL formation is amplified upon treatment of pBCEC with LXR agonists. pBCEC were cultured on 75 cm^2 flasks and incubated in the absence or presence of TO901317 (TO) (5 μM) and/or HDL_3 (50 μg/ml) for 24 h in serum-free medium. Supernatants were collected and concentrated (45-fold) using Amicon Ultra-10K centrifugal filters. Samples (5 μL) were analyzed by two-dimensional crossed immunoelectrophoresis with anti-human apoA-I antibody. A-D, representative patterns of α-HDL and/or pre-β-HDL generated under different conditions are shown. E, relative area of pre-β-HDL and α-HDL peaks was calculated by multiplication of peak height and peak width at half height. The pre-β-HDL amount is expressed as percentage of the sum of α-HDL and pre-β- HDL peak areas. F, PLTP activity in supernatants was determined as described under "methods". Means ± SE of three independent experiments performed in triplicates (** $p< 0.01$ *versus* pBCEC+HDL_3).

4.11. Nascent HDL formation is amplified upon LXR activation in pBCEC

To evaluate the formation of apoA-I containing nascent particles in pBCEC, we examined supernatants of primary pBCEC cultured in serum-free medium for 24 h. We immuno-detected apoA-I-containing nascent particles in pBCEC supernatants, and as expected, the amount of endogenous HDL particles formed was increased by LXR activation (Fig. 15). Because of the heterogeneity in size of apoA-I containing particles, no distinct bands were visible. However, the size of the major fraction of nascent apoA-I containing particles produced by pBCEC varied between 12-19 nm (Fig. 15).

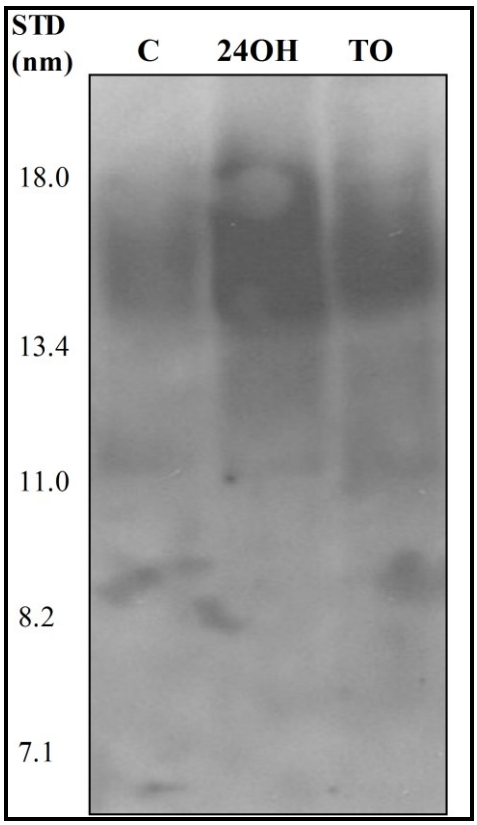


FIGURE 15: LXR activation stimulates nascent HDL formation in pBCEC. Primary pBCEC were cultured on 75 cm^2 flasks and incubated in the absence or presence of TO901317 (TO; 5 μM) or 24OH-C (24OH; 10 μM) for 24 h in serum-free medium. Supernatants were collected and concentrated using Amicon Ultra-10K centrifugal filters. The presence of HDL-sized particles was assessed by NDGGE followed by immunoblotting using anti-human apoA-I antibody. Image shown is representative of three independent experiments. Ctrl, control. (STD, protein standards of known diameter).

4.12. PLTP contributes to both apoA-I and HDL_3- mediated cholesterol removal from pBCEC

To get further insights into the role of endogenous PLTP in apoA-I- and HDL_3-mediated cholesterol removal from pBCEC, we silenced the gene using RNA interference (RNAi) technology (Fig. 16). Real time PCR and immunoblotting analyses confirmed that transfection with siRNA (25 nM) targeting human PLTP sequence, resulted in 75% (Fig. 16A) and 60% (Fig. 16B) reduction in PLTP mRNA and protein levels, respectively.

Furthermore, PLTP activity assays confirmed a significant reduction (73%) in PL transfer activity in PLTP-silenced cells (Fig. 16C). PLTP silencing had no effect on mRNA expression levels of ABCA1 (Fig. 16D).

