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Original Article

Vehicle-dependent Effects of Sphingosine 1-phosphate on Plasminogen Activator Inhibitor-1 Expression

Chiharu Takahashi[1, 2], Makoto Kurano[1, 2], Masako Nishikawa[1, 2], Kuniyuki Kano[2, 3], Tomotaka Dohi[4], Katsumi Miyauchi[4], Hiroyuki Daida[4], Tomo Shimizu[5], Junken Aoki[2, 3] and Yutaka Yatomi[1, 2]

[1]Department of Clinical Laboratory Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[2]CREST, Japan Science and Technology Corporation (JST), Tokyo, Japan
[3]Laboratory of Molecular and Cellular Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Miyagi, Japan
[4]Department of Cardiovascular Medicine, Juntendo University School of Medicine, Tokyo, Japan
[5]Tsukuba Research Institute, Research & Development Division, Sekisui Medical Co., Ltd., Ibaraki, Japan

***Aim*: Sphingosine 1-phosphate (S1P) has been suggested to be a positive regulator of plasminogen activator inhibitor 1 (PAI-1) in adipocytes, while some studies are not consistent with this pro-thrombotic property of S1P. Since S1P is bound to apolipoprotein M (apoM) on HDL or to albumin in plasma, we compared the properties of these two forms on the PAI-1 induction.**
***Methods*: We investigated the associations of S1P, apoM, and PAI-1 concentrations in the plasma of normal coronary artery (NCA), stable angina pectoris (SAP), and acute coronary syndrome (ACS) subjects (n=32, 71, and 38, respectively). Then, we compared the effects of S1P with various vehicles on the PAI-1 expression in 3T3L1 adipocytes. We also investigated the modulation of the PAI-1 levels in mice infected with adenovirus coding apoM.**
***Results*: Among ACS subjects, the PAI-1 level was positively correlated with the S1P level, but not the apoM level. In adipocytes, S1P bound to an apoM-rich vehicle induced PAI-1 expression to a lesser extent than the control vehicle, while S1P bound to an apoM-depleted vehicle induced PAI-1 expression to a greater extent than the control vehicle in 3T3L1 adipocytes. Additionally, apoM overexpression in mice failed to modulate the plasma PAI-1 level and the adipose PAI-1 expression level. S1P bound to albumin increased PAI-1 expression through the S1P receptor 2-Rho/ROCK-NFκB pathway.**
***Conclusion*: S1P bound to albumin, but not to apoM, induces PAI-1 expression in adipocytes, indicating that S1P can exert different properties on the pathogenesis of vascular diseases, depending on its vehicle.**

Key words: Sphingosine 1-phosphate, Plasminogen activator inhibitor 1, Apolipoprotein M

Introduction

Thrombotic diseases, such as acute coronary syndrome (ACS), pulmonary embolism, and cerebral artery infarction, are causes of mortality and morbidity among human subjects worldwide until now[1, 2], and elucidation of regulatory factors for hemostasis and fibrinolysis to overcome these diseases remains an important task. Among these factors, the adipokine plasminogen activator inhibitor-1 (PAI-1) exerts important pro-thrombotic effects: PAI-1 inhibits conversion of plasminogen into plasmin and thus promotes clot formation and the resultant thrombus formation[3-7]. In reality, PAI-1 has been demonstrated to be associated with cardiovascular diseases in several clinical studies[8, 9]. Considering these crucial roles of PAI-1, the mechanisms and physiological factors regulating PAI-1 expression should be elucidated. Until now, various candidate molecules have been proposed as regu-

Address for correspondence: Yutaka Yatomi, Department of Clinical Laboratory Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
E-mail: yatoyuta-tky@umin.ac.jp
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lators for PAI-1, including tumor necrosis factor-α, tumor growth factor-β, glucose, insulin, hypoxia, and sphingosine 1-phosphate (S1P)[10-14]. Among them, S1P is a bioactive lysophospholipid mediator that has been reported to be involved in the pathogenesis of many diseases in various fields, including malignant tumors, autoimmune diseases, diabetes, kidney diseases, liver diseases, and vascular diseases[15]. S1P has been proposed to be a positive regulator of PAI-1 based on the results of several basic studies[14, 16], suggesting that S1P has a pro-thrombotic property.

