Apolipoprotein M supports S1P production and conservation and mediates prolonged Akt activation via S1PR1 and S1PR3

アポリポタンパク質 M は S1P 産生促進および保護に寄与し、S1PR1 および S1PR3 を介した Akt の持続的な活性化を引き起こす

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Abbreviations

ApoA4: Apolipoprotein A4

ApoM: Apolipoprotein M

BSA: Bovine serum albumin

CHO: Chinese hamster ovary

ERK: Extracellular signal-regulated kinase

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LPP: Lipid phosphate phosphatase

Mfsd2b: Multiple facilitator superfamily domain containing 2B

PBS: Phosphate-buffered saline

S1P: Sphingosine 1-phosphate

S1PR1: Sphingosine 1-phosphate receptor 1

S1PR3: Sphingosine 1-phosphate receptor 3

SphK: Sphingosine kinase

Spns2: Spinster homolog 2

TBS-T: Tris-buffed saline containing 0.1% Tween20

Summary

Sphingosine 1-phosphate (S1P) is one of the lipid mediators involved in diverse physiological functions. S1P circulates in blood and lymph bound to carrier proteins. Three S1P carrier proteins have been reported, albumin, apolipoprotein M (ApoM) and apolipoprotein A4 (ApoA4). The carrier-bound S1P exerts its functions via specific S1P receptors (S1PR1-5) on target cells. Previous studies showed several differences in physiological functions between albumin-bound S1P and ApoM-bound S1P. However, molecular mechanisms underlying the carrier-dependent differences have not been clarified. In addition, ApoA4 is a recently identified S1P carrier protein, and its functional differences from albumin and ApoM have not been addressed. Here, we compared the three carrier proteins in the processes of S1P degradation, production and receptor activation. ApoM retained S1P more stable than albumin and ApoA4 in the cell culture medium when compared in the equimolar amounts. ApoM facilitated the S1P release from endothelial cells most efficiently. Furthermore, ApoM-bound S1P showed a tendency to induce prolonged activation of Akt via S1PR1 and S1PR3. These results suggest that the carrier-dependent functional differences of S1P are partly ascribed to the differences in the S1P stability, S1P-producing efficiency and signaling duration.

Key word: Sphingosine 1-phosphate, Apolipoprotein M, Apolipoprotein A4, LC-MS/MS

Introduction

Sphingosine 1-phosphate (S1P) is one of the lysophospholipidic bioactive molecules involved in diverse physiological functions (*1*), such as proliferation and migration of various types of cells (*2, 3*), lymphocyte trafficking (*4-7*), and regulation of angiogenesis and vascular permeability (*8-11*). S1P is produced intracellularly by phosphorylation of sphingosine by S1P-producing enzymes, sphingosine kinase 1 and 2 (SphK1/2) (*12, 13*), and released into the blood via S1P-specific transporters spinster homolog 2 (Spns2) (*14, 15*) and major facilitator superfamily domain containing 2B (Mfsd2b) (*16-18*). SphK1/2 are widely expressed in many types of cells, while Spns2 and Mfsd2b show limited expression in vascular endothelial cells and red blood cells (RBCs), respectively (*18, 19*). Endothelial cells and RBCs are recognized as major supply sources of S1P in blood, with the expression of both S1P-producing enzymes (SphK1/2) and S1P-transporters (Spns2 or Mfsd2b) while little or no expression of S1P-degrading enzymes such S1P phosphatases and S1P lyases (*20-22*). After the release via the transporters from endothelial cells and RBCs, S1P binds to carrier proteins and circulates in blood and lymph, then stimulates specific S1P receptors (S1PR1-5) (*1, 23-26*) on the surface of target cells to exert physiological functions. There are three types of S1P carrier proteins reported so far, albumin (*1*), apolipoprotein M (ApoM) (*27-29*) and apolipoprotein A4 (ApoA4) (*30*). Albumin is one of the most abundant proteins in the blood which functions as a carrier for small molecules such as

endogenous hormones, ions and various species of lipids (*31*). ApoM is bound primarily to high-density lipoproteins (HDLs) and retains S1P specifically in its hydrophobic pocket (*27, 32*), and ApoA4 is the third most abundant apolipoprotein in human HDLs and shows inhibition of vascular inflammation (*33, 34*). Recently, our group reported that ApoA4 served as an alternative S1P carrier in the absence of albumin and ApoM (*30*).

It has been reported that there are several differences in the physiological functions exerted by S1P depending on the carrier proteins. For example, albumin-bound S1P (albumin-S1P) activated Gαi and suppressed forskolin-induced increases in intracellular cAMP concentration in human umbilical vein endothelial cells (HUVECs), while ApoM-bound S1P (ApoM-S1P) did not (*35*). Intercellular adhesion molecule-1 expression induced by tumor necrosis factor-α stimulation was suppressed by ApoM-S1P, but not by albumin-S1P (*35*). Vascular barrier function enhanced by ApoM-S1P was more prolonged compared to albumin-S1P (*36*). Lymphopoiesis was inhibited only by ApoM-S1P (*37*). However, molecular mechanisms underlying these carrier-dependent functional differences have not been clarified yet. In addition, ApoA4 is a recently identified S1P carrier protein (*30*), and its functional differences from albumin and ApoM have not been addressed.

In this study, we compared these three S1P carrier proteins in the processes of S1P degradation, S1P production and receptor activation. ApoM retained S1P more stable than

albumin and ApoA4 in the culture medium of endothelial cells when compared in the equimolar

amounts. ApoM also facilitated the S1P release from endothelial cells most efficiently.

Furthermore, ApoM-bound S1P showed a tendency to induce prolonged activation of Akt via

S1PR1 and S1PR3. These results suggest that the carrier-dependent differences in physiological

S1P functions are partly ascribed to the differences in the S1P stability, S1P-producing efficiency

and signaling duration.

Materials and Methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee of Gunma University (approval number #21-067). C57BL/6JJcl male mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and maintained on normal chow diet with 12 hours light/dark cycles.

Materials

Flp-In^{TM}-CHO cells (ThermoFisher Scientific, Massachusetts, U.S.A.) stably expressing sphingosine kinase 1 (referred to as Flp-In-CHO-SphK1) and a plasmid vector for human Spns2 expression (pcDNA5-HA-hSpns2) were provided by Dr. T. Nishi (Osaka university). A lentivirus vector for human ApoM fused with the constant domain (Fc) of immunoglobulins (pCDH-ApoM-Fc-puro) (38) was provided by Dr. T. Hla (Harvard Medical School). A lentivirus vector for human Mfsd2b (pLV-hMfsd2b-puro) was purchased from Vector Builder (Chicago, U.S.A.). Lentivirus vectors for human S1PR1 and S1PR3 are described elsewhere (30). Antibodies against Akt (#9272), phospho-Akt (Ser473, #9271), ERK1/2 (#9102) and phospho-ERK1/2 (T202/Y204, #9106) were purchased from Cell Signaling Technology (Massachusetts, U.S.A.). S1P, deuterium-labeled S1P (S1P-d7), S1P (C17) and deuterium-labeled sphingosine (sphingosine-d7) were purchased from Avanti Polar Lipids (Alabama, U.S.A.).

Lyophilized powder of albumin from bovine serum (essentially fatty acid-free) was purchased

from Merck (Darmstadt, Germany).

Cell culture

Flp-In-CHO-SphK1 cells and human embryonic kidney (HEK293T) cells were

maintained in Ham's F12 medium (Wako, Osaka, Japan) and Dulbecco's modified Eagle's

medium (Wako), respectively, supplemented with 10% fetal bovine serum (FBS) and antibiotics

(62.5 µg/ml penicillin and 100 µg/ml streptomycin). For Flp-In-CHO-SphK1 cells, zeocin (200

µg/ml) was also added to maintain the stable transformants. Human umbilical vein endothelial

cells (HUVECs) were cultured on fibronectin-coated dishes in EGM-2 medium (Lonza, Basel,

Switzerland) supplemented with 10 % FBS. All the cells were maintained under a humidified

atmosphere in a 37°C incubator with 5% CO_2.

Lentivirus production

HEK293T cells were transfected with the lentivirus vectors along with lentiviral

packaging plasmids pVSV-G, pMDL/pPRE and pRSV-REV (Addgene, Massachusetts, U.S.A.)

using a calcium phosphate method. The lentiviral-containing media were collected 72 hours after

the transfection, filtered through a 0.45 µM filter, then aliquoted and stored at –130°C until use.

Establishment of the stable transformants in CHO and HEK293T cells

HEK293T cells were infected with lentivirus for the expression of ApoM-Fc or human

albumin, and maintained under the selection with 10 µg/ml puromycin. Flp-In-CHO-SphK1 cells were transfected with a pcDNA5-HA-hSpns2 vector by FuGENE HD Transfection Reagent (Promega, Madison, U.S.A.) along with an Flp-recombinase expression vector (pOG44) to induce the recombination at the FRT sites, and maintained under the selection with 200 µg/ml hygromycin (CHO-SphK1/Spns2 cells). Flp-In-CHO-SphK1 cells were infected with lentivirus for the expression of Mfsd2b, and maintained under the selection with 5 µg/ml puromycin (CHO-SphK1/Mfsd2b cells). CHO cells were infected with lentivirus for the expression of S1PR1 or S1PR3, and maintained under the selection with 5 µg/ml puromycin (CHO-S1PR1 or -S1PR3 cells).

RBC preparation from mouse blood

Blood was collected from C57BL/6JJcl male mice (retired, 20-35 weeks old) into the isotonic Tyrode-HEPES buffer (10 mM HEPES-2NaOH, 12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM D-glucose, 2.9 mM KCl, 1 mM $MgCl_2$, pH 7.4) containing 100 µg/ml heparin from mouse heart under the anesthesia with isoflurane, and centrifuged at 900 g for 20 minutes at 20°C. The pellets (RBC fraction) were washed three times with the Tyrode-HEPES buffer supplemented with 0.5% BSA.

