Apolipoprotein M modulates erythrocyte efflux and tubular reabsorption of sphingosine-1-phosphate

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Abstract: Sphingosine-1-phosphate (S1P) mediates several cytoprotective functions of HDL. ApoM acts as an S1P binding protein in HDL. Erythrocytes are the major source of S1P in plasma. After glomerular filtration apoM is endocytosed in the proximal renal tubules. Human or murine HDL elicited time- and dose-dependent S1P efflux from erythrocytes. Compared to HDL of wild type (wt) mice, S1P efflux was enhanced in the presence of HDL from apoM transgenic mice but not diminished in the presence of HDL from apoM knock-out (Apom-/-) mice. Artificially reconstituted and apoM-free HDL also effectively induced S1P efflux from erythrocytes. S1P and apoM were not measurable in the urine of wt mice. Apom-/- mice excreted significant amounts of S1P. ApoM was detected in the urine of mice with defective tubular endocytosis because of knock-out of LDL receptor related protein, chloride-proton exchanger ClC-5 (Clcn5-/-) or the cysteine transporter cystinosin. Urinary levels of S1P were significantly elevated in Clcn5-/- mice. In contrast to Apom-/- mice, these mice showed normal plasma concentrations for apoM and S1P. In conclusion, HDL facilitates S1P efflux from erythrocytes by both apoM-dependent and apoM-independent mechanisms. Moreover, apoM facilitates tubular reabsorption of S1P from the urine, however with no impact on S1P plasma concentrations.

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Apolipoprotein M modulates erythrocyte efflux and tubular reabsorption of sphingosine-1-phosphate

Short title: ApoM modulates cellular efflux and urinary excretion of S1P

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ABSTRACT

Sphingosine-1-phosphate (S1P) mediates several cytoprotective functions of HDL. ApoM acts as an S1P binding protein in HDL. Erythrocytes are the major source of S1P in plasma. After glomerular filtration apoM is endocytosed in the proximal renal tubules. Human or murine HDL elicited time- and dose-dependent S1P efflux from erythrocytes. Compared to HDL of wild type (wt) mice, S1P efflux was enhanced in the presence of HDL from apoM transgenic mice but not diminished in the presence of HDL from apoM knock-out (Apom\(^{-/-}\)) mice. Artificially reconstituted and apoM-free HDL also effectively induced S1P efflux from erythrocytes. S1P and apoM were not measurable in the urine of wt mice. Apom\(^{-/-}\) mice excreted significant amounts of S1P. ApoM was detected in the urine of mice with defective tubular endocytosis because of knock-out of LDL receptor related protein, chloride-proton exchanger CIC-5 (Clcn5\(^{-/-}\)) or the cysteine transporter cystinosin. Urinary levels of S1P were significantly elevated in Clcn5\(^{-/-}\) mice. In contrast to Apom\(^{-/-}\) mice, these mice showed normal plasma concentrations for apoM and S1P. In conclusion, HDL facilitates S1P efflux from erythrocytes by both apoM-dependent and apoM-independent mechanisms. Moreover, apoM facilitates tubular reabsorption of S1P from the urine, however with no impact on S1P plasma concentrations.
INTRODUCTION

Sphingosine-1-phosphate (S1P) acts both as an intracellular signalling molecule and an extracellular agonist of at least five different G-protein coupled receptors. By its dual functions S1P regulates the survival, proliferation and migration as well as the functionality of many cells, eventually in opposite directions [1-4]. Therefore the absolute and relative abundance of S1P in intracellular and extracellular compartments appears to be important for its biological functionality[4].

Most cells form S1P by the phosphorylation of sphingosine, a degradation product of ceramides, through sphingosine kinase and degrade S1P to phosphoethanolamine and fatty aldehyde through S1P-lyase [1-4]. By contrast, erythrocytes and platelets but also other cells, which have low or no lyase activity, release S1P [4-6], probably by an as yet unidentified ATP binding cassette transporter (ABC) [4, 7, 8]. Within the plasma compartment the majority of S1P is transported by high-density lipoproteins (HDL) in which it exerts many cyto-protective and anti-inflammatory effects, for example on endothelial cells [8-10]. The enrichment of S1P in HDL has been explained by the presence of a specific S1P binding protein, namely apolipoprotein M (apoM) [10]. Purified and recombinant apoM binds S1P with an IC50 of 0.9 µmol/L, which is in the range of physiological S1P plasma concentrations [11]. X-ray crystallography of apoM identified an S1P binding domain which was confirmed by the recombinant generation of non-S1P-binding apoM mutants [11]. In agreement with these physicochemical data, S1P was co-purified with apoM containing HDL but not apoM-free HDL from both human and murine plasma. Moreover, S1P concentrations were dramatically decreased in HDL of apoM knock-out (Apom<sup>−/−</sup>) but increased in HDL of mice transgenic for apoM (Apom<sup>tg</sup>). As an in vitro indication of functional relevance the stimulatory effects of S1P on nitric oxide production by endothelial cells were mimicked by apoM-containing HDL but not by apoM-free HDL. Finally, the physiological relevance of S1P binding by apoM was indicated by the reduced basal endothelial barrier function in lungs of apoM knock-out mice [12]. Despite this very strong in vitro and in
vivo evidence for the limiting effect of apoM on the transport and function of S1P in plasma and HDL, concentrations of S1P and apoM in either plasma or HDL do not correlate with each other [12, 13]. Moreover, stoichiometric calculations revealed that apoM is not saturated with S1P but present at an up to eight fold molar excess [12, 13]. We therefore investigated the impact of apoM on two other potential pathways of S1P metabolism, namely efflux from erythrocytes and urinary excretion.