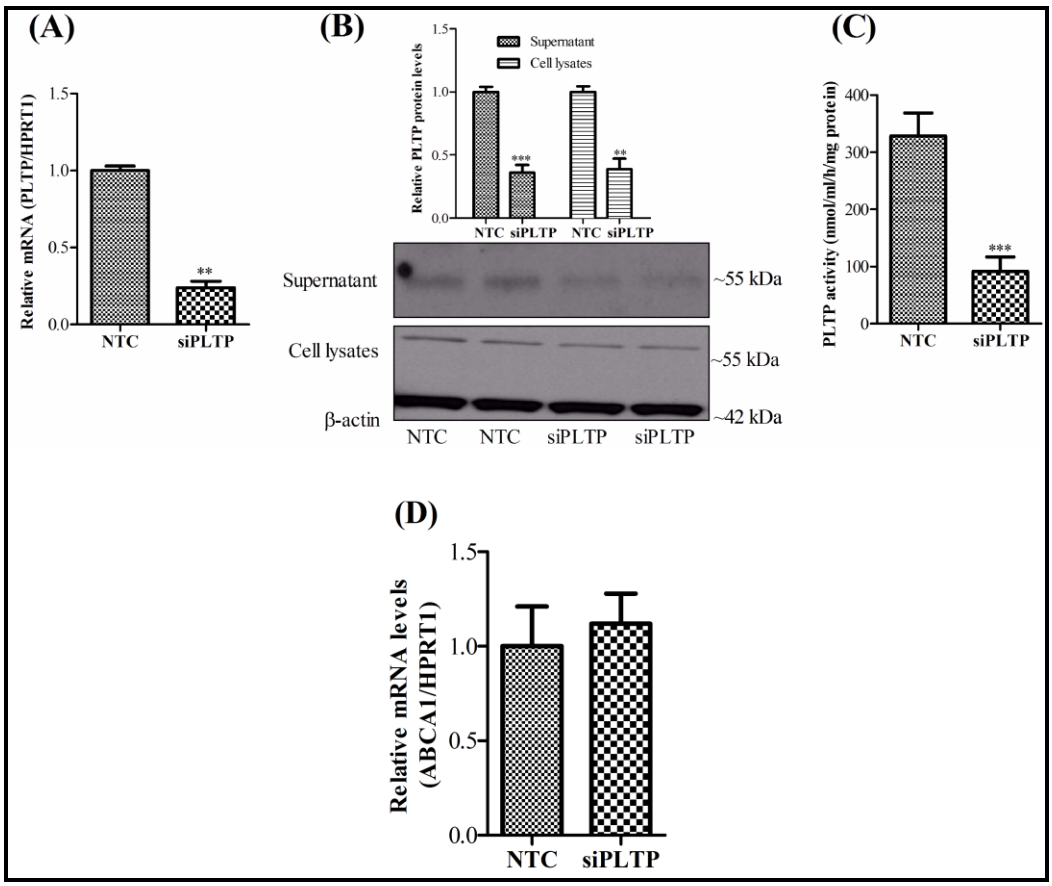


FIGURE 16: Targeted silencing of PLTP is achieved in pBCEC using RNA interference technology. pBCEC were cultured on 12-well plates and transfected with hPLTP siRNA (25 nM) for 40 h. Non targeting control (NTC) siRNA was used as the negative control. A and D, RNA was isolated, reverse transcribed, and real-time PCR was performed using SYBR Green technology. PLTP (A) and ABCA1 (D) mRNA expression levels were normalized to HPRT1 (means ± SE of 4 experiments performed in triplicate; ** $p< 0.01$ *versus* NTC). B, proteins were TCA-precipitated from supernatants; cells were lysed, and immunoblotting was performed using rabbit polyclonal anti-PLTP and β-actin antibodies as described under "methods". Image shown is a representative immunoblot. Band intensities were evaluated by densitometric scanning. Data shown are means ± SE of three experiments performed in duplicates (**, $p < 0.01$; *** $p< 0.001$ *versus* NTC. C, phospholipid transfer activity in the supernatants was determined after *PLTP* silencing as described under "methods". (Means ± SD of one experiment are representative of at least three independent experiments performed in triplicates. ***, $p< 0.001$ *versus* NTC).

Silencing of PLTP resulted in an up to 67% (at 24 h) reduction in time-dependent cholesterol release to apoA-I (Fig. 17A). We also evaluated HDL_3 -mediated cholesterol release in PLTP-silenced cells and observed a reduction by up to 32% in cholesterol removal capacity from PLTP-silenced as compared to the non-silenced cells (Fig. 17B), whereby the PLTP silencing effects became more evident at the later time points (4 and 24 h) investigated (Fig. 17B).

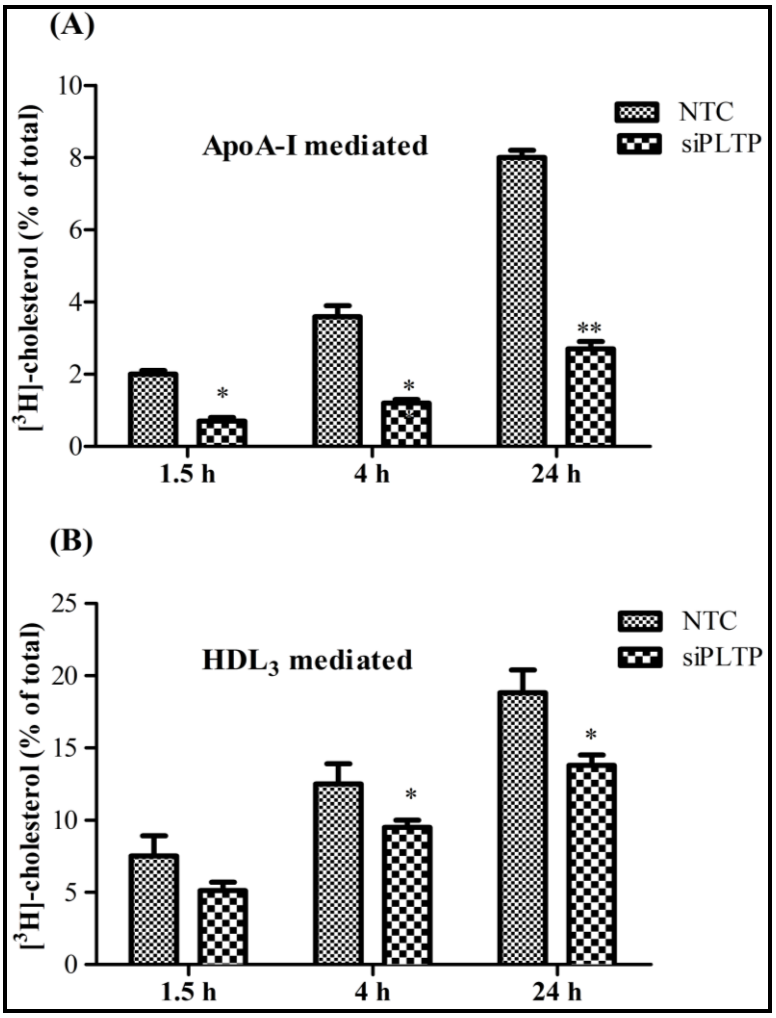


FIGURE 17: Both apoA-I and HDL_3 mediated cholesterol removal from pBCEC are diminished upon PLTP silencing. pBCEC were cultured on 12-well plates. Cells were labeled with [^{3}H]-cholesterol (0.5 µCi/ml) and simultaneously transfected with hPLTP siRNA (25 nM) for 40 h. Cellular cholesterol pools were equilibrated for 2 h and time-dependent cellular [^{3}H]-cholesterol release to apoA-I (A; 10 µg/ml) or HDL_3 (B; 50 µg/ml) was measured. (Means ± SD of one experiment representative of at least four independent experiments performed in triplicates, *, $p< 0.05$, **, $p< 0.01$ *versus* NTC).

4.13. PLTP modulates amyloid beta (Aβ) oligomerisation in pBCEC

To investigate whether PLTP might have a direct or indirect role in Aβ metabolism, we treated pBCEC with HDL_3, PLTP-modified HDL particles or with increasing amounts of active plasma PLTP. Analyses of intracellular Aβ oligomers by immunostaining revealed a reduction of Aβ octamers by 63% with HDL_3, 30% with PLTP-modified HDL particles, 47% with 50 nmol/ml active PLTP and 56% with 1000 nmol/ml active PLTP (Fig. 18A). Albeit not significant, we also observed a trend towards decreased Aβ tetramers levels with the above mentioned treatment (Fig. 18B), while we were unable to detect any changes in intracellular amyloid precursor protein (APP) protein levels (Fig. 18C).