However, contrary to these possible pro-thrombotic properties of S1P, a basic study has suggested that S1P is an anti-atherosclerotic mediator; S1P has cardio-protective properties, such as anti-apoptosis[17], anti-inflammation[18], vasorelaxation[19], and the maintenance of vascular permeability[20-22]. S1P is mainly carried on HDL (about 65%), followed by albumin (about 30%)[23, 24], and a recent elegant study elucidated that S1P is carried on apolipoprotein M (apoM)[25], which is a minor apolipoprotein riding mainly on HDL[26]. In fact, in some clinical studies, the level of S1P bound to HDL was reported to be lower, while S1P bound to albumin was somewhat higher in patients with coronary artery disease than in healthy subjects[27, 28]. Considering these results together with the report that the plasma PAI-1 level possesses an inverse correlation with the HDL-cholesterol level[29], it seems natural to speculate that albumin-linked S1P, but not HDL-linked S1P, may specifically induce PAI-1 expression.

In many basic studies investigating physiological properties of S1P, cells are treated with S1P using albumin as a vehicle. However, some reports have demonstrated a difference between S1P bound to albumin and S1P bound to HDL/apoM; S1P bound to HDL sustained the endothelial cell barrier longer than S1P bound to albumin[30]; S1P bound to apoM-lipoproteins or recombinant apoM enhanced the insulin secretion from a β-cell line to a greater extent than S1P bound to albumin[31]; and S1P bound to HDL, but not bound to albumin, restrained lymphopoiesis and neuro-inflammation[32]. In addition to these reports, Galvani *et al.* recently elucidated that S1P bound to HDL is a biased agonist against S1PR1[33]. With regard to the effects of S1P on PAI-1, one report utilized albumin as a vehicle[16] and the other report utilized HDL_3 as a source of S1P[34]. Although both reports demonstrated that S1P induced PAI-1 expression in 3T3L1 adipocytes, they did not directly compare this property of S1P between when S1P is bound to albumin and when it is bound to HDL.

Aim

We aimed to compare the properties of inducing PAI-1 between S1P bound to albumin and S1P bound to HDL/apoM in this study.

Methods

Clinical Study

Samples were obtained from subjects who underwent coronary angiography at Juntendo University Hospital (J-Bacchus trial) between July and December 2009[35, 36]. Patients without significant stenosis were placed in a normal coronary arteries (NCA) group, while those with significant stenosis were placed in an acute coronary syndrome (ACS) or a stable angina pectoris (SAP) group. Patients with acute myocardial infarction and unstable angina were included in the ACS group. The ethics review committee at Juntendo University Hospital approved the study, all the participants signed informed consent forms, and the study was registered in the UMIN protocol registration system (#UMIN000002103). This study was also approved by the institutional review boards of both the University of Tokyo and Juntendo University School of Medicine. The preparation of blood samples was described in a previous study[36]. Plasma samples were stored at −80℃, and freeze-thaw treatments were limited to twice prior to the measurement of S1P, PAI-1, and apoM levels. The plasma S1P concentration was determined using LC−MS/MS[37]. The plasma PAI-1 level was measured using human active PAI-1 ELISA (IHPIKT; Innovative Research, Novi, MI). The plasma apoM level was determined using human apoM ELISA, which we developed and described in a previous study[31]. In brief, 96-well plates (Thermo Fisher Scientific Inc. Allentown, PA) were coated with 250 ng/well of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), followed by blocking with PBST containing 1% BSA. Then, we added mouse anti-human apoM monoclonal antibody developed against recombinant human apoM (ATGen Co., Ltd., Gyeonggi-do, Korea) to the plate, which was left for 1 hour. The samples were denatured with PBS containing 2% SDS and 0.2 M DTT (pH 7.2), diluted in PBST containing skimmed milk (1:600), and added on the plate. After 1 h, the samples were replaced for biotinylated rabbit anti-human apoM antibody (developed against the C-terminal peptide of human apoM). Human apoM bound to the plate was detected with horseradish peroxidase-labeled streptavidin and o-phenylenediamine (OPD). A dilution series of the standard prepared from a serum pool from healthy subjects was included on each plate to calculate the apoM concentration. The validation of this ELISA was described in