Plasma concentrations of S1P were recently shown to correlate with red blood cell counts [5, 6, 14], probably because the lack of the S1P degrading lyase makes erythrocytes to the main source of S1P in plasma [4, 5]. Because HDL was previously found to induce S1P efflux from erythrocytes [5], we compared the S1P efflux capacity of HDL from wt, Apom−/−, and Apom+ mice [15].

After glomerular filtration, apoM is reabsorbed from the primary urine into proximal tubular epithelial cells by binding to the endocytic receptor megalin (low density lipoprotein receptor related protein 2, LRP2). Accordingly, mice with a conditional renal knock-out of megalin excrete apoM in urine [16]. Megalin and its co-receptor cubilin also mediate the tubular reabsorption of several small plasma proteins which carry small molecules and are filtrated through the glomeruli [17, 18]. Not only megalin and cubilin but also endosomal and lysosomal proteins such as chloride-proton exchanger ClC-5 (mutated in Dent’s disease) and the cystine transporter cystinosin (mutated in cystinosis), respectively, are key components of the machinery that rescues essential molecules such as vitamin B12 and vitamin D from inappropriate urinary loss [17, 19, 20]. To test whether this is also of relevance for the metabolism of S1P, we compared the urinary excretion of S1P and apoM in wt and Apom−/− mice [15] as well as mice with dysfunctional megalin (Lrp2−/−) [21], ClC-5 (Clcn5−/−) [22], or cystinosin (Ctns−/−) [23].
METHODS

Plasma and urine collections from mice

Mice with knock-out of apoM (\textit{Apom} \textsuperscript{-/-}) or transgenic overexpression of human apoM (\textit{Apom}\textsuperscript{tg}) [15] as well as mice with defective expression of megalin (\textit{Lrp2}\textsuperscript{-/-}) [21], CIC-5 (\textit{Cln5}\textsuperscript{-/-}) [22], or cystinosin (\textit{Ctns}\textsuperscript{-/-}) [23] were previously described. Blood samples were obtained by cardiac puncture immediately after sacrifice. Plasma was prepared by 15 minutes of centrifugation of blood at 2000 g. Urine samples were collected in metabolic cages for 8 hours (\textit{Cln5}\textsuperscript{-/-}, \textit{Ctns}\textsuperscript{-/-} and their littermate controls), 16 hours (\textit{Lrp2}\textsuperscript{-/-} and their littermate controls), or 24 hours (\textit{Apom}\textsuperscript{-/-}, \textit{Apom}\textsuperscript{tg}, and their littermate controls) according to standard protocols. Each drop of urine was immediately cooled down to -20°C in the collector of the metabolic cages. All plasma and urine samples were kept frozen at -80°C before further use or analysis. The age of mice, at which samples were obtained, is reported in the Results section. All animal procedures were approved by the appropriate National Research Council Guide for the Care and Use of Laboratory Animals/Animal Ethics Committee.

Isolation and reconstitution of HDL

Human HDL was isolated from plasma of healthy blood donors (Kantosspital Schaffhausen, Switzerland) or mouse plasma by stepwise ultracentrifugation \((d = 1.063-1.21 \text{ kg/L})\) at 360’000 x g, 15°C for 15h as described previously [24] using solid potassium bromide (SigmaAldrich, Buchs, Switzerland) for density adjustment. ApoA-I was further purified from delipidated HDL as described previously [24]. Discoidal rHDL particles were produced by the cholate dialysis method and contained apoA-I, 2-oleoyl-1-palmitoyl-sn-glycero-3 phosphocholine (POPC) (Sigma), and sodium cholate (Sigma) in a molar ratio of 1/100/100 [24].
**S1P efflux from erythrocytes**

Erythrocytes were isolated from the blood of healthy adult volunteers. The blood was anticoagulated with sodium citrate and then centrifuged at 2000 g for 5 min at 4°C. After removing the plasma, the sedimented erythrocytes were washed three times with sterile PBS and resuspended 1:1 in PBS (v/v) containing either BSA, human or murine HDL, reconstituted HDL or lipid-free apoA-I at the concentrations indicated in the results section and incubated at 37°C. Aliquots were removed at different time points (as indicated in the results section) and immediately centrifuged at 2000g for 3 min at 4°C to sediment erythrocytes. The supernatants were carefully transferred into new tubes avoiding any contamination with erythrocytes. For S1P measurement, 25 µl aliquots of the supernatant were taken and processed as described below.