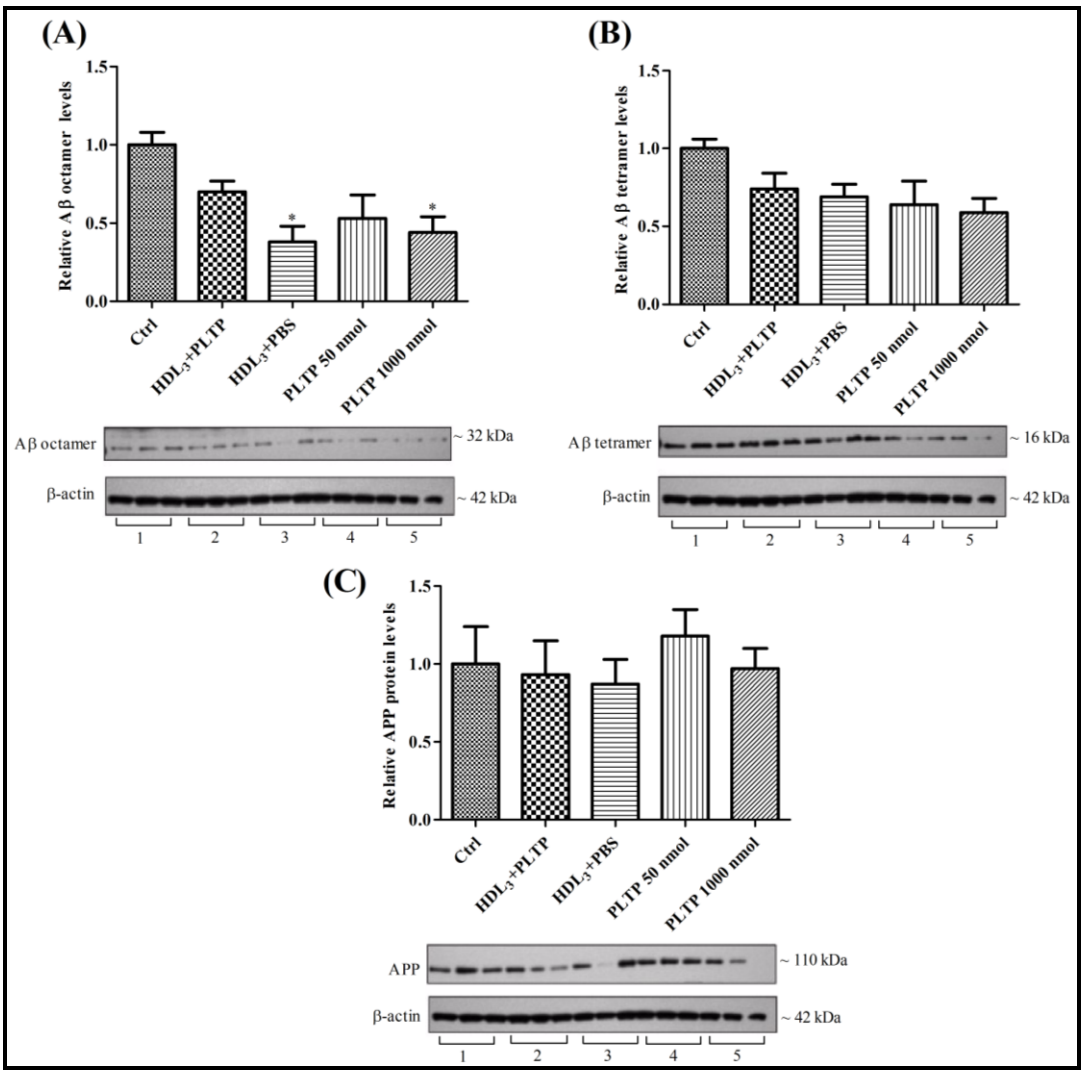


FIGURE 18: Decreased Aβ oligomerisation in pBCEC treated with HDL_3 and active PLTP. A-C, confluent pBCEC were incubated for 24h in serum-free medium containing HDL_3, PLTP-modified HDL particles, increasing amounts of active plasma PLTP, or vehicle only (Ctrl). Intracellular proteins were extracted from the cells, separated by SDS-PAGE (4-12%) and blotted onto PVDF membranes. Intracellular Aβ oligomers (A and B) and APP (C) were immunodetected using appropriate rabbit polyclonal antibodies. Image shown is a representative immunoblot of three independent experiments. Band intensities were evaluated by densitometric scanning. Data shown are means ± SEM of three experiments performed in triplicate (*, $p< 0.05$ *versus* controls; 1, 2, 3, 4 and 5 represents control (Ctrl), HDL_3 + PLTP, HDL_3 + PBS, 50 nmol/ml PLTP and 1000 nmol/ml PLTP respectively).

In parallel, we further analysed the mRNA levels of β-secretase (β-site APP cleavage enzyme 1, BACE1) and APP in PLTP-silenced pBCEC. Interestingly, there was a significant increase (1.8-fold) in β-secretase mRNA levels in PLTP-silenced cells compared to the non-silenced cells (Fig. 19A), while the APP mRNA levels remains unchanged (Fig. 19B).