Purification of the S1P carrier proteins

HEK293T cells stably expressing ApoM-Fc or albumin were seeded on 150 mm dishes

and cultured to 80% confluency. The cells were washed with phosphate-buffered saline (PBS) to remove FBS-derived proteins, then cultured in Opti-MEM (ThermoFisher Scientific) for 24 hours. The culture supernatant was collected and centrifuged at 1,500 rpm for 10 minutes to remove cell debris. Recombinant ApoM-Fc and albumin in the culture supernatant were purified by anion exchange chromatography on a RESOURSE Q column (1 ml, GE Healthcare Life Science, Chicago, U.S.A.) with a liner gradient of NaCl from 0 to 350 mM by an AKTA purifier 10 liquid chromatography system (GE Healthcare Life Science). The fractions containing the recombinant proteins were collected and dialyzed against PBS using Amicon Ultra-15 centrifugal filter units (Merck).

Histidine-tagged ApoA4 proteins (ApoA4) were expressed in *E.coli* (BL-21 strain), and recovered in the soluble fractions of the cell lysate. The recombinant proteins were purified by affinity chromatography on a HisTrap FF column (5 ml, GE Healthcare Life Science) with a liner gradient of imidazole from 5 mM to 250 mM by the AKTA purifier 10 liquid chromatography system and dialyzed against PBS using Amicon Ultra-15 centrifugal filter units.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

S1P and S1P-d7 in the culture supernatant and plasma were extracted by mixing with 4 volumes of methanol containing 25 nM S1P (C17) as an internal standard. After the centrifugation at 15,000 rpm for 5 minutes, the supernatant was evaporated, re-dissolved in 60% methanol and

applied to LC-MS/MS analyses using an LCMS-8050 system (Shimadzu, Japan, Kyoto) as described before (*39*).

S1P-d7 degradation assay in HUVECs

Prior to assays, S1P-d7 (1 µM) was loaded to S1P carrier proteins dissolved in Opti-MEM for 1 week at 4°C (*32*). HUVECs were seeded on 24 well plates (1.0×10^5 cells/well) and incubated for 24 hours. The cells were washed with Opti-MEM twice and incubated with the carrier-bound S1P-d7 for indicated times. The culture supernatant was collected, centrifuged at 15,000 rpm for 5 minutes at 4°C, then applied to the LC-MS/MS analyses.

S1P-d7 degradation assay in mouse blood

C57BL/6JJcl male mice at 8-9 weeks old were used for the experiment. A 15 µM stock solution of S1P-d7 was prepared with either of the carrier proteins (600 µM BSA, 15 µM ApoM, and 45 µM ApoA4) and administered into retro-orbital venous so that the S1P-d7 concentration in blood would be approximately 1 µM, assuming the blood volume of mice to be 1 ml per 14 g mouse body weight (*40*). Blood was collected with heparin at indicated times. Plasma was prepared from the blood samples and applied to the LC-MS/MS analyses.

S1P production assay in cultured cells and RBCs

Cells (HUVECs, CHO-SphK1/Spns2 or CHO-SphK1/Mfsd2b) were seeded on 24 well plates (1.5×10^5 cells/well) and incubated for 24 hours. The cells were washed with Opti-MEM

twice and incubated with the S1P carrier proteins dissolved in Opti-MEM for indicated times. In some experiments, CHO-SphK1/Mfsd2b cells were pre-incubated with 1 µM sphingosine-d7 in Opti-MEM containing 0.5% BSA for 1 hour at 37°C, then washed three times with the buffer to remove un-incorporated sphingosine-d7. The culture supernatant was collected, centrifuged at 15,000 rpm for 5 minutes at 4°C, and applied to the LC-MS/MS analyses.

RBCs (5.0×10^7 cells/tube) were incubated with 1 µM sphingosine-d7 in the Tyrode-HEPES buffer containing 0.5% BSA for 1 hour at 37°C, then washed three times with the buffer to remove un-incorporated sphingosine-d7. The RBCs were incubated with the S1P carrier proteins dissolved in the Tyrode-HEPES buffer for indicated times. After the centrifugation at 1,500 rpm for 10 minutes, the supernatant was applied to the LC-MS/MS analyses.

Western blot analysis

Total cell lysates were prepared in a cell lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2mM EDTA, 10 mM 2-glycerophosphate, 1 mM Na_3VO_4, 5 mM NaF, 1% Triton X-100) containing cOmplete protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined by Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific), and equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim-milk in Tris-buffed saline containing 0.1% Tween20 (TBS-T), and

incubated with primary antibodies diluted in the blocking solution at 4°C overnight. After

washing with TBS-T three times, the membranes were incubated with HRP-conjugated secondary

antibodies in the blocking solution for 1 hour at room temperature. After the wash with TBS-T,

the membranes were incubated with Immobilon Forte Western HRP Substrate (Merck) according

to the manufacturer's instructions and visualized using an ImageQuant LAS 4010 image analyzer

system (GE Healthcare Life Science).

Quantitative PCR analysis

Total RNA was isolated from cells using ISOGEN II (Nippon Gene, Tokyo, Japan) and

purified using RNA spin columns (GE Healthcare Life Science). Total RNA was reverse-transcribed

to cDNA using ReverTra Ace™ (Toyobo, Osaka, Japan). Quantitative PCR was performed using

Thunderbird SYBR Green qPCR mix (Toyobo) and a StepOne Plus Real-Time PCR System

(ThermoFisher Scientific) according to the manufacturer's instructions. The primers for PCR

analyses were as follows. Mouse SphK1, forward 5'-ACAGTGGGCACCTTCTTTC-3' and

reverse 5'-CTTCTGCACCAGTGTAGAGGC-3', human Spns2, forward 5'-
CATGTGGATCCCGCTCTACC-3' and reverse 5'-AGGTGATGGCCCCAAAGATG-3', human

Mfsd2b, forward 5'-CCACCGCCTACCGGATGA-3' and reverse 5'-
GCCGCAATGCAGTAGAGATG-3', glyceraldehyde 3-phosphate dehydrogenase, forward 5'-
GCCAAGGTCATCCATGACAACT-3' and reverse 5'-GAGGGGCCATCCACAGTCTT-3'.

Statistical analysis

Results were expressed as mean +/– standard deviation (SD). Sample sizes were indicated in figure legends. Statistical analysis was performed using R software (version 4.2.1). One-way analysis of variance followed by Tukey's test was performed for comparing more than 2 groups. Two-tailed Student's *t*-test was used to directly compare two groups. A p-value < 0.05 was considered to be statistically significant.

Results and Discussion

ApoM retains S1P more stable than albumin and ApoA4 in the HUVEC culture medium

To compare the S1P stability among the 3 carrier proteins both *in vitro* and *in vivo*, we prepared recombinant human albumin, human ApoM fused with the Fc domain of immunoglobulins (ApoM-Fc) and human ApoA4. Albumin and ApoM-Fc were expressed in HEK293T cells, and ApoA4 was expressed in *E.coli* BL-21, then purified by column chromatography (Figure 1). S1P labeled with deuterium (S1P-d7) was loaded onto each of the S1P carrier proteins dissolved in Opti-MEM for 1 week at 4°C (*32*) prior to the experiments and added to the culture medium of HUVECs at the final concentration of 1 µM. First, we compared the S1P decreases in the culture medium between the recombinant human albumin and bovine serum albumin. Since there were no discernible differences between human and bovine albumin (Figure 2), we used bovine albumin thereafter.

When S1P-d7 (1 µM) was loaded onto equal amounts of the carrier proteins (1 µM each), ApoM-Fc-bound S1P-d7 was retained most stably in the HUVEC culture medium with more than 90% of the S1P-d7 still remaining after 60 minutes (Fig.3A). In contrast, albumin- and ApoA4-bound S1P-d7 decreased to less than 50% within 10 minutes, and only about 10% of S1P-d7 was retained after 60 minutes (Fig.3A). Phospholipids like S1P are mainly dephosphorylated in blood by lipid phosphate phosphatases (LPPs) present on the cell membrane of endothelial cells, and

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LPP3 is the major type of LPPs expressed in HUVECs (*1, 41*). The results shown in Fig. 3A

indicate that ApoM protects S1P most efficiently from the degradation by LPPs or from the loss

to the culture environment (e.g., absorption to plasticware) and that albumin and ApoA4 show

similar S1P retainability, when compared at the equimolar ratio.

Albumin is the most abundant protein in the blood which is contained at around 600

µM. Thus, we carried out the experiments in the conditions that 1 µM S1P-d7 was mixed with

physiological concentrations of the carrier proteins in blood; 600 µM albumin, 1 µM ApoM, and

3 µM ApoA4. As shown in Fig. 3B, albumin retained more than 90% of S1P-d7 at similar levels

as ApoM after 60 minutes. As for ApoA4, the S1P-d7 decrease was slowed down compared to 1

µM ApoA4 in Fig. 1A (20% vs. 35% at 10 min), but still less than 10% of S1P-d7 remained after

60 minutes. These results demonstrated that excess amounts of albumin retained S1P at a

comparable level as ApoM at least in the culture medium of endothelial cells, although the S1P

retainability at the equimolar amounts was weaker than ApoM.

To examine the carrier-dependent S1P retainability *in vivo*, we next administered the

carrier protein-bound S1P-d7 to mice and compared the S1P-d7 decreases in the blood. S1P-d7

was pre-bound to the carrier proteins at the physiological concentrations, and the final S1P-d7

concentration in blood was adjusted to be around 1 µM. Administration of S1P-d7 and each blood

collection were performed from one side of the retro-orbital venous alternately as indicated in Fig.

4A. In contrast to the results in HUVECs, we did not observe the differences in the S1P-d7

decreases among the carrier proteins (Fig. 4B). The half-life of the S1P-d7 was less than 10 min

regardless of the types of the carrier proteins, which is in accordance with the previously reported

values in the albumin-bound S1P (*42*). It is reported that overexpression of ApoM or

administration of the recombinant ApoM-Fc resulted in increased S1P concentration in blood (*32,

38*). Thus, we also measured the endogenous S1P concentrations in the blood after the

administration of the carrier-bound S1P-d7. As shown in Fig. 4C, the mice administered with

ApoM-Fc-bound S1P-d7 showed higher S1P concentrations by around 200 nM than the other 2

groups at all the time points measured. These results indicate that S1P-d7 bound to ApoM-Fc was

replaced with endogenously produced S1P, which resulted in increased S1P concentration. In

contrast, albumin and ApoA4 did not show such S1P increases.