**Quantification of S1P in plasma, HDL, and urine**

S1P was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after derivatization with acetic anhydride. The S1P concentrations in plasma or erythrocyte supernatants (25µl), HDL (50 µg), urine (500µl) were analysed after adding 10 pmol internal standard (D<sub>7</sub>S1P, Avanti Polar Lipids, Alabaster, AL, USA). For calibration S1P (Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in DMSO/concentrated-HCl (100:2, v/v) at a concentration of 0.28 mmol/L stock solution. Each series of measurements was calibrated with 1, 2.5, 5, 10, 15, 20, and 25 pmol of S1P supplemented with 10 pmol of D<sub>7</sub>S1P (IS). Quality control samples with 7.5 and 22.5 pmol S1P were evaluated at the beginning and at the end of each sample series. Double blank and blank samples for carry-over control were prepared by adding methanol and internal standard, respectively to 25 µl of water and processed as plasma samples.
Lipids were extracted with 1 ml of an organic solution consisting of ethyl acetate/2-propanol (6:1, v/v) and 50µl of concentrated formic acid added for phase separation [25]. The upper organic phase was separated and evaporated to dryness under a stream of nitrogen. For the derivatisation of the primary amino and the secondary alcohol groups of S1P [26] the dried lipids were dissolved in 100µl of pyridine and 50µl of acetic anhydride and incubated at 40°C for 20 min. After evaporating the acetylation reagents, the reaction products were dissolved in 100µl of methanol and transferred to glass vials prior to LC-MS/MS analysis.

Acetylated S1P (S1P (Ac)_2) was analyzed on an LC-MS system, consisting of an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), a Rheos 2200 HPLC pump (Flux Instruments, Reinach, Switzerland) and a TSQ Quantum Access mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic conditions for reverse-phase separation of S1P(Ac)_2 have been modified from Berdyshev et al. [26]. Separation of S1P(Ac)_2 was done on a Nucleosil C18 HD column (125x2 mm, 100 Å, 5 µm) at 40°C. As mobile phases we used (A) water/methanol/formic acid (20:80:0.5, v/v) and (B) methanol/acetonitrile/formic acid (59:40:0.5, v/v), both containing 5mM ammonium formate. Elution started with 100% (A) for 1.0 min (0.25 ml/min) and increased to 100% (B) within 4.0 min and was then kept constant for 4 min. Finally the column was re-equilibrated with 100% mobile phase A for 5.5 min. The injection volume was 10 µL. The injection system and syringe was washed twice with methanol/acetone/2-propanol (1:1:1, v/v), containing HCOOH 0.1% and acetone/methanol/water (2:2:1, v/v) solutions after every injection. S1P(Ac)_2 and D_7S1P(Ac)_2 eluted at t_r ~6.3 min.

Double blank and blank samples were analysed before each set of calibrators and samples to exclude carryover.

For ionisation ESI was used and detection was performed in the positive mode, monitoring [M-H_2O]^+ ions using Selective Reaction Monitoring (SRM) for the transitions of m/z 446.2 → 264.2 (30V) for S1P(Ac)_2 and m/z 453.2 → 271.2 (27 V) for D_7S1P(Ac)_2 (spray voltage 5000 V,
skimmer offset 10 V and ion transfer capillary temperature 300°C). Further ionisation and
detection parameters were optimized by tuning the system with S1P(Ac)_2 standard. Data analysis
was performed on XCalibur 2.0.6 (Thermo Scientific).

The standard curve was constructed by plotting the S1P/IS peak area ratios against the
concentrations of the S1P standards. The S1P concentration in samples was determined by linear
regression obtained from the standard curve. The lower level of quantification was defined as the
10% functional assay sensitivity and amounted to 1 pmol extracted S1P corresponding to 40
nmol/l plasma or 2 nmol/l urine. At amounts of 7.5 pmol and 22.5 pmol, the intra-day
imprecision was 5.8% and 2.3%, respectively, and the inter-day imprecision 4.3% and 8.1%,
respectively.

**Western blotting of apoM in plasma and urine**

Proteins were separated on 14% SDS polyacrylamide gels and transferred onto nitrocellulose
membrane. After blocking the membranes were blocked in 5% milk Tris-Buffered Saline Tween
(TBS-T), 0.1% for 1h at room temperature. Thereafter, the membranes were incubated over night
at 4°C with a commercially available apoM antibody (LC-C51665; LifeSamp BioSciences). The
1:1000 dilution of antibody was made in 5% milk TBS-T. The membranes were then washed
three times with TBS-T and incubated with secondary antibody anti-rabbit coupled with HRP
(1:10000). After washing three times for 10 minutes, the membrane was developed with ECL
Plus Western blotting detection reagent (Pierce) according to the manufacturer’s instructions.

**Statistical Analyses**

Statistical analyses were performed by using Graph-Pad. Normality of the data was determined
by using the Kolmogorov-Smirnov test. Normally distributed data were analyzed by the two-
tailed unpaired Student’s test, not normally distributed data by Mann-Whitney U. Differences in prevalences were analysed by Chi-square test.