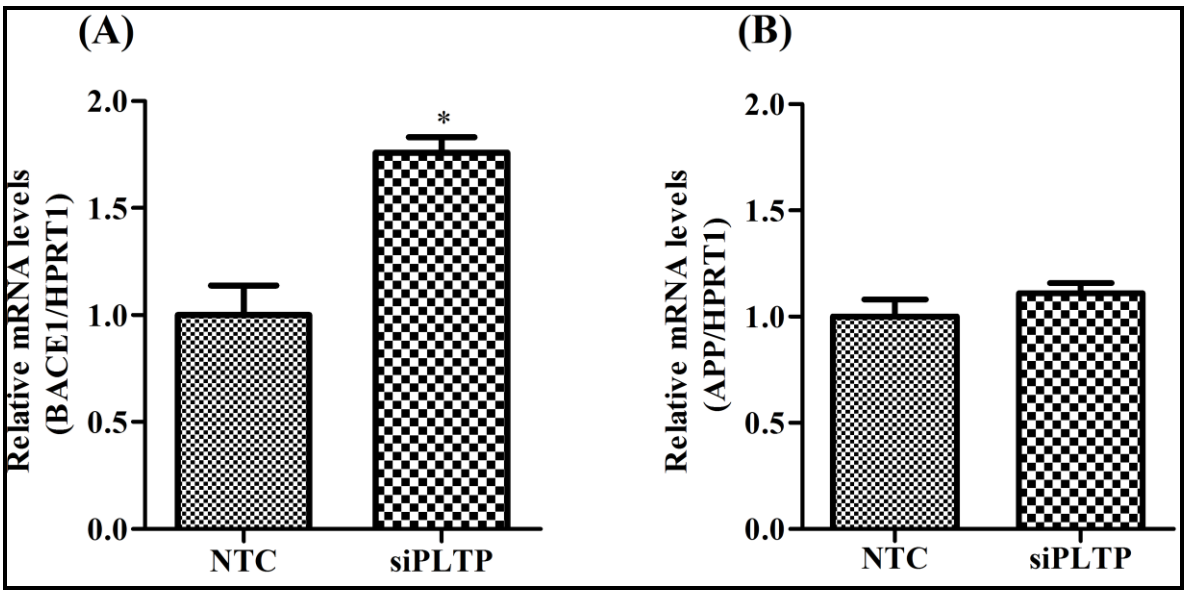


FIGURE 19: Increased expression of BACE1 mRNA in PLTP-silenced cells. pBCEC were transfected with hPLTP siRNA (25 nM) for 40 h and non-targeting control (NTC) siRNA was used as the negative control. RNA was isolated, reverse transcribed, and real-time PCR was performed using SYBR Green technology. BACE1 (A) and APP (B) mRNA expression levels were normalized to HPRT1 (Means ± SE of at least three experiments performed in triplicates, *, $p< 0.05$ *versus* NTC).

4.14. LXR activation up-regulates PLTP activity in murine BCEC in vivo

It has been previously reported that PLTP expression in mouse liver is up-regulated by LXR activation *in vivo* (170, 171). To explore whether PLTP activity in BCEC could be regulated by LXR activation *in vivo*, we administered C57BL6 mice with synthetic LXR agonist TO901317 for 7 days and assayed the PLTP activity in plasma, mBCEC, whole brain, and liver. Plasma lipid analysis revealed a significant increase in total cholesterol (1.4-fold) and phospholipids (1.9-fold) content in TO901317 treated animals as compared to vehicle control (Table. 3).

Table 3: Plasma lipid profile of C57/BL6 mice treated with LXR agonist TO901317 or vehicle.
Data are expressed as mean ± SD; n = 7 per group

Lipid profile	Vehicle	TO901317 (50 mg/kg)
Triglycerides (mg/dl)	86.8 ± 24	77.3 ± 19.1
Total cholesterol (mg/dl)	46.2 ± 14	64.3 ± 7.9 *
Free cholesterol (mg/dl)	23.4 ± 5.3	28.7 ± 5.9
Phospholipids (mmol/L)	1.6 ± 0.5	3.0 ± 0.9 **

* $p< 0.05$, ** $p< 0.01$, vs vehicle

Plasma PL-transfer activity in TO901317 fed mice was increased to 1.7-fold compared to vehicle control (Fig. 20A). Whole brain (1.3-fold) and liver (1.9-fold) PLTP activity was also increased in TO901317-fed mice (Fig. 20B). More importantly, isolated mBCEC were highly enriched in PL transfer activity and displayed a 2.5-fold increase in PLTP activity in TO901317-treated animals compared to vehicle control (Fig. 20B).

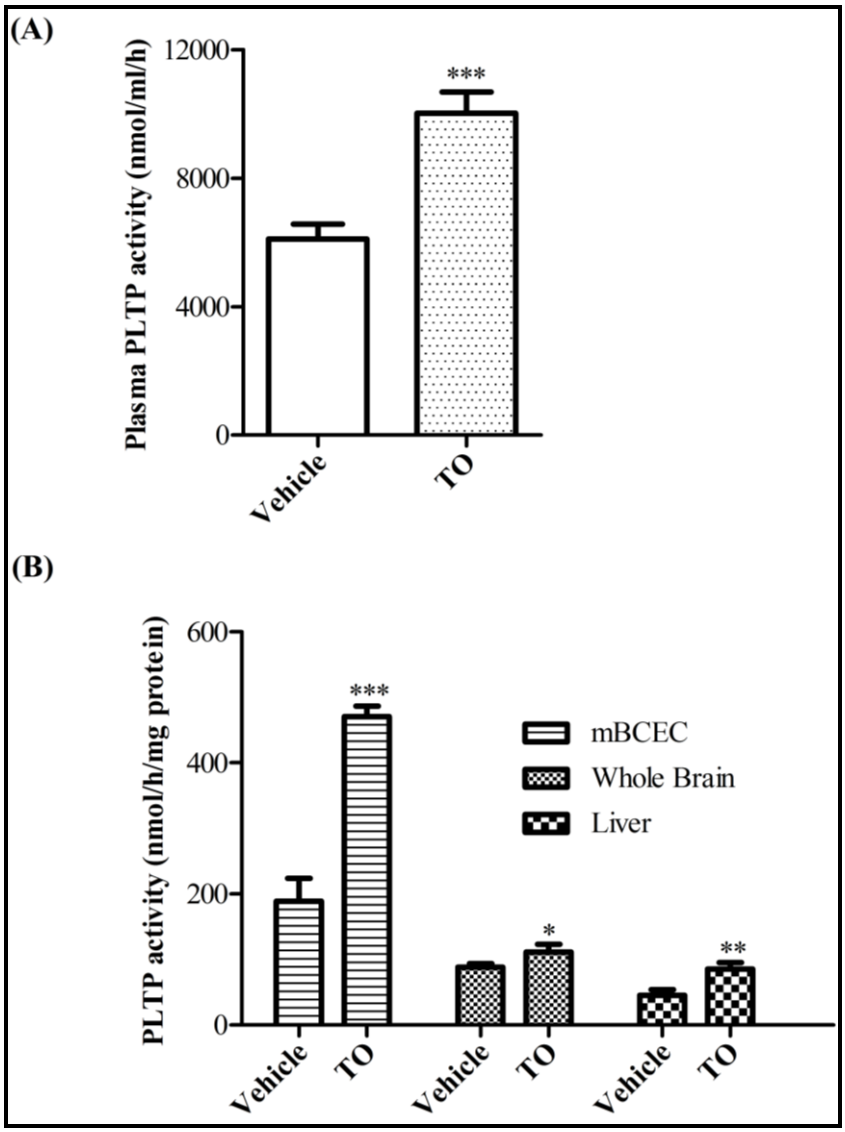


FIGURE 20: PLTP activity in BCEC is regulated by LXR agonist administration *in vivo*. C57/BL6 mice were administered orally either vehicle or TO901317 (50 mg/kg/d) for one week. PLTP activity in plasma (A), isolated mBCEC, whole brain, and liver (B) was measured based on the transfer rate of [^{3}H]-phosphatidylcholine (PC) from liposomes to human HDL_3. Data represent mean values ± SD, (n = 7). * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ *versus* vehicle, mBCEC- mouse brain capillary endothelial cells.