In contrast to the degradation assays in HUVECs, the stability of albumin- and ApoM-

Fc-bound S1P-d7 in mouse blood was very low, with a decrease to less than 10% after 60 minutes.

The exact reasons for this difference remain unknown, but one of the possibilities is that S1P-

degrading activity is much higher in blood than in cultured HUVECs. Another possibility is that

there are high levels of various lipids contained in blood including free fatty acids, phospholipids,

lysophospholipids like S1P and lysophosphatidic acids, and so on. S1P-d7 on albumin and ApoM

can be replaced by these endogenous free fatty acids and S1P in a certain equilibrium. The S1P

increases observed in the ApoM-Fc-administered mice partially support this idea.

Spns2-mediated S1P release is facilitated by ApoM

Endothelial cells and RBCs are major supply sources of S1P in blood in homeostatic

conditions. Platelets also release a massive amount of S1P during the coagulation processes. S1P

is produced intracellularly and released into the blood by an endothelial-specific S1P transporter

Spns2 or by an RBC/platelet-specific S1P transporter Mfsd2b. Next, we examined if the

efficiency of S1P release is different depending on the types of the S1P carrier proteins outside of

the S1P-producing cells. Physiological concentrations of the carrier proteins were added to the

culture medium, and the culture supernatants were collected over time for the S1P measurement

by LC-MS/MS.

When examined in HUVECs, S1P release was facilitated most efficiently by ApoM-Fc

at earlier time points up to 12 hours (Fig. 5A). S1P-release was almost comparable between

ApoM-Fc and albumin after 24 hours, while ApoA4 did not contribute to the S1P release at any

time points (Fig. 5A). Since the amount of endogenous S1P release from RBCs was extremely

low, RBCs were pre-loaded with deuterium-labeled sphingosine (sphingosine-d7), and the release

of S1P-d7 was evaluated as described (*16*). In contrast to the results in HUVECs, S1P-d7 release

was facilitated most efficiently by albumin at earlier time points up to 3 hours (Fig. 5B). Although

the exact mechanisms remain unclear, endothelial cells and RBCs might have different

preferences for the carrier proteins for the efficient S1P release.

S1P-release was also examined in reconstituted systems in CHO cells stably overexpressing SphK1 (CHO-SphK1 cells) and either of Spns2 (CHO-SphK1/Spns2 cells) or Mfsd2b (CHO-SphK1/Mfsd2b cells). The expression levels of SphK1, Spns2 and Mfsd2b were confirmed by qPCR analyses (Figure 6A). No S1P release was observed in the parental cell line CHO-K1 nor CHO-SphK1 cells regardless of the carrier proteins (Figure 6B). In CHO-SphK1/Spns2 cells, S1P was efficiently released in the presence of ApoM-Fc, but much less in albumin and ApoA4 (Fig. 5C). However, S1P release from CHO-SphK1/Mfsd2b cells was extremely low again (Figure 6C). It is probably because Mfsd2b proteins could not be efficiently folded into the right conformation when ectopically expressed in CHO cells, since Mfsd2b is predicted to have 12 transmembrane domains (*17*) and endogenously-produced S1P was not released efficiently. Thus, CHO-SphK1/Mfsd2b cells were pre-loaded with sphingosine-d7, and the release of S1P-d7 was evaluated. As a result, S1P-d7 release was detected in all of the carriers, with the order of ApoM > albumin >> ApoA4 (Fig. 5D). While albumin showed better S1P-d7 release from RBCs (Fig. 5B), ApoM was better in CHO- SphK1/Mfsd2b cells. The efficiency of S1P release might be determined not only by simple interactions between the transporter proteins and the carrier proteins, but also by some other factors in a cell-type-specific manner. Overall, our data show that ApoM facilitates the Spns2-mediated S1P release most efficiently.

ApoM-bound S1P has a tendency to induce prolonged activation of Akt via S1PR1 and S1PR3

Among the 5 types of S1P receptors (S1PR1-5), S1PR1 is the most abundantly expressed in vascular endothelial cells, followed by S1PR3. Stimulation of S1PR1 and S1PR3 by S1P activates mainly Gαi protein and subsequent downstream signaling pathways such as Akt and ERK1/2. Endothelial cell migration and angiogenesis induced by S1P-S1PR1 signaling have been ascribed to the activation of PI3K/Akt/endothelial NO synthase pathway (*43*), while pro-survival/anti-apoptotic signaling from S1PR1 is closely correlated with increased activation of ERK1/2 (*44, 45*). However, most of the signaling studies for S1P receptors have been conducted with albumin-bound S1P, and almost no studies paid close attention to the ratio of S1P to its carrier proteins. Since our results above showed that S1P stability in the cell culture medium was different depending on the concentrations of the carrier proteins, we examined the S1P-induced activation of Akt and ERK1/2 via S1PR1 and S1PR3 both in the equimolar and physiological concentrations of the carrier proteins.

CHO cells that express almost no endogenous S1P receptors were transfected with S1PR1 or S1PR3 (CHO-S1PR1 or -S1PR3 cells) and stimulated with S1P to examine the activation of Akt and ERK1/2 by Western blot analyses. At first, we stimulated the cells for 5 minutes with varying concentrations of S1P pre-loaded with the equimolar amounts of the carrier proteins. As shown in Figures 7 and 8, Akt and ERK1/2 were activated in an S1P-dose-dependent manner regardless of the carrier proteins both in CHO-S1PR1 and CHO-S1PR3 cells. In CHO-

S1PR3 cells, ApoM-Fc-S1P showed a tendency to activate Akt and ERK1/2 more efficiently than the other carriers, as little as 1 nM (Figure 8, indicated by arrows). Since 10 nM S1P was enough to induce the sub-saturation levels of phosphorylation of Akt and ERK1/2, we fixed the S1P concentration at 10 nM in the following time-course experiments with different concentrations of the carrier proteins.

When stimulated with 10 nM S1P with the equimolar amounts of the carrier proteins (10 nM each), both Akt and ERK1/2 showed activation with peaks around 5~10 minutes both in CHO-S1PR1 (Fig. 9A) and CHO-S1PR3 (Fig. 9B) cells. There was no obvious difference in the peak activation levels of Akt and ERK1/2 among the carrier proteins. However, ApoM-Fc-S1P showed a tendency to induce prolonged activation of Akt at 30 minutes compared to albumin- and ApoA4-S1P both in CHO-S1PR1 (Fig. 9A, arrows) and CHO-S1PR3 cells (Fig. 9B, arrows), although a statistically significant difference was not obtained.

Since we observed that albumin at the physiological concentration retained S1P with comparable levels as ApoM-Fc in the cell culture medium (Fig. 3B), we examined if S1P pre-loaded on the excess amount of albumin also induces prolonged activation of Akt like ApoM-Fc-S1P. However, albumin-S1P (10 nM S1P with 600 μM albumin) did not show such prolonged Akt activation in CHO-S1PR1 (Fig. 9C) nor -S1PR3 (Fig. 9D) cells, while ApoM-Fc-S1P (10 nM S1P with 1 μM ApoM-Fc) showed the trend again in CHO-S1PR1 (Fig. 9C, arrows) and -S1PR3 cells

(Fig. 9D, arrows). The trend of prolonged Akt activation by ApoM-Fc-S1P was observed even after 60 minutes both in CHO-S1PR1 (Figure 10, arrows) and in CHO-S1PR3 cells (Figure 11, arrows).

These results together indicate that ApoM-bound S1P induces prolonged Akt activation via S1PR1 and S1PR3 while albumin and ApoA4 do not exert such effects even if they exist in the excess amounts over S1P. One of the reported functional differences between ApoM and albumin is that ApoM-S1P enhances the vascular barrier function in a prolonged manner compared to albumin-S1P (*36*). Vascular barrier functions are regulated by complex mechanisms which involve the formation of adherens junctions and cytoskeletal re-organization, and Akt is one of the important intermediate-signaling molecules in both processes. Prolonged Akt activation by ApoM-S1P observed in this study might be one of the underlying mechanisms for the enhanced vascular barrier function by ApoM-S1P.

Although the S1P retainability in the cell culture medium was improved by the excess amount of albumin (Fig. 3B), it did not lead to improved receptor activation, suggesting that the functional differences among the S1P carrier proteins are not determined only by the S1P stability outside of the target cells. It is possible that ApoM directly interacts with S1P receptors for efficient transfer of S1P and prolonged activation of the S1P receptors, which should be determined in future studies.

In summary, we showed that ApoM had the best retainability of S1P among the 3 carrier proteins *in vitro* when compared in the equimolar amounts. ApoM also promoted the S1P release most efficiently from the S1P-producing cells such as endothelial cells and CHO cells reconstituted with SphK1 and Spns2. Furthermore, ApoM-S1P had a tendency to induce the prolonged activation of Akt via S1PR1 and S1PR3. Excess amounts of albumin showed improved S1P retainability *in vitro*, but it did not result in prolonged Akt activation (Figure 12). Functional differences of S1P depending on the carrier proteins might be partly explained by the ApoM's abilities to retain S1P stably and to facilitate S1P release from the producer cells, but further studies are required to fully understand the underlying mechanisms.