RESULTS

HDL and apoM promote S1P efflux from erythrocytes

To confirm previous data that HDL induces S1P efflux from erythrocytes [5], 1 ml of washed human erythrocytes were incubated with two different concentrations of human HDL for increasing time. Figures 1a and 1b show the time-dependent accumulation of S1P in human HDL without and with correction for the endogenous S1P content, respectively. Maximal efflux, which doubled the concentration of S1P pre-existing in HDL, was reached after 4 hours. Half-maximal efflux was reached after 1 to 2 hours of incubation. Albumin (500 µg/ml), which is the second most important carrier of S1P in plasma [4, 10] elicited very little S1P efflux under the same conditions.

Next we monitored the time-dependent S1P efflux from erythrocytes in the presence of 300 µg/ml HDL which were isolated from plasmas of either wt, Apom<sup>−/−</sup> or Apom<sup>+/−</sup> mice in comparison with 300 µg/ml albumin. As reported previously, HDL of these three mouse strains differ by endogenous S1P content (see also baseline data in figure 1c). Therefore we present S1P efflux before and after correction for the endogenous S1P content of HDL (figures 1c and 1d, respectively). During 8 hours of incubation S1P concentrations increased steadily in HDL of all three mouse strains. S1P efflux capacity of HDL from wt mice resembled that of human HDL. Maximal net S1P efflux measured was significantly increased in the presence of HDL from Apom<sup>+/−</sup> mice but not decreased in the presence of HDL from Apom<sup>−/−</sup> mice (figure 1d). Also of note S1P efflux in the presence of HDL from both wt and Apom<sup>−/−</sup> mice reached saturation after 2
to 4 hours whereas S1P efflux in the presence of HDL from Apom<sup>tg</sup> mice did not reach saturation within 8 hours, the maximal time the experiment could be performed without hemolysis.

To provide further evidence that HDL can elicit S1P efflux independently of apoM, we compared the capacity of native HDL, reconstituted HDL, lipid-free apoA-I and albumin to induce S1P efflux. Whereas lipid-free apoA-I was not capable to induce S1P efflux, reconstituted apoM-free HDL were even more active in stimulating S1P efflux than native HDL at a concentrations of 200 µg/mL (figure 2).

**Urinary excretion of S1P is increased in apoM knock-out mice as well as in mice with dysfunctional tubular protein reabsorption**

As reported previously [12, 13] and as compared to their wt littermates, S1P levels in plasma and HDL are significantly decreased by 20% and 50%, respectively, in 12-14 weeks old Apom<sup>−/−</sup> mice and significantly increased by factors three and five, respectively in plasma and HDL of age-matched Apom<sup>tg</sup> mice (figures 3a and 3b)

In 21 urine samples of wt mice we measured S1P at very low concentration below or close to the lower level of quantification of our method (10% functional assay sensitivity: 1 pmol S1P corresponding to 2 nmol S1P per liter urine). In fact, only five of 21 samples of wt mice but four of five samples Apom<sup>−/−</sup> mice had S1P levels above this threshold (P = 0.018, chi-square test). In the direct comparison, urine concentrations of S1P were significantly higher in 6 and 16 weeks old Apom<sup>−/−</sup> mice than in 6 and 16 weeks old wt controls (P < 0.05, figure 3c).

Next we investigated whether also disturbances of tubular apoM reabsorption are associated with increased urinary S1P excretion. By Western blotting we found apoM present in the urine samples of mice with non-functional megalin (Lrp2<sup>−/−</sup>), ClC-5 (Clcn5<sup>−/−</sup>), or cystinosin (Ctns<sup>−/−</sup>) (figure 4a). ApoM was not detectable in the urine of wt littermates.
In the urine samples of 21 wt mice from three different labs, S1P was below or close to the level of quantification (2 nmol/L). By contrast, 3 of 4 urine samples from 11 weeks old Lrp2\(^-/-\) mice and 6 of 7 samples from 12-16 weeks old Clcn5\(^-/-\) mice contained clearly quantifiable concentrations of S1P (figures 5a and 5b). Upon direct comparison of mutant mice and littermate controls S1P excretion was significantly increased in Clcn5\(^-/-\) mice (figure 5b), which are known to suffer from a severe dysfunction of the proximal tubule [27]. At ages of 18 weeks, 20-24 weeks or 30-38 weeks, neither male nor female Ctns\(^-/-\) mice showed increased S1P excretion.

Western Blotting did not provide any evidence for grossly altered concentrations of apoM in plasmas of Lrp2\(^-/-\), Clcn5\(^-/-\), or Ctns\(^-/-\) mice (figure 4b). Neither did the three mouse models with tubular apoM proteinuria show any statistically significant or consistent differences in plasma concentrations of S1P (figures 5d, 5e, 5f).