DISCUSSION

5. DISCUSSION

The protective role of HDL against Alzheimer's disease and/or dementia is under extensive investigation because, strikingly, prospective studies point towards an inverse relationship between plasma HDL levels and the risk of cognitive decline or AD development (237, 238). Cholesterol metabolism in the brain appears to be completely separated from that in the periphery due to the presence of tight junctions established by brain microvascular endothelial cells. However, these cells are also actively involved in HDL metabolism (40, 41) at the blood-brain interface. PLTP is an LXR target gene involved in the regulation of HDL biogenesis in the periphery and is also expressed in neurons and glial cells (23, 217). Recent *in vivo* data demonstrated the association of PLTP in Aβ metabolism (221, 222). These findings, collectively, prompted us to investigate the potential role of PLTP in HDL and Aβ metabolism at the BBB using an *in vitro* model consisting of primary pBCEC.

The key findings of our investigation are that (i) active PLTP is highly expressed in brain capillary endothelial cells, (ii) both *in vitro* and *in vivo* brain PLTP is regulated by LXR activation, (iii) under simulated diabetic conditions *in vitro* PLTP expression and activity is reduced, (iv) PLTP-mediated HDL_3 remodeling increases cholesterol efflux from pBCEC, and pre-β-HDL formation is augmented upon LXR activation, (v) PLTP is involved in HDL biogenesis at the BBB, and (vi) PLTP modulates Aβ oligomerisation at the BBB.

5.1. Regulation of PLTP in brain capillary endothelial cells

The results presented here demonstrate for the first time that PLTP is synthesized by BCEC of the BBB (Fig. 2 and 3). One of the most interesting findings is that polarized pBCEC secrete substantial amounts of PLTP into both apical and basolateral compartments but more favorably towards the basolateral (i.e., parenchymal or brain)

side (Fig. 6). PLTP secreted to both compartments is active in transferring phospholipids, strongly supporting a role of BCEC-derived PLTP in lipid transport and metabolism at the blood-brain interface and possibly also in deeper regions of the brain. Intriguingly, and unlike reported recently in human fetoplacental endothelial cells [HPEC;(230, 239)], PLTP protein and activity was detectable (Fig 4 and 5) also in pBCEC lysates, indicating that pBCEC retain a fraction of the produced protein in intracellular pool(s), whereas HPEC release the entire amount of synthesized PLTP into the culture medium. Differentiated intracellular levels of PLTP have also been detected in neurons, and active PLTP has been found (trans)located in the nucleus, however the possible function(s) of intracellular PLTP are thus far unknown (219).

Furthermore, we established that expression and activity of PLTP in pBCEC is under control of LXR (Fig. 4 and 5). In line with this, a direct regulatory influence of LXR on PLTP has been reported in HepG2 cells, macrophages, and in mouse liver (170, 171). The PLTP promoter region contains a high-affinity LXR response element (DR-4A and DR-4B) that binds LXR/RXR heterodimers and the regulatory effects of LXR ligands on PLTP expression/activity were lost in animals or cells lacking LXR (170, 171). Our present findings are of physiological relevance because under *in vivo* conditions BCEC are in contact with 24*(S)*OH-cholesterol, the major brain cholesterol metabolite which can readily cross the BBB to be transported by HDL and LDL to the liver for metabolism/elimination (48).

Our animal studies confirm that LXR activation modulates PLTP activity *in vivo* and for the first time show that this effect applies also to BCEC (Fig. 20). Following LXR activation, the PLTP activity was up-regulated in plasma as well as in all the tissues/cell samples analysed, i.e. liver, whole brain and isolated mBCEC. It is important to note that PLTP activity detected in mBCEC was higher than in whole brain, confirming a significant role of PLTP at the BBB (Fig. 20B). Since LXR agonist modulated PLTP activity in BCEC both *in vitro* and *in vivo*, it is very tempting to state that PLTP regulates lipid metabolism at the blood-brain interface.

5.2. Effect of simulated diabetic conditions on PLTP

Plasma PLTP activity has been reported to be elevated in patients with both Type 1 and Type 2 diabetes. However, Type 1 diabetic patients treated with short-term high glucose as well as high insulin infusion showed decreased plasma PLTP activity (240). We (in primary human placental endothelial cells) and others (in HepG2) have previously reported an increased PLTP activity, when using high glucose concentrations *in vitro* (172, 230). In contrast, when we mimicked *in vitro* diabetic conditions (high glucose, high insulin or their combination) in pBCEC both PLTP mRNA expression and activity was significantly reduced (Fig. 7 and 8). Our *in vitro* BBB model showed a 35% decrease in PLTP activity with supraphysiological concentration of insulin, which is in line with that reported in cultured HepG2 cells (241). However, the underlying mechanism behind the decreased PLTP activity and expression found in pBCEC under simulated diabetic condition is presently unknown, and warrants further investigation. In contrast to reduced PLTP mRNA expression and activity, high glucose and insulin exposure of pBCEC leads to an increase in PLTP mass levels (Fig. 9). It is also important to note that, PLTP activity is not only determined by its protein mass but also governed by HDL particle size and interaction with apolipoproteins (174, 242, 243).

5.3. Effect of PLTP-mediated HDL_3 remodelling on cholesterol efflux

It has been well described that PLTP modulates HDL size and composition thereby enhancing the ability of HDL to remove (excess) cholesterol and phospholipid from cells (23, 184). The smaller HDL particles, termed pre-β-HDL, are formed as a result of PLTP-mediated HDL remodeling and serve as the ideal initial acceptors for cellular cholesterol (226, 244). The role of PLTP in HDL-mediated reverse cholesterol transport has been established (245). We previously reported an auto-regulatory reverse sterol transport mechanism operating in pBCEC at the BBB (40). In this study we established a firm role of pBCEC-derived PLTP in this process. When using PLTP-modified HDL particles as compared to native HDL_3 as exogenous cholesterol acceptors, pBCEC responded with enhanced cholesterol release, and LXR activation augmented the effect

further (Fig. 12). It is interesting to note that, addition of PLTP-modified HDL_3 to the basolateral compartment of polarized pBCEC resulted in an enhanced removal/transfer of cholesterol to the brain parenchymal side as compared to native HDL_3 acceptor particles (Fig. 13 A and B). Furthermore, LXR activation resulted in enhanced removal/transfer of cholesterol to both basolateral as well as to apical compartments as compared to native HDL_3 acceptor particles (Fig. 13 C and D). Neurons require high amounts of cholesterol, but cannot efficiently produce all required cholesterol, and thus rely on external sources such as glial cells (49). Based on our findings we speculate that BCEC may form an additional source of external cholesterol. pBCEC efficiently release cholesterol into the basolateral direction and PLTP present at the brain parenchymal side remodels HDL particles thereby enhancing the transfer of cholesterol from the BBB to the brain. In addition, we immuno-detected pre-β-HDL formation in pBCEC supernatants incubated with native HDL_3 and the relative amount of pre-β-HDL was also augmented by LXR activation (Fig. 14). These results together strongly suggest that PLTP, of either exogenous (i.e., plasma) or endogenous (i.e., BCEC) origin, mediates HDL remodeling at the BBB and thereby markedly enhances cholesterol release from pBCEC.