Acknowledgements

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Figures

Carrier protein	Albumin	ApoM-Fc	ApoA4
Host	293T	293T	E.Coli (BL21-A1)
Column	RESOURCE Q	RESOURCE Q	HisTrap
Separation	Anion exchanger	Anion exchanger	Affinity

Albumin: 66.5 kDa   ApoM-Fc: 50 kDa   ApoA4: 46 kDa
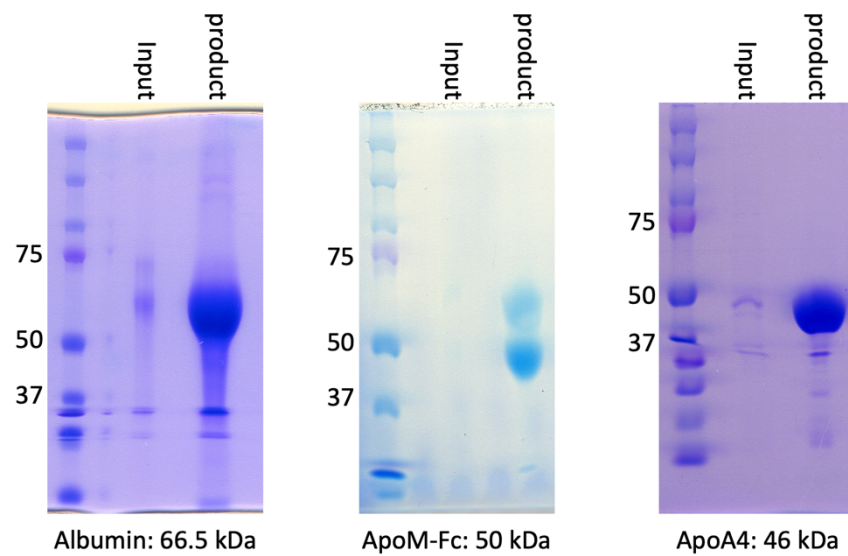
Figure 1. Preparation of the S1P carrier proteins

Albumin and ApoM-Fc were expressed in HEK293T cells, and ApoA4 was expressed in *E.coli*

BL-21, then purified by column chromatography as indicated. The proteins before (Input) and after

(Product) the purification were separated by 10% sodium dodecyl sulfate-polyacrylamide gel

electrophoresis, and revealed by Coomassie Brilliant Blue G-250 staining.
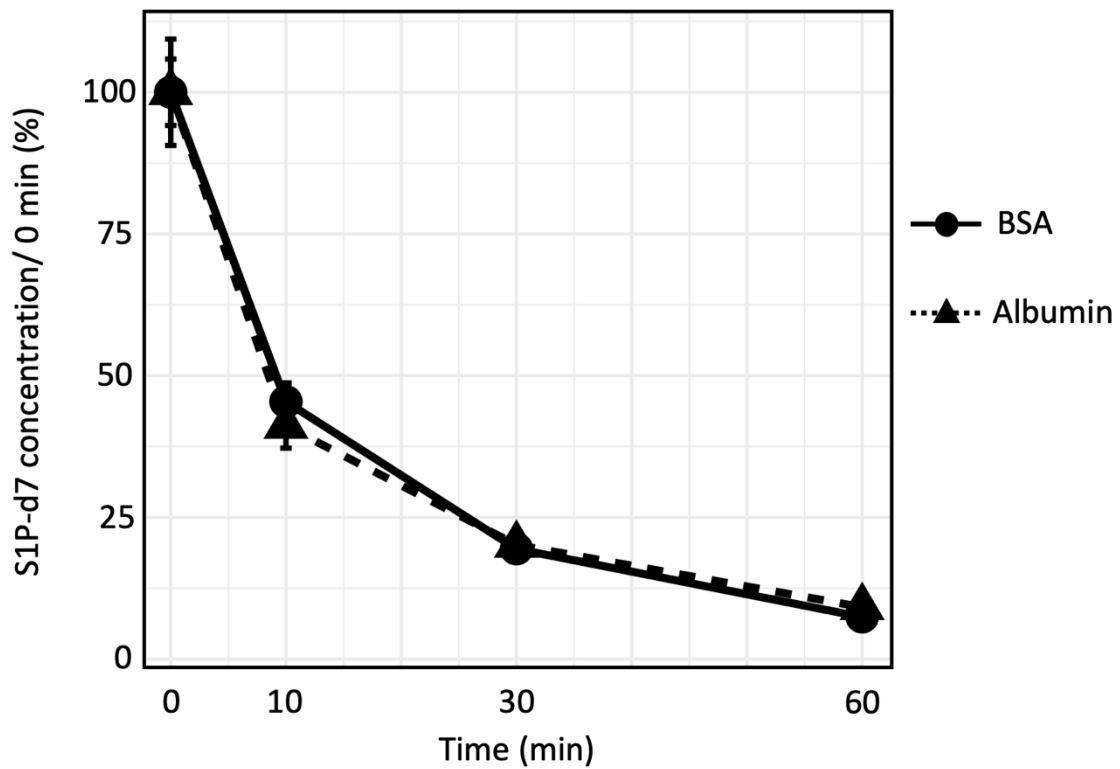
Figure 2. Comparison of the S1P degradation in HUVECs between the recombinant human albumin and bovine serum albumin

Line plots of S1P-d7 concentrations in the culture supernatant in HUVECs. S1P-d7 (1 µM) was bound to recombinant human albumin (Albumin) or bovine serum albumin (BSA) at equal amounts (1 µM each), and added to the culture medium. The culture supernatants were collected at indicated times, and S1P-d7 concentration was determined by LC-MS/MS analyses. Each data point is expressed as the percentages of the initial concentrations (mean +/– SD, n = 3).

(A)
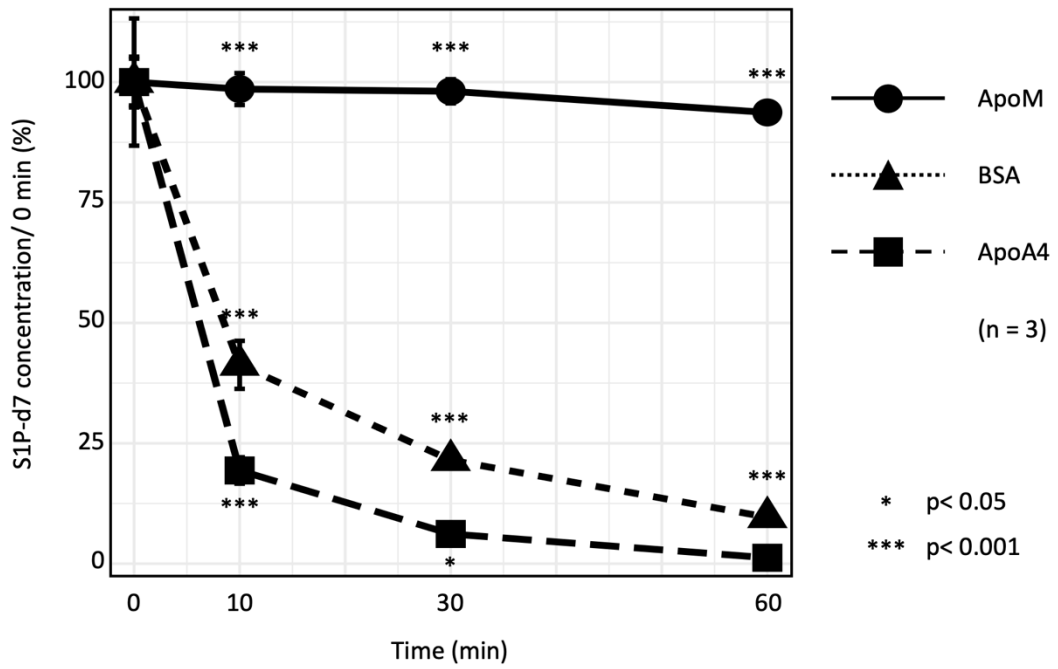
(B)
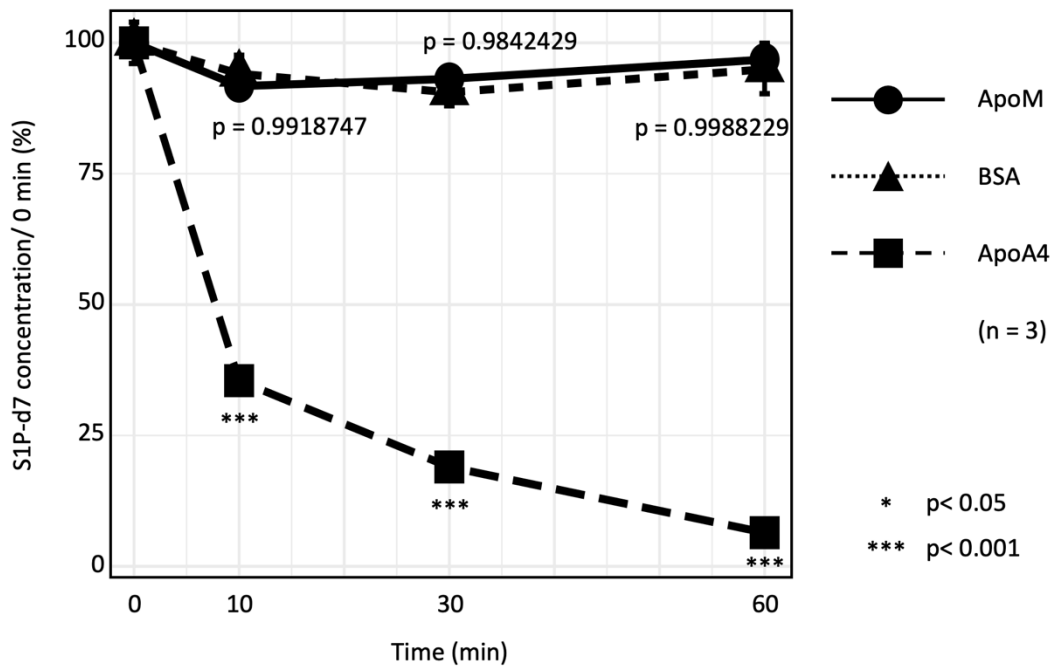
Figure 3. Analyses of the carrier protein-dependent differences in S1P degradation in HUVECs

Line plots of S1P-d7 concentrations in the culture supernatant in HUVECs. S1P-d7 (1 µM) was

bound to the carrier proteins at equal amounts (1 µM each) (A) or at the physiological

concentration (600 µM albumin, 1 µM ApoM and 3 µM ApoA4) (B), and added to the culture

medium. The culture supernatants were collected at indicated times, and S1P-d7 concentration

was determined by LC-MS/MS analyses. Each data point is expressed as the percentages of the

initial concentrations (mean +/– SD, n = 3). The data are representative of at least 3 independent

experiments with essentially the same results. Statistically significances were analyzed by