**DISCUSSION**

Previous work by the laboratories of Dahlbäck and Nielssen [11, 12] identified ApoM as a physiologically relevant binding protein of S1P in HDL of plasma. Our lab previously confirmed the limiting effect of apoM on S1P levels in plasma but also found indications for more complex relationships between HDL, apoM, and S1P [13]. Most notably concentrations of apoM and S1P in total or apoB-depleted plasma did not correlate with each other [13], possibly due to the molar excess of apoM compared to its ligand S1P which varies inter-individually between factors 1.2 and 8 and/or the presence of alternative ligands, which compete with S1P for binding to apoM [10, 13]. However, we also observed statistically significant correlations of S1P concentrations with concentrations of HDL-cholesterol, apoA-I and other measures of HDL [13]. This raised the questions whether HDL can handle S1P also independently of apoM and whether apoM may influence S1P plasma levels independently of its transport function in plasma.
To answer the first question, we investigated the capacity of HDL to induce S1P efflux from erythrocytes. HDL of both humans and wt mice were found to induce time-dependent and saturable S1P efflux leading to maximally doubling of the endogenous S1P concentration in normal human or murine HDL (400 pmol/l S1P per mg HDL protein). Assuming that 80% of HDL proteins correspond to apoA-I, i.e. the predominant protein of HDL which has a molecular mass of 28 kDa, the molar concentration of S1P per apoA-I amounts to 1/72. Assuming that the HDL particles contain in average 3 molecules of apoA-I [28, 29], every 24th HDL particle in our experiments contained one molecule of S1P at baseline. The doubling of S1P content by efflux indicates the saturation of S1P efflux much below the HDL particle concentration. This can be explained by the presence of a specific S1P binding site in HDL which is not saturated in HDL isolated from plasma, such as apoM [10-12]. At first sight in agreement with this explanation, we found the net S1P efflux capacity of HDL from Apom\textsuperscript{tg} mice significantly increased without reaching saturation. By contrast, both HDL from wt and Apom\textsuperscript{-/-} mice elicited time-dependent and saturable S1P efflux from erythrocytes, which did not differ from each other and that was markedly greater than in the presence of albumin. Moreover, reconstituted HDL consisting only of apoA-I and POPC induced S1P efflux from erythrocytes. The apparent 2 to 3 fold higher efficacy of rHDL as compared to native HDL is probably reflecting the higher particle concentration at identical protein mass concentrations, because rHDL contains 2 molecules of apoA-I whereas native HDL contains at least three molecules of apoA-I plus other proteins. Our finding of rHDL-induced S1P efflux does also explain the previous observation that initially S1P-free rHDL exert cytoprotective effects on cardiomyocytes in a S1P receptor dependent fashion.[30]

Taken together our findings indicate the presence of an additional apoM-independent mechanism by which HDL can induce efflux and/or mediate the binding of S1P. Because of the saturation much below the concentration of HDL particles or phospholipids, it is unlikely that this apoM-independent fraction of HDL-induced S1P efflux is unspecific, for example as the result of
association with phospholipids. The non-saturation of S1P efflux in the presence of HDL from Apom
"tg" mice indicates that apoM rather than phosphatidylcholine acts as the slow determinant of S1P binding capacity. The saturable S1P efflux in the presence of HDL from Apom
"/-
mice points to the presence of an as yet unknown relatively fast inducer of S1P efflux. Since the reconstituted HDL only contain apoA-I in addition to phospholipids, apoA-I is the prime candidate for this activity. In this respect, it is noteworthy that ATP binding cassette transporters (ABC) A1 and G1 as well as scavenger receptor B1, which are important cellular interaction partners for apoA-I or HDL-induced efflux of cholesterol or phosphatidylcholine, have been suspected to promote or modulate cellular S1P efflux as well [4, 8]. In fact glyburide, a pharmacological inhibitor of ABC transporters including ABCA1, was previously found to inhibit S1P efflux from red blood cells. However, although present in erythrocytes, functional experiments excluded ABCA1 and ABCA7 as the mediators of S1P efflux from these cells [7]. In agreement with the lacking involvement of ABCA1, we did not find any S1P efflux induced by lipid-free apoA-I which as the primary interaction partner of ABCA1 stimulates efflux of cholesterol and phosphatidylcholine from many cells. The roles of ABCG1 and SR-BI, which are typically interacting with both native and artificially lipidad HDL [29], for S1P efflux are as yet unknown.

To address the second question, whether apoM may regulate S1P plasma concentrations beyond transport in plasma, we compared the urinary excretion of S1P of wt and Apom
"/-
mice. Not unexpected, urine of wt mice did not contain much S1P. However, quantifiable amounts of S1P were excreted with the urine by Apom
"/-
mice. This data indicated that apoM plays some role for preventing urinary S1P excretion but does not inform whether the excreted S1P is of plasmatic or renal origin. On the one hand and next to hepatocytes, the epithelial cells lining the proximal tubules of the kidney are the only cells which express apoM [31]. Hence, kidney-derived apoM may play an important role in handling S1P, for example for the interaction with S1P receptors. In fact S1P and S1P receptors were reported to convey protection of the proximal tubule
epithelium against oxidative stress induced by ischemia/reperfusion injury [32]. On the other hand, the plasma-derived 22 kDa large murine apoM or 26 kDa large human apoM is ultrafiltrated through glomeruli and reabsorbed from the primary urine by the endocytic receptor megalin into the proximal tubule epithelial cells [16] so that the lack of apoM could interfere with the tubular recovery of S1P. To discriminate between these two explanations we investigated the urinary excretion of apoM and S1P by mice which lack megalin or the chloride-proton exchanger ClC-5 or the lysosomal cystine transporter cystinosin, which all play important roles for the tubular reabsorption of carrier proteins and their cargo from the primary urine [17, 19, 20]. We confirmed the previously observed urinary apoM excretion by megalin knock-out mice [16]. In addition, also mice without CLC-5 or cystinosin showed a urinary loss of apoM. Like Apom−/− mice, Lrp2−/− and Clcn5−/− mice showed clearly increased urinary S1P excretion. This difference in urinary S1P excretion was much less prominent if not absent in Ctns−/− mice. This may reflect the fact that the cystinosis model develops tubular dysfunction later in life [33]. In our hands, even at the age of 30-38 weeks, when tubular dysfunction is manifested, we did not see any increase in S1P excretion although apoM is excreted with the urine. Nevertheless the unusual urinary excretion of S1P in the urine of Apom−/−, Lrp2−/− and Clcn5−/− mice suggests that tubular reabsorption of apoM interferes with the urinary loss of S1P. However, in Lrp2−/− and Clcn5−/− mice the urinary loss of apoM and S1P appears too low to substantially decrease the plasma concentrations of apoM and S1P. Therefore it is also unlikely, that the urinary loss contributes to the lower plasma and HDL-S1P levels observed in Apom−/− mice [12, 13]. However, cubilin deficiency was previously reported to increase the turnover and decrease plasma albumin and apoA-I concentrations, slightly but significantly [18]. Quantitative studies in larger numbers of mice may hence unravel subtle differences.