5.4. PLTP in HDL biogenesis at the BBB

pBCEC express ABCA1, SR BI and apoA-I, all involved in sterol transport at the BBB (40, 41). The LXR/ABCA1/apoA-I cholesterol efflux pathway facilitates the assembly of particles possessing a density corresponding to that of HDL particles preferentially at the basolateral side (brain side) of the BBB (40). PLTP is known to interact with and stabilize ABCA1 in peripheral cells, thus enhancing cholesterol efflux to apoA-I (198, 246). Since PLTP, apoA-I and ABCA1 are all more abundant at the basolateral side, it is reasonable to hypothesize that PLTP expressed in BCEC could similarly interact with and/or stabilize ABCA1 and in concert with apoA-I promote HDL genesis at the brain side of the BBB.

Assembly of cellular phospholipids and cholesterol with apoA-I form a crucial step in HDL formation (236). Our RNA interference studies to knock-down PLTP in pBCEC resulted in a 67% reduction (Fig. 17A) of cholesterol release to apoA-I, confirming a key role of PLTP in the process of HDL genesis. Characterization of apoA-I containing particles in pBCEC conditioned media by NDGGE/immunoblotting analyses also clearly pointed towards the formation of nascent HDL particles at the BBB, which was amplified upon LXR activation (Fig. 15). In previous studies nascent apoA-I containing particles with diameters varying from 8 -20 nm have been detected in various cell lines (247). The nascent HDL particles detected in pBCEC supernatants have a similar size as that reported for human CSF lipoproteins (11-20 nm) that are also mainly enriched in apoA-I (57). Because previous studies employed different separation methods, further characterization of nascent HDL particles formed by pBCEC is required to draw firm conclusions on their composition. However, based on our current findings it is reasonable to assume that 24(*S)*OH-cholesterol while crossing the BBB can induce PLTP expression and activity together with ABCA1 and apoA-I to enhance lipid efflux and HDL formation. Hence, PLTP expressed in pBCEC together with apoA-I may serve to integrate plasma and cerebrovascular lipid metabolism. Efflux of cellular cholesterol is also mediated by HDL_3 representing a ligand for SR BI and ABCG1 (40, 248). Present PLTP gene-silencing experiments in pBCEC suggest an association of PLTP also with HDL_3 mediated cholesterol efflux, accounting for approximately 30% (Fig. 17B). Our findings, collectively, underline a crucial role for PLTP in HDL metabolism at the BBB.

5.5. PLTP in Aβ metabolism at the BBB

It has been documented that HDL particles mediate clearance of Aβ from cells (249, 250). Because we here revealed a critical role of PLTP in HDL biogenesis at the BBB, we assumed that it might be also involved in the regulation of Aβ homeostasis. Indeed, incubation of pBCEC in the presence of active plasma PLTP significantly reduced intracellular levels of Aβ oligomers (Fig. 18). Furthermore, PLTP-modifiedHDL_3 particles reduced intracellular levels of Aβ oligomers, although to a lesser extent than unmodified HDL_3 (Fig. 18). Although here we have not addressed the underlying

mechanism, one potential explanation is that HDL binds and removes Aβ thereby reducing intracellular Aβ/oligomers. According to our results, Aβ would bind more efficiently to unmodified than to PLTP-modified HDL_3. The mechanism on how PLTP itself reduces Aβ oligomers in pBCEC is presently unknown. Interestingly, we found increased levels of BACE1 mRNA in PLTP-silenced pBCEC (Fig. 19A). This is intriguing as increased levels of BACE1 may enhance generation of Aβ by redirecting the APP processing toward the amyloidogenic pathway (130). Recent study in PLTP-deficient aged mice also reported an increased expression and activity of BACE1 in various brain regions (222). Further investigation is required to explore the mechanism(s) behind these findings.

5.6. Potential role of PLTP and HDL in preserving cerebrovascular health

Numerous human population studies have identified that low levels of plasma HDL cholesterol (HDL-C) is a major risk factor for the development of cardiovascular events and currently HDL is being intensively investigated as a promising therapeutic target to reduce cardiovascular diseases (251). It has been increasingly evident that plasma HDL-C levels also have a close association with AD pathology and cognitive performance (237, 238). Lower concentrations of circulating HDL-C and apoA-I are highly correlated with the severity of AD, and also the level of apoA-I is low in the CSF and brain tissue of AD patients, while high serum HDL-C levels reduce the risk of AD (102, 103, 238, 252). A recent epidemiological study by Bowman *et al*., (253) indicates that high plasma triglyceride and low HDL-C levels significantly correlate with BBB impairment in patients with mild to moderate AD. However, the mechanistic explanation behind these associations is largely obscure.

Apolipoproteins documented in the brain are mainly apoE, apoA-I, apoA-IV, apoD, apoH and apoJ, and lipoproteins detected have a density resembling HDL in plasma (58, 87). Despite the presence of BBB it is widely accepted that a small fraction of circulating HDL particles is getting access to CNS through transcytosis or selective

uptake by HDL-receptors. In the brain, HDL-like particles are mainly seen in two compartments; as apoE/apoA-I-containing HDL particles in CSF and as apoE/apoJ/apoA-I-containing HDL particles in ISF. Continuous supplies of lipids are required for neurons mainly for membrane synthesis and they depend greatly on exogenous sources. Lipoprotein-mediated clearance mechanisms to eliminate excess lipids are also functioning in neurons (56). Previous studies undoubtedly established that brain HDL has a crucial role in cholesterol turnover in the CNS (250). In this study we have established the critical role of PLTP in HDL formation at the BBB. In recent years, several neuro-protective properties of HDL have been reported. HDL can exert its neuroprotective roles through different mechanisms. HDL is an important factor for the maturation of synapses and the preservation of synaptic plasticity and hippocampal volume (237). HDL can reduce the toxicity of Aβ and assist in its clearance through BBB. It has been shown that in AD animal models, the overexpression or deletion of apoA-I significantly attenuates or increases CAA respectively and thus HDL/apoA-I contributes to the cognitive functions (112, 113). In both plasma and CSF, Aβ is normally present in association with HDL-like particles and based on density gradient ultracentrifugation in CSF the HDL-like particles can be separated in to three subfractions namely HDL_1-like, HDL_2-like and HDL_3-like particles (97, 254). Compared to age-matched controls in CSF, Koudinov *et al*., (255) observed a variation in the distribution of Aβ with different HDL subfractions in AD subjects, indicating that the binding of Aβ to different HDL subfractions have important structural/functional consequences. HDL can directly bind Aβ and maintain Aβ in a soluble state to inhibit its assembly into fibrils, thereby reducing the neurotoxicity of Aβ and also might remove the excess Aβ piled up in the vessels (249, 256).