Turkey's test (***$p < 0.001$, *$p < 0.05$).
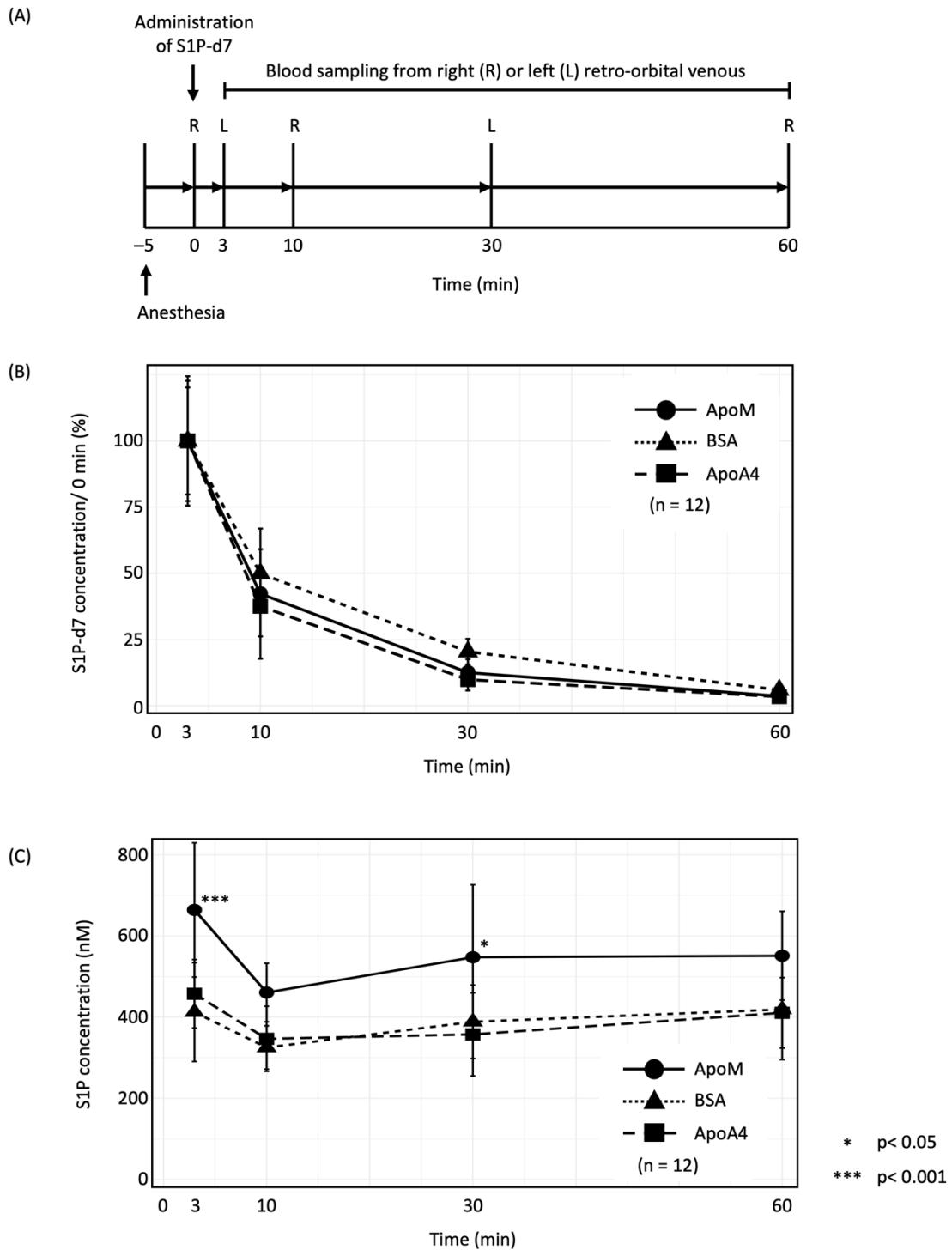
Figure 4. Analyses of the carrier protein-dependent differences in S1P degradation in mouse blood

(A) Schematic diagram illustrating the S1P-d7 degradation assay in mouse blood. Line plots of

S1P-d7 (B) and S1P (C) concentrations in mouse plasma. S1P-d7 bound to the carrier proteins were prepared as described in the Materials and Methods, and administered into retro-orbital venous so that the S1P-d7 concentration in blood would be approximately 1 µM. Blood was collected from retro-orbital venous at indicated times. Administration of S1P-d7 and each blood collection were performed from one side of the retro-orbital venous alternately as indicated in (A). Plasma was prepared from the collected blood, and S1P-d7 and S1P concentrations were determined by LC-MS/MS. Each data point is expressed as the percentages of the initial concentrations (mean +/– SD, n = 12). Statistically significances were analyzed by Turkey's test (***$p < 0.001$, *$p < 0.05$).
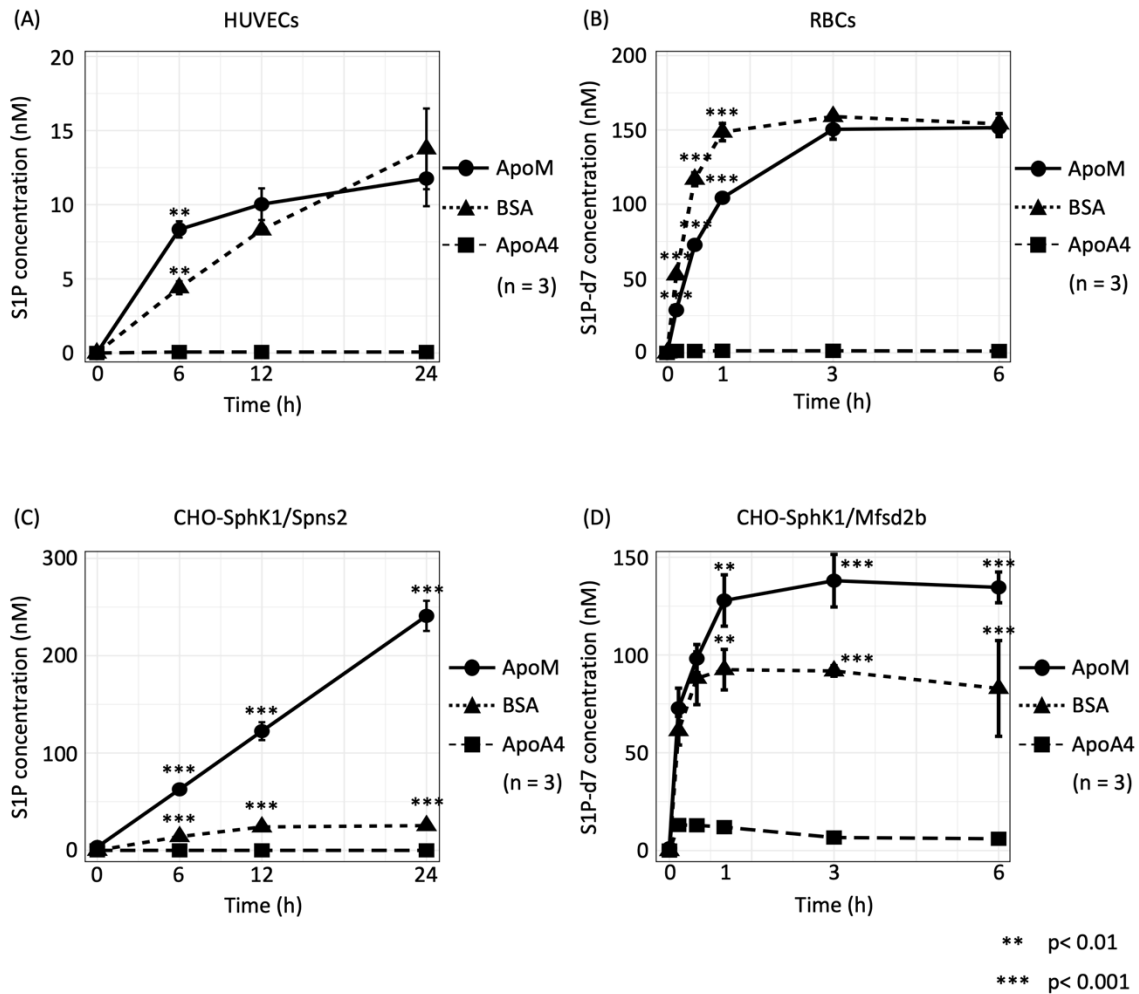
Figure 5. Analysis of the carrier protein-dependent differences in S1P release

Line plots of S1P (A and C) and S1P-d7 (B and D) concentrations in the culture supernatant from

HUVECs (A), mouse RBCs (B), CHO-SphK1/ Spns2 cells (C) and CHO-SphK1/ Mfsd2b cells

(D). Physiological concentration of the carrier protein (600 μM albumin, 1 μM ApoM and 3 μM

ApoA4) were added to the culture medium, and the culture supernatants were collected at

indicated times. The cells were loaded with sphingosine-d7 (1 μM) for 1 hour prior to the assays

in B and D. S1P and S1P-d7 concentrations were determined by LC-MS/MS analyses. Each data

point is expressed as mean +/– SD (n = 3). The data represent at least 3 independent experiments

with essentially the same results. Statistically significances were analyzed by Turkey's test (***p<