In conclusion, our studies of mice differing by the expression of apoM or the activity of proteins involved in the tubular endocytosis of apoM indicate that binding of S1P by apoM plays at least two further roles in S1P metabolism beyond mediating S1P transport in HDL. First, HDL
facilitates S1P efflux from erythrocytes by both apoM-dependent and apoM-independent mechanisms. Second, apoM facilitates the reabsorption of S1P from the primary urine into tubular epithelial cells, however with no impact on S1P plasma concentration. By the two newly identified activities, apoM may modulate the paracrine and autocrine interactions of S1P with vascular endothelial and renal tubular epithelial cells, respectively. In addition we provide evidence for an additional HDL-associated factor which contributes to S1P efflux and transport by HDL.
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REFERENCES

1. Fyrst, H., and J. D. Saba. 2010. An update on sphingosine-1-phosphate and other sphingolipid mediators. Nature chemical biology 6: 489-497.

2. Hla, T., and A. J. Dannenberg. 2012. Sphingolipid signaling in metabolic disorders. Cell metabolism 16: 420-434.

3. Hornemann, T., and T. S. Worgall. 2013. Sphingolipids and atherosclerosis. Atherosclerosis 226: 16-28.

4. Nishi, T., N. Kobayashi, Y. Hisano, A. Kawahara, and A. Yamaguchi. 2014. Molecular and physiological functions of sphingosine 1-phosphate transporters. Biochimica et biophysica acta 1841: 759-765.

5. Bode, C., S. C. Sensken, U. Peest, G. Beutel, F. Thol, B. Levkau, Z. Li, R. Bittman, T. Huang, M. Tolle, M. van der Giet, and M. H. Graler. 2010. Erythrocytes serve as a reservoir for cellular and extracellular sphingosine 1-phosphate. Journal of cellular biochemistry 109: 1232-1243.

6. Ono, Y., M. Kurano, R. Ohkawa, H. Yokota, K. Igarashi, J. Aoki, M. Tozuka, and Y. Yatomi. 2013. Sphingosine 1-phosphate release from platelets during clot formation: close correlation between platelet count and serum sphingosine 1-phosphate concentration. Lipids in health and disease 12: 20.

7. Kobayashi, N., N. Kobayashi, A. Yamaguchi, and T. Nishi. 2009. Characterization of the ATP-dependent sphingosine 1-phosphate transporter in rat erythrocytes. The Journal of biological chemistry 284: 21192-21200.

8. Liu, X., S. L. Xiong, and G. H. Yi. 2012. ABCA1, ABCG1, and SR-BI: Transit of HDL-associated sphingosine-1-phosphate. Clinica chimica acta; international journal of clinical chemistry 413: 384-390.

9. Sattler, K., and B. Levkau. 2009. Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection. Cardiovascular research 82: 201-211.
10. Christoffersen, C., and L. B. Nielsen. 2013. Apolipoprotein M: bridging HDL and endothelial function. Current opinion in lipidology 24: 295-300.

11. Sevvana, M., J. Ahnstrom, C. Egerer-Sieber, H. A. Lange, B. Dahlback, and Y. A. Muller. 2009. Serendipitous fatty acid binding reveals the structural determinants for ligand recognition in apolipoprotein M. Journal of molecular biology 393: 920-936.

12. Christoffersen, C., H. Obinata, S. B. Kumaraswamy, S. Galvani, J. Ahnstrom, M. Sevvana, C. Egerer-Sieber, Y. A. Muller, T. Hla, L. B. Nielsen, and B. Dahlback. 2011. Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. Proceedings of the National Academy of Sciences of the United States of America 108: 9613-9618.