Neuronal production of Aβ depends on the membrane content of cholesterol and hence HDL can reduce the production of Aβ by activating reverse cholesterol transport with the help of ABC transporters, a process that can be also induced by LXRs activation (250). HDL can also mediate the removal of potentially neurotoxic 24*(S)*OH-cholesterol out of brain through BBB with the help of SR-BI, thus maintaining the sterol homeostasis in the brain (40). The origin of Aβ deposits in CAA is still mysterious it can be plasma-derived, brain parenchymal-derived or even from the local production i.e.

brain endothelium (130). Based on our results, it is very tempting to speculate that, HDL particles modified by PLTP can reduce the Aβ deposits in the microvessels by modulating/removing the cholesterol content of BCEC (Fig. 12 and 13). According to the recent reports, ABCA1 and SR-BI-mediated cholesterol efflux to apoA-I and HDL is significantly impaired in AD patient's plasma and may cause the accumulation of intracellular cholesterol in AD patients, favoring amyloidogenesis (257).

In contrast to CSF, there is only very limited information available about the maturation/lipidation state of HDL-like particles in the brain ISF. However, a recent report from Stukas *et al*., (258) supports that nascent discoidal apoA-I/apoE bearing HDL-like particles are mainly involved in the clearance of Aβ rather than the mature spherical HDL-like particles. The report from Stukas *et al*., (258) emphasizes the importance of PLTP-mediated remodeling of HDL particles in Aβ clearance at the BBB or even deeper regions of brain, as the remodeling generates more discoidal HDL-like particles (Fig. 14 and 15). LCAT is the key enzyme involved in the esterification and maturation of discoidal HDL to spherical HDL in the CNS. Stukas *et al*., (258) observed no significant change in both parenchymal as well as vascular amyloid burden in LCAT-deficient AD murine models, indicating that LCAT-mediated maturation of HDL is not a prerequisite for its role in Aβ metabolism.

AD is considered also as a vascular disorder (259) and there is an increasing evidence about the beneficial effects of HDL on the vascular endothelium through anti-inflammatory, anti-thrombotic and anti-oxidative properties attributed by HDL (260). Furthermore, depending upon the pathophysiological background HDL can differentially regulates angiogenesis, which is altered in AD (261). EC of the BBB are exposed to HDL particles from both sides i.e. circulating HDL at the apical side and brain parenchymal HDL at the basolateral side and hence HDL can exert its endothelial protective functions from both sides. Both apoA-I and HDL have potent anti-inflammatory and anti-oxidative properties, and the enzyme associated with HDL, paraoxonase 1 (PON1) is the important contributor for these properties (262). It has been shown that both *in vitro* and *in vivo* that in EC HDL can prevent the expression of

cellular inflammatory adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecules -1 (ICAM-1), which are reportedly over expressed in AD brain EC (263) . In activated EC, HDL inhibits the release of monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNFα), which lowers the adhesion and activation of monocyte. It is very important to note that, the expression and secretion of these pro-inflammatory molecules are significantly increased in the case of AD patients (251, 263). According to Khalil *et al*., (257) report, the anti-inflammatory effect of HDL is significantly lowered in AD patient's serum compared to the healthy subjects and intriguingly, AD patient's HDL even exhibits a pro-inflammatory phenotype. It is also interesting to note that, the overexpression of human apoA-I attenuates neuroinflammation in an AD mouse model, as evident by the reduced level of microglial and astrocytes activation (113). In a recent investigation, Dang *et al*., (264) reported that in an *in vitro* model of BBB, HDL prevents the elastase and MMP 9-induced breakdown of BBB under ischemic conditions.

HDL maintains vascular endothelial tone by inducing endothelial nitric oxide synthase (eNOS) activity, which increases the production of strong a vasodilator, nitric oxide (NO). HDL can directly activates eNOS via SR-BI and sphingosine-1 phosphate receptor 3 (S1P3), consequently stimulate the production of endothelial NO (262). Another mechanism by which HDL stimulates the production of endothelial NO is by mediating cholesterol efflux to ABCG1, thereby reducing the inhibitory interaction of eNOS with caveolin-1 and restoring eNOS activity (262). HDL inhibits apoptosis (HDL-associated apoJ is an anti-apoptotic agonist) of EC and enhances endothelial integrity and repair (260). HDL stimulates endothelial cell migration, proliferation and recruitment of circulating endothelial progenitor cells to the damaged site of endothelium via SR-BI and SIP3, while sphingosine-1 phosphate (SIP) and eNOS also contribute to this repair process (262). ApoM/apoE is as an important carrier of S1P in HDL particles and HDL-associated S1P and PON1 are the key mediators for some of these endothelial protective effects of HDL (265). However, the serum PON1 activity and brain tissue S1P (SIP is also seen in association with CSF HDL) levels are significantly lower in AD and VD, indicating that the function of HDL is significantly impaired under these conditions (266-269).

The results from two different animal models of cerebral stroke suggest that reconstituted HDL exhibits protection from neuronal damage after the onset of ischemic stroke, possibly by HDL's anti-oxidative mechanisms (270). Alpha tocopherol (αTocH)/vitamin E is a potent antioxidant associated with HDL and it has been shown that decrease in vitamin E levels significantly increases Aβ accumulation by decreasing its clearances from brain and blood in AD animal model (271, 272). In the cultured EC of BBB we have reported that SR-BI facilitates the selective uptake of HDL-associated αTocH (43). PLTP plays a significant role in the transfer of αTocH (203, 204), indicating that αTocH with the help of PLTP might protect the BBB and also the deeper regions of brain against oxidative damage. It has been shown that in mice dietary supplementation of vitamin E increases the BBB integrity and tight junction protein expression, and also prevents Aβ-induced memory deficits by reducing cerebrovascular oxidative stress (221, 223). Even more strikingly, a recent randomized clinical trial reported that αTocH medication significantly delayed clinical progression in mild to moderate AD patients taking acetylcholinesterase inhibitor (273).