0.001, **p< 0.01).
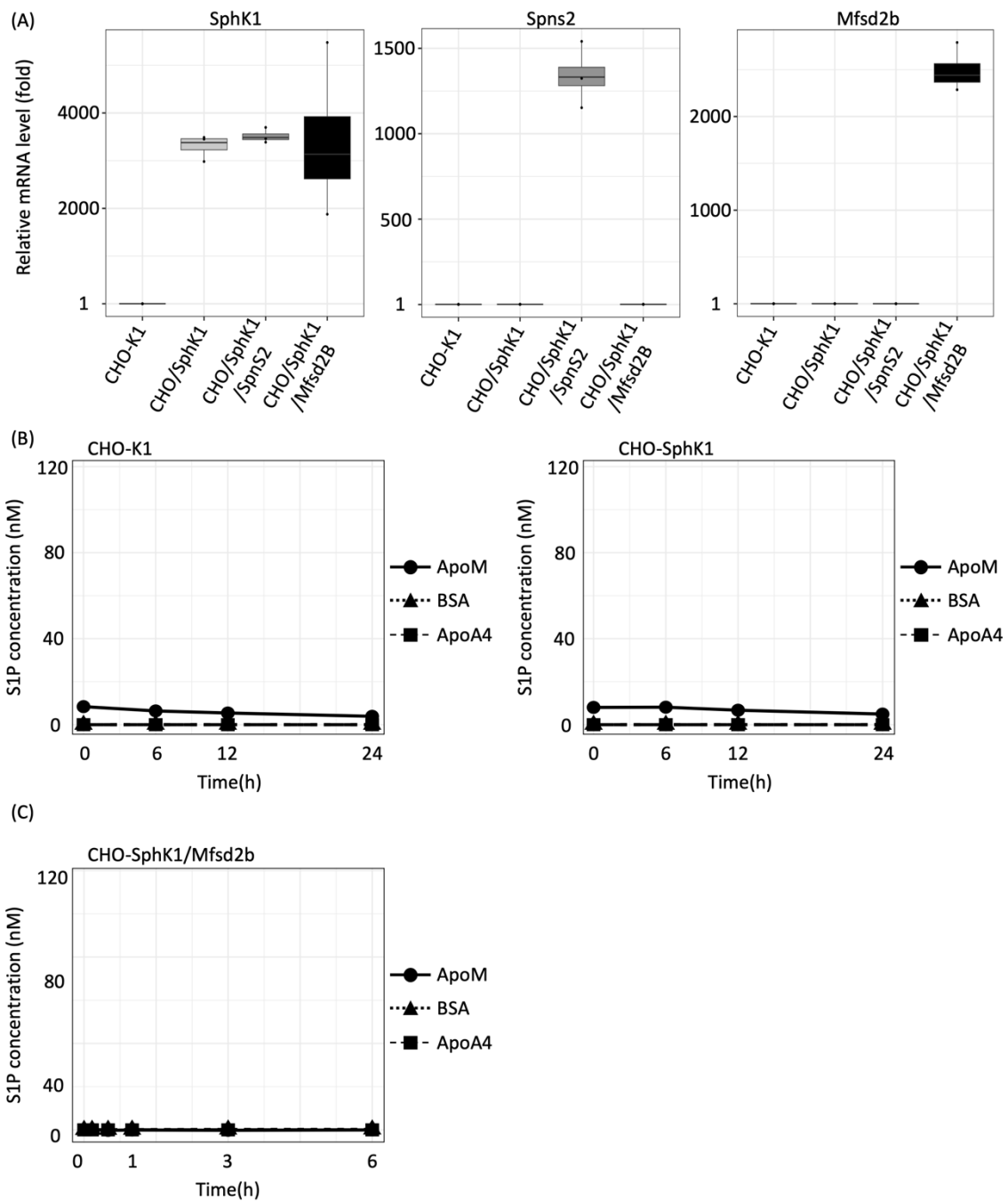
Figure 6. Analysis of the carrier protein-dependent differences in S1P production

(A) Expression levels of SphK1, Spns2 and Mfsd2b mRNA were compared by qPCR analyses in

CHO-K1, Flp-In-CHO-SphK1, CHO-SphK1/Spns2 and CHO-SphK1/Mfsd2b cells. Data are

normalized to the expression levels of glyceraldehyde 3-phosphate dehydrogenase and expressed

as fold changes to CHO-K1 cells.

Line plots of S1P concentrations in the culture supernatant from CHO-K1 (B, left), CHO-SphK1

(B, right) and CHO-SphK1/ Mfsd2b cells (C). Physiological concentration of the carrier protein

(600 µM albumin, 1 µM ApoM and 3 µM ApoA4) were added to the culture medium, and the

culture supernatants were collected at indicated times. S1P concentrations were determined by

LC-MS/MS analyses (mean +/– SD, n = 3).

Figure 7. Analyses of Akt and ERK activation by the carrier-bound S1P via S1PR1 (S1P dose-dependency)

The results of Western blot analyses from 3 independent experiments are shown separately,

together with the statistically processed graphs on the bottom (mean +/– SD). CHO-S1PR1 cells

were stimulated for 5 minutes with increasing concentrations of S1P bound to the equal

concentration of the carrier proteins. The total cell lysates were prepared, and the activation of

Akt and ERK1/2 was examined by Western blot analyses with the phospho-specific antibodies

(left side). The band intensity was digitized using Image J software, and the ratio of the

phosphorylated forms to total amounts was calculated (right side). The data are expressed in the
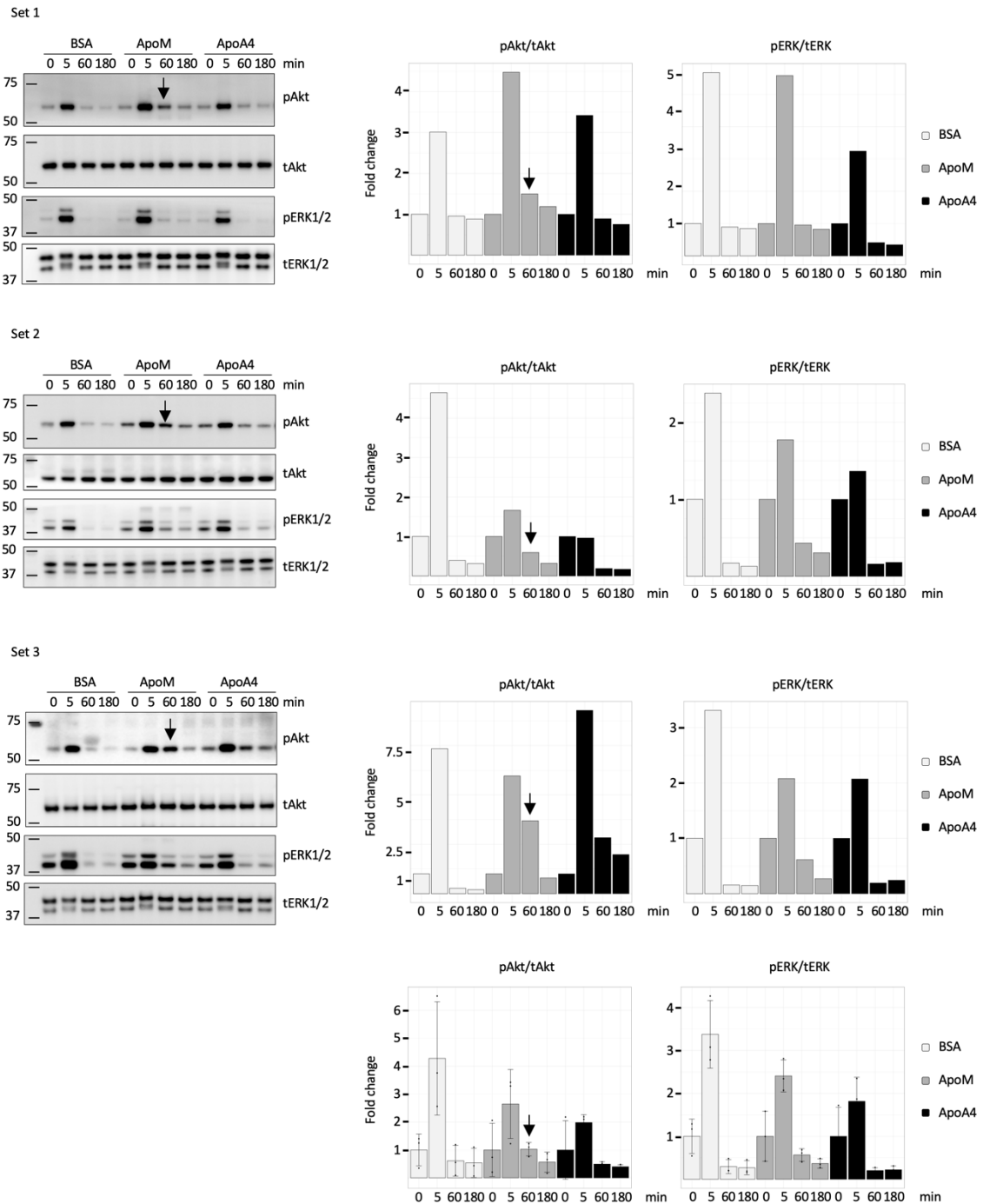
fold change from the no-stimulation control.

Figure 8. Analyses of Akt and ERK activation by the carrier-bound S1P via S1PR3 (S1P dose-dependency)

The results of Western blot analyses from 3 independent experiments are shown separately,

46

together with the statistically processed graphs on the bottom (mean +/− SD). CHO-S1PR3 cells

were stimulated for 5 minutes with increasing concentrations of S1P bound to the equal

concentration of the carrier proteins. The total cell lysates were prepared, and the activation of

Akt and ERK1/2 was examined by Western blot analyses with the phospho-specific antibodies

(left side). The band intensity was digitized using Image J software, and the ratio of the

phosphorylated forms to total amounts was calculated (right side). The data are expressed in the

fold change from the no-stimulation control.