13. Karuna, R., R. Park, A. Othman, A. G. Holleboom, M. M. Motazacker, I. Sutter, J. A. Kuivenhoven, L. Rohrer, H. Matile, T. Hornemann, M. Stoffel, K. M. Rentsch, and A. von Eckardstein. 2011. Plasma levels of sphingosine-1-phosphate and apolipoprotein M in patients with monogenic disorders of HDL metabolism. Atherosclerosis 219: 855-863.

14. Ohkawa, R., K. Nakamura, S. Okubo, S. Hosogaya, Y. Ozaki, M. Tozuka, N. Osima, H. Yokota, H. Ikeda, and Y. Yatomi. 2008. Plasma sphingosine-1-phosphate measurement in healthy subjects: close correlation with red blood cell parameters. Annals of clinical biochemistry 45: 356-363.

15. Wolfrum, C., M. N. Poy, and M. Stoffel. 2005. Apolipoprotein M is required for prebeta-HDL formation and cholesterol efflux to HDL and protects against atherosclerosis. Nature medicine 11: 418-422.

16. Faber, K., V. Hvidberg, S. K. Moestrup, B. Dahlback, and L. B. Nielsen. 2006. Megalin is a receptor for apolipoprotein M, and kidney-specific megalin-deficiency confers urinary excretion of apolipoprotein M. Molecular endocrinology 20: 212-218.

17. Christensen, E. I., H. Birn, T. Storm, K. Weyer, and R. Nielsen. 2012. Endocytic receptors in the renal proximal tubule. Physiology 27: 223-236.
18. Aseem, O., B. T. Smith, M. A. Cooley, B. A. Wilkerson, K. M. Argraves, A. T. Remaley, and W. S. Argraves. 2013. Cubilin Maintains Blood Levels of HDL and Albumin. Journal of the American Society of Nephrology: JASN.

19. Wilmer, M. J., F. Emma, and E. N. Levchenko. 2010. The pathogenesis of cystinosis: mechanisms beyond cystine accumulation. American journal of physiology. Renal physiology 299: F905-916.

20. Devuyst, O., and R. V. Thakker. 2010. Dent's disease. Orphanet journal of rare diseases 5: 28.

21. Nykjaer, A., D. Dragun, D. Walther, H. Vorum, C. Jacobsen, J. Herz, F. Melsen, E. I. Christensen, and T. E. Willnow. 1999. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell 96: 507-515.

22. Christensen, E. I., O. Devuyst, G. Dom, R. Nielsen, P. Van der Smissen, P. Verroust, M. Leruth, W. B. Guggino, and P. J. Courtoy. 2003. Loss of chloride channel ClC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules. Proceedings of the National Academy of Sciences of the United States of America 100: 8472-8477.

23. Nevo, N., M. Chol, A. Bailleux, V. Kalatzis, L. Morisset, O. Devuyst, M. C. Gubler, and C. Antignac. 2010. Renal phenotype of the cystinosis mouse model is dependent upon genetic background. Nephrology, dialysis, transplantation: official publication of the European Dialysis and Transplant Association - European Renal Association 25: 1059-1066.

24. Ohnsorg, P. M., L. Rohrer, D. Perisa, A. Kateifides, A. Chroni, D. Kardassis, V. I. Zannis, and A. von Eckardstein. 2011. Carboxyl terminus of apolipoprotein A-I (ApoA-I) is necessary for the transport of lipid-free ApoA-I but not prelipidated ApoA-I particles through aortic endothelial cells. The Journal of biological chemistry 286: 7744-7754.
25. Bielawski, J., J. S. Pierce, J. Snider, B. Rembiesa, Z. M. Szulc, and A. Bielawska. 2009. Comprehensive quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. Methods in molecular biology 579: 443-467.
26. Berdyshev, E. V., I. A. Gorshkova, J. G. Garcia, V. Natarajan, and W. C. Hubbard. 2005. Quantitative analysis of sphingoid base-1-phosphates as bisacetylated derivatives by liquid chromatography-tandem mass spectrometry. Analytical biochemistry 339: 129-136.
27. Wang, S. S., O. Devuyst, P. J. Courtoy, X. T. Wang, H. Wang, Y. Wang, R. V. Thakker, S. Guggino, and W. B. Guggino. 2000. Mice lacking renal chloride channel, CLC-5, are a model for Dent's disease, a nephrolithiasis disorder associated with defective receptor-mediated endocytosis. Human molecular genetics 9: 2937-2945.
28. Segrest, J. P., M. C. Cheung, and M. K. Jones. 2013. Volumetric determination of apolipoprotein stoichiometry of circulating HDL subspecies. Journal of lipid research 54: 2733-2744.
29. Annema, W., and A. von Eckardstein. 2013. High-density lipoproteins. Multifunctional but vulnerable protections from atherosclerosis. Circulation journal : official journal of the Japanese Circulation Society 77: 2432-2448.
30. Frias, M. A., U. Lang, C. Gerber-Wicht, and R. W. James. 2010. Native and reconstituted HDL protect cardiomyocytes from doxorubicin-induced apoptosis. Cardiovascular research 85: 118-126.
31. Zhang, X. Y., X. Dong, L. Zheng, G. H. Luo, Y. H. Liu, U. Ekstrom, P. Nilsson-Ehle, Q. Ye, and N. Xu. 2003. Specific tissue expression and cellular localization of human apolipoprotein M as determined by in situ hybridization. Acta histochemica 105: 67-72.
32. Koch, A., J. Pfeilschifter, and A. Huwiler. 2013. Sphingosine 1-phosphate in renal diseases. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 31: 745-760.
33. Raggi, C., A. Luciani, N. Nevo, C. Antignac, S. Terryn, and O. Devuyst. 2014. Dedifferentiation and aberrations of the endolysosomal compartment characterize the early stage of nephropathic cystinosis. Human molecular genetics 23: 2266-2278.
Figure legends