To the best of our knowledge there is only very limited information available about the functional status of HDL and/or the diversity/variations of HDL subpopulations in plasma, CSF and brain ISF under neurodegenerative conditions like AD. Further clear investigation about lipoprotein metabolism in the brain and the association between plasma and CNS HDL is critically essential. However, the available data indicates that the functionality of plasma HDL is significantly impaired in AD and may contribute to AD pathology via the cerebrovasculature. There is no doubt that HDL possesses multiple vascular endothelial protective functions, however, HDL-raising clinical trials have been disappointing and failed to demonstrate any significant effects on cardiovascular events indicating that the quality of HDL is important than the quantity (29). Also we need to clearly understand how HDL function relates to HDL subpopulations and also need to know more about which functions and subpopulations of HDL are altered during HDL-based therapies. Finally, we need to design smarter pharmaceutical approaches which can integrate the concept of HDL proteome and lipidome to increase the functionality of HDL thereby protecting the cerebrovasculature that can eventually diminish the incidence or severity of AD.

In summary, the results of the present study strongly suggest that PLTP produced by BCEC forms an additional source of PLTP to the brain pool. As an LXR target and key determinant of HDL metabolism at the BBB, PLTP might be involved in regulating APP/Aβ homeostasis. A model of the potential roles of PLTP and HDL at the BBB is outlined in Figure 21. The present study identified for the first time that phospholipid transfer protein is expressed in cerebrovascular endothelial cells, regulated by LXRs in BCEC, and actively involved in cholesterol efflux, HDL genesis and remodeling, and Aβ metabolism at the blood brain barrier *in vitro*. Further investigation is needed to fully disclose the prospective roles of PLTP at the BBB in particular under conditions of lipid-related neurodegenerative diseases, like AD.

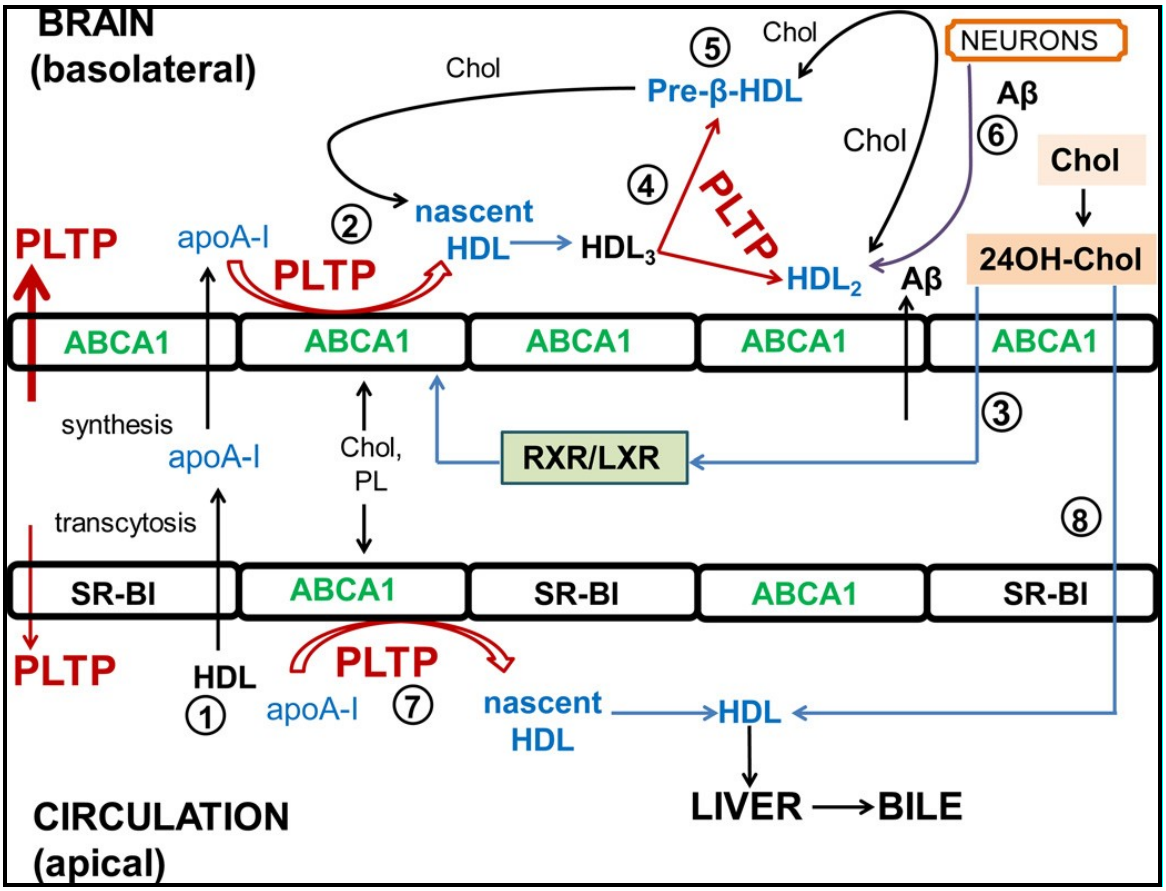


FIGURE 21: Proposed model of PLTP roles in HDL metabolism and HDL functions at the BBB. 1, small fraction of apoA-I from circulating HDL can transcytose across cerebrovascular endothelial cells (43). In addition, apoA-I is expressed by BCEC and released bidirectionally but mainly to the basolateral

compartment (40). 2, active PLTP is also secreted bidirectionally but mainly to the basolateral compartment. Nascent HDL particles are formed at the brain side with the aid of ABCA1 and PLTP. 3, LXR activation by 24OH-cholesterol (or synthetic agonists) promotes the formation of nascent apoA-I containing particles. 4, nascent HDL particles mature to form spherical HDL_3, and PLTP further remodels HDL_3 into HDL_2 and pre-β-HDL particles. 5, pre-β-HDL (and also HDL_2) accepts excess cellular cholesterol. 6, HDL may directly bind excess Aβ (from neurons and from BCEC); both PLTP and HDL may reduce intracellular Aβ oligomers, and HDL may facilitate the elimination of excess Aβ into the circulation (87, 250, 274). 7, HDL particles are also formed and remodeled at the apical side, through endogenous and exogenous (plasma) PLTP. 8, HDL in the circulation with the help of SR-BI accepts 24*(S)*-hydroxycholesterol (40, 41), which will be finally removed through bile. (HDL, high density lipoproteins; ABCA1, ATP-binding cassette transporter A1; RXR, retinoid X receptor; PL, phospholipid; 24OH-Chol, 24*(S)*-hydroxycholesterol; Chol, cholesterol; SR-BI, scavenger receptor, class B, type I).

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