(A) CHO-S1PR1 cells: Equal concentration (BSA: ApoM: ApoA4 = 10: 10: 10 nM)
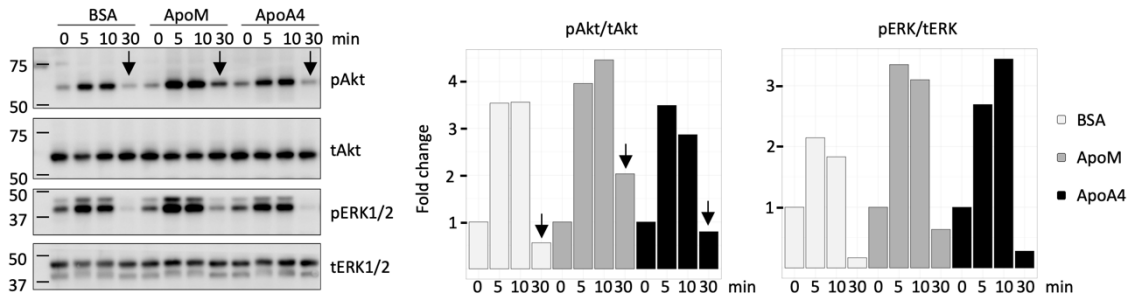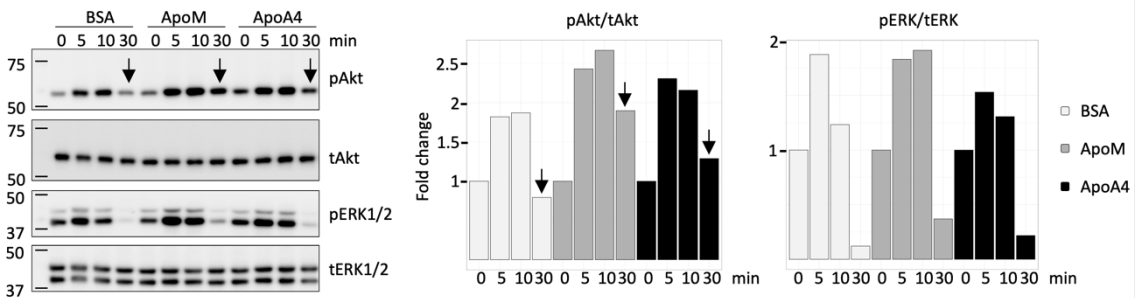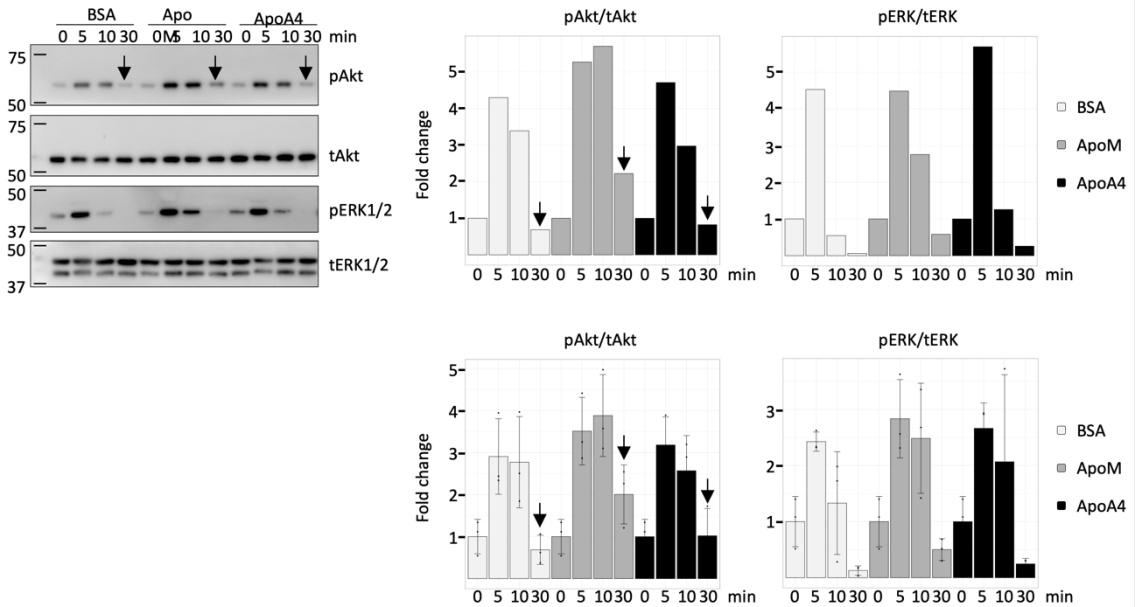
(B) CHO-S1PR3 cells: Equal concentration (BSA: ApoM: ApoA4 = 10: 10: 10 nM)
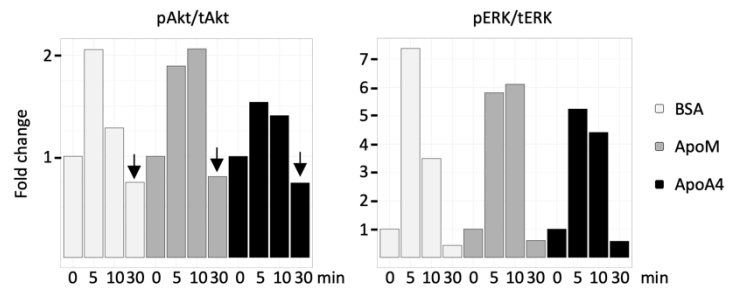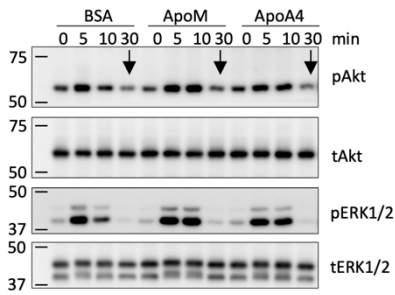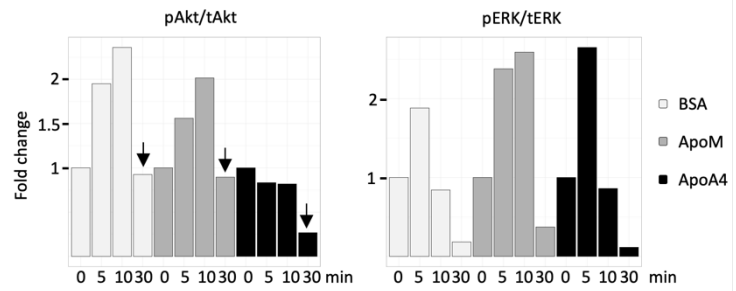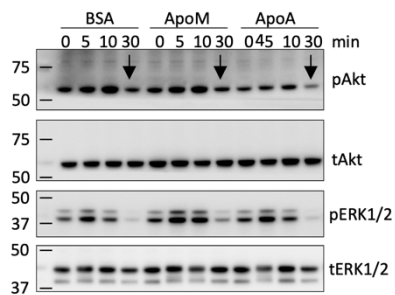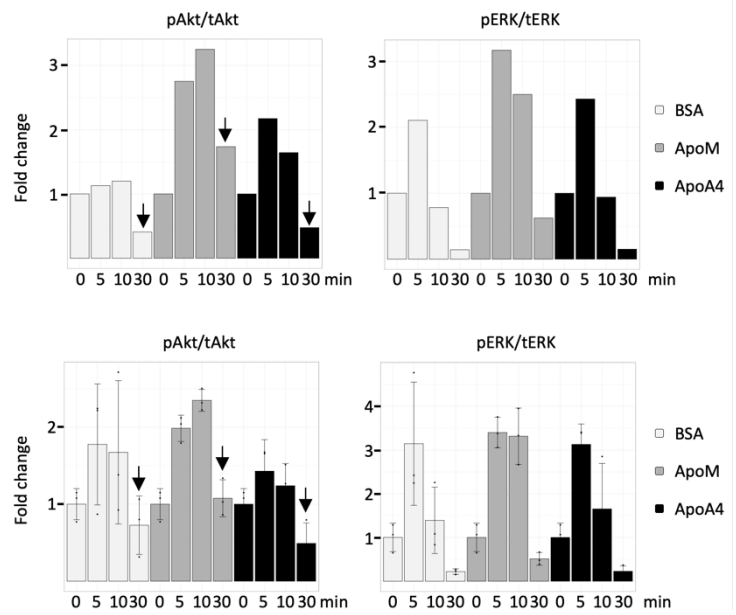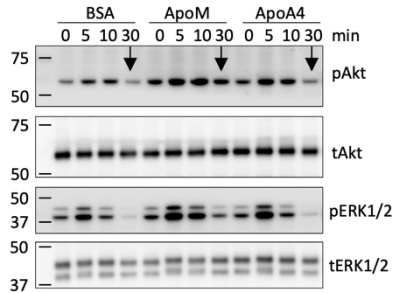
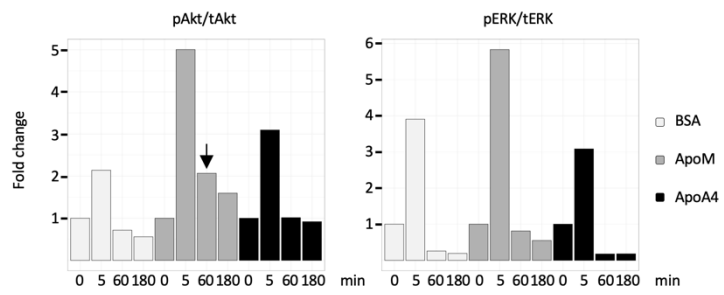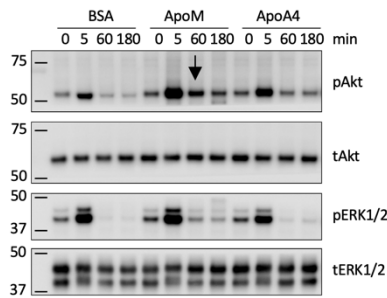**(C)** CHO-S1PR1 cells: Physiological concentration (BSA: ApoM: ApoA4 = 600: 1: 3 μM)