Figure 1. Time-dependent efflux of S1P from washed human erythrocytes in the presence of 500 μg/ml bovine serum albumin (BSA, figures a, b, c, d), 250 or 500 μg/ml human HDL (a, b) or 300 μg/ml HDL from wt, Apom−/− or Apom+/+ mice (c,d). Figures 1a and 1c show absolute S1P amounts in one millilitre of supernatant after incubation of HDL or albumin with erythrocytes for the indicated time intervals and subsequent removal of erythrocytes by centrifugation. Figures 1b, 1d show data on net S1P efflux which were obtained by subtracting the S1P concentrations of the various HDL preparations at baseline from the S1P concentrations at the indicated time points. Values are mean±SD. Statistically significant differences between HDL of Apom−/− and wt mice as well as between HDL of Apom+/+ and wt mice are indicated by asterisks (*, p≤0.05; **, p≤0.01; ***, p≤0.001; Student-t-test).

Figure 2. Net S1P efflux from washed human erythrocytes in the presence of native and reconstituted HDL, lipid-free apoA-I, or bovine serum albumin. Presented are net amounts of S1P released by erythrocytes in one millilitre of supernatant after four hours incubation with 20 μg/ml or 200 μg/ml HDL isolated either from human plasma or artificially reconstituted by cholate dialysis of apoA-I and POPC (rHDL), 20 μg/ml lipid-free apoA-I, or 200 μg/ml BSA. Values are mean±SD.

Figure 3. ApoM determines S1P concentration in plasma (a), HDL (b) and urine (c) of wt, Apom−/−, Apom++ mice. Figures 2a and 2b represent S1P levels in plasma and HDL samples from 12-14 weeks old wt, Apom−/−, Apom++ mice. Figure 2c shows urinary S1P excretion by 6 and 16 weeks old Apom−/− mice and their wt littermates. Each point identifies data from individual mice. Solid lines indicate median values. The dashed line indicates the lower level of quantification of S1P by LC-MS in urine samples. (*, p≤0.05; **, p≤0.01, ***, p≤0.001; Mann-Whitney U-test).
Figure 4. Western Blotting of apoM in urine (a) and plasma (b) of Apom$^{-/-}$, Apom$^{+/+}$ mice and mice with defective megalin (Lrp2$^{-/-}$), ClC-5 (Clcn5$^{-/-}$) and cystinosin (Ctns$^{-/-}$) and their wt controls. 20 µl of urine or 11.5 µl of 1/10 diluted plasma were separated by SDS-PAGE and immunoblotted as described in the methods section. Note the absence of apoM from urine samples of wt mice but presence in urine samples of mice with defective tubular transport (a). By contrast apoM plasma levels appear indistinguishable between wt mice and the different tubular proteinuria models.

Figure 5. Concentrations of S1P in urine (a-c) or plasma (d-f) of mice with defective megalin (Lrp2$^{-/-}$) (a,d), ClC-5 (Clcn5$^{-/-}$) (b,e), or cystinosin (Ctns$^{-/-}$) (c,f) as compared to their wt controls. Each point represents data from an individual mouse sample. Figures 4a and 4d show urine and plasma levels of S1P in 11 weeks old Lrp2$^{-/-}$ mice (open symbols). Figures 4b and 4e show urine and plasma levels of S1P in 12-16 weeks old Clcn5$^{-/-}$ mice (open symbols). Figures 4c and 4f show urine and plasma S1P levels in 18 weeks old Ctns$^{-/-}$ mice (open symbols), in 20-24 weeks old Ctns$^{-/-}$ mice (closed symbols) and in 30-38 weeks old Ctns$^{-/-}$ mice (crossed symbols). Dashed lines indicate the lower level of quantification of S1P by LC-MS in urine samples. (**, p≤0.01; Mann-Whitney U-test). Note the higher medians of S1P excretion in mice with defective tubular protein reabsorption as compared to wt controls.
Figure 1

- Panel A: Graph showing the effect of HDL 500 µg/ml, HDL 250 µg/ml, BSA 500 µg/ml, and Blank on S1P pmol over time (0-6 hours).
- Panel B: Graph showing the effect of HDL Apom1, HDL wt, HDL Apom-/-, BSA, and Blank on S1P pmol over time (0-8 hours).
- Panel C: Graph showing the effect of HDL 500 µg/ml, HDL 250 µg/ml, and BSA on S1P pmol over time (0-6 hours).
- Panel D: Graph showing the effect of HDL Apom1, HDL wt, HDL Apom-/-, and BSA on S1P pmol over time (0-8 hours).