50
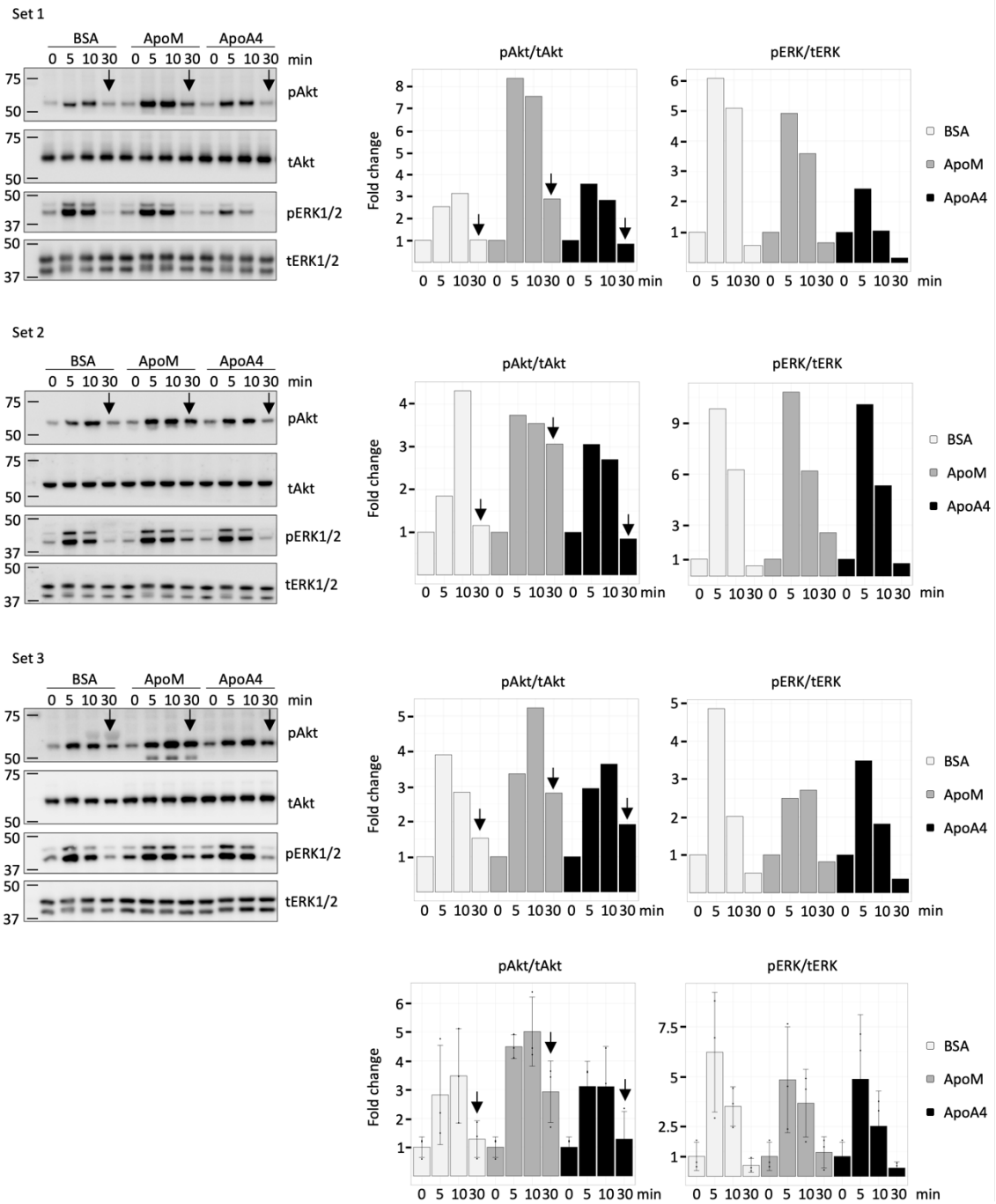
Figure 9. Analyses of Akt and ERK activation by the carrier-bound S1P via S1PR1 and

S1PR3

The results of Western blot analyses from 3 independent experiments are shown separately,

together with the statistically processed graphs on the bottom (mean +/– SD). CHO-S1PR1 (A

and C) and CHO-S1PR3 (B and D) were stimulated with 10 nM S1P bound to the equal

concentration (A and B) or the physiological concentration (C and D) of the carrier proteins for

indicated times. The total cell lysates were prepared, and the activation of Akt and ERK1/2 was

examined by Western blot analyses with the phospho-specific antibodies (left side). The band

intensity was digitized using Image J software, and the ratio of the phosphorylated forms to total

amounts was calculated (right side). The data are expressed in the fold change from the time 0

(no stimulation control).

Figure 10. Analyses of Akt and ERK activation by the carrier-bound S1P via S1PR1 (longer time course)

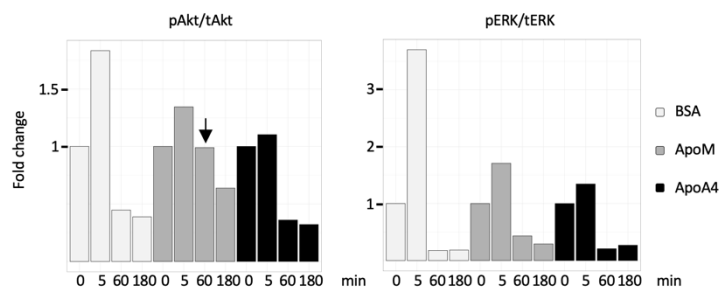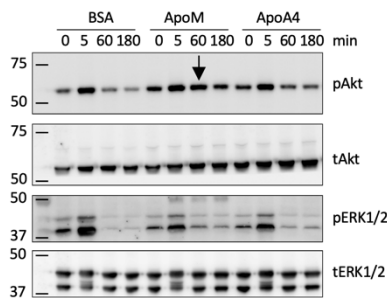The results of Western blot analyses from 3 independent experiments are shown separately,

together with the statistically processed graphs on the bottom (mean +/– SD). CHO-S1PR1 cells

were stimulated with 10 nM S1P bound to the physiological concentration of the carrier proteins

for indicated times. The total cell lysates were prepared, and the activation of Akt and ERK1/2

was examined by Western blot analyses with the phospho-specific antibodies (left side). The band

intensity was digitized using Image J software, and the ratio of the phosphorylated forms to total

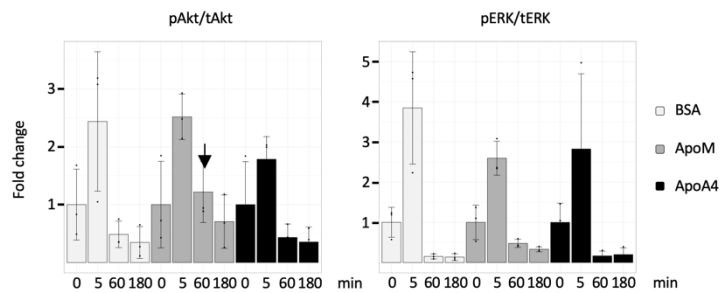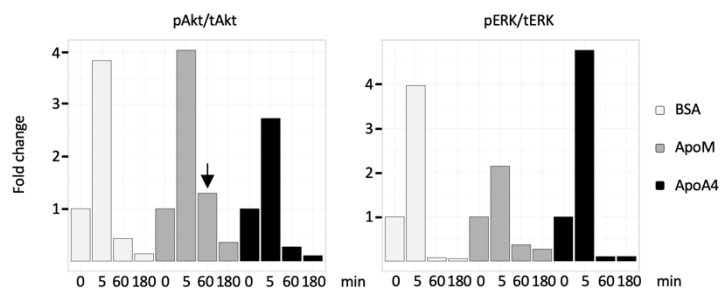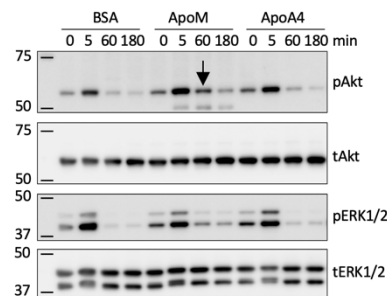amounts was calculated (right side). The data are expressed in the fold change from the time 0
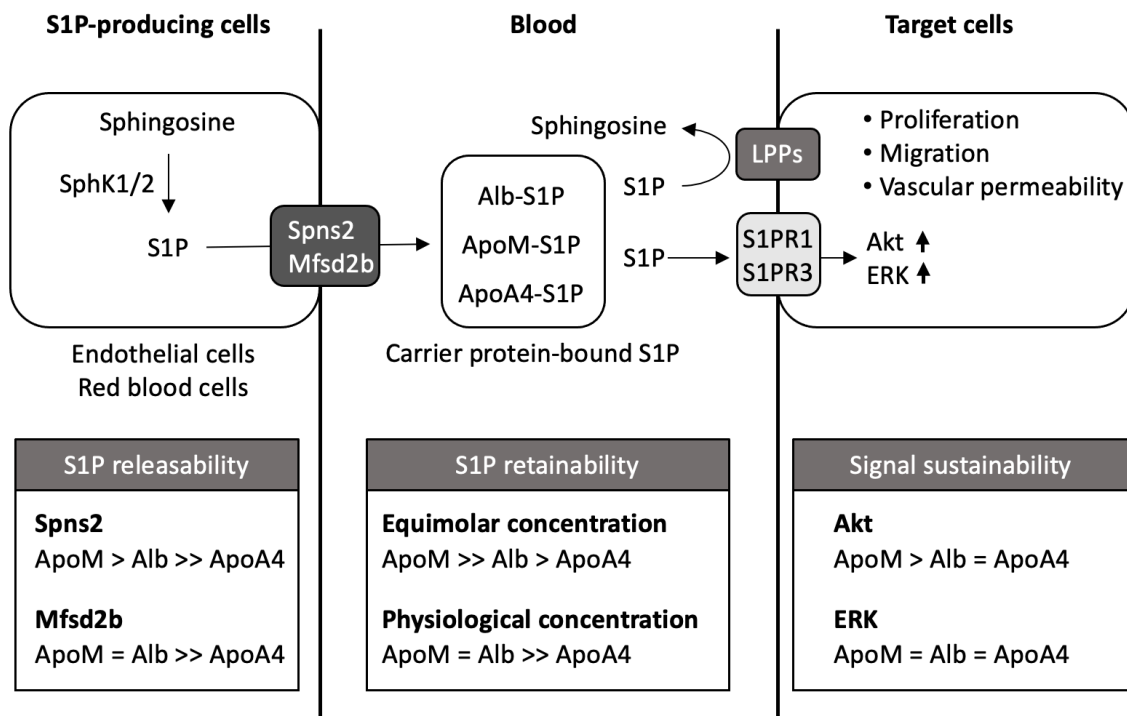
(no stimulation control).

Figure 11. Analyses of Akt and ERK activation by the carrier-bound S1P via S1PR3 (longer time course)

The results of Western blot analyses from 3 independent experiments are shown separately,

together with the statistically processed graphs on the bottom (mean +/– SD). CHO-S1PR3 cells

were stimulated with 10 nM S1P bound to the physiological concentration of the carrier proteins

for indicated times. The total cell lysates were prepared, and the activation of Akt and ERK1/2

was examined by Western blot analyses with the phospho-specific antibodies (left side). The band

intensity was digitized using Image J software, and the ratio of the phosphorylated forms to total

amounts was calculated (right side). The data are expressed in the fold change from the time 0

(no stimulation control).

Figure 12. Graphical abstract of the whole result in this paper

ApoM showed the best retainability of S1P among the 3 carrier proteins *in vitro* when compared

in the equimolar amounts. ApoM also promoted the S1P release most efficiently from the S1P-

producing cells such as endothelial cells. ApoM-S1P showed a tendency to induce the prolonged

activation of Akt via S1PR1 and S1PR3. Excess amounts of albumin showed improved S1P

retainability *in vitro*, but it did not result in prolonged Akt activation.