a previous study[31].

Materials

D-erythro-S1P (SL-140; Enzo Life Science, Plymouth Meeting, PA) was dissolved in methanol. Immediately before use, the methanol was evaporated and the reagent was resolved in PBS containing 0.4% fatty acid-free BSA (A8806; Sigma-Aldrich Co., St. Louis, MO) or other vehicles prepared as described below. VPC23019 (857360P; Avanti Polar Lipids, Alabaster, AL), JTE013 (10009458; Cayman Chemical, Ann Arbor, MI), Y27632 (257-00511; WAKO Pure Chemical Industries, Osaka, Japan), wortmannin, YC-1 (W1628, Y102; Sigma-Aldrich Co), and SIS3 and BAY11-7082 (sc-222318, sc-200615; Santa Cruz Biotechnology, Inc. TX) were dissolved in DMSO.

Preparation of Platelet-Rich Plasma and Platelet Stimulation

Blood was collected with 10% citrate from healthy donors after obtaining written informed consent and was centrifuged (150×*g*, 20 minutes, 4℃) to obtain platelet-rich plasma (PRP). PRP was then divided into two batches: One batch was stimulated with collagen at 10 μg/ml (collagen reagent Horm; Nycomed, Munich, Germany) for 15 minutes under stirring at 1000 rpm and the supernatant was collected after centrifugation (10000 rpm, 1 minute, 4℃), while the other batch was not stimulated and was stirred under the same conditions. Platelet aggregation of the collagen-stimulated PRP was measured using an aggregation meter (PA-200C; Kowa Company, Ltd.).

Isolation of HDL and Lipoprotein-Depleted Plasma

HDL_2 (1.063＜d＜1.125 g/mL), HDL_3 (1.125＜d＜1.21 g/mL), and total HDL (1.063＜d＜1.25 g/mL) were isolated from PRP samples by an ultracentrifugation method with a Ti70 rotor (Beckman-Coulter Instruments, Palo Alto, CA). The remaining samples were utilized as lipoprotein-depleted plasma (LDP). The samples were dialyzed against PBS (pH 7.4) at 4℃ for 48 hours and stored at 4℃ until use. To investigate the effect of HDL or LDP on PAI-1 expression, the final concentration of HDL or LDP was adjusted to 800 μg/mL in serum-free DMEM.

Measurement of S1P

S1P contents in HDL and LDP were determined using two-step lipid extraction followed by HPLC separation as described in a previous study[38].

Cell Experiments

3T3L1 fibroblasts (JCRB cell bank) were cultured in DMEM (D5796; Sigma-Aldrich Co.) containing 10% fetal bovine serum (FBS, 10099-141; Gibco BRL, Eggstein, Germany) and 1% penicillin/streptomycin (15070-063; Gibco, Grand Island, NY) in an incubator containing 5% CO_2. Two days after confluence, the differentiation of 3T3L1 fibroblasts into adipocytes was induced using 0.5 mM 3-isobutyl-1-methylxanthine (099-03411, WAKO Pure Chemical Industries), 1 μM dexamethasone (047-18863; WAKO Pure Chemical Industries), and 10 μg/mL of insulin (I1882; Sigma-Aldrich Co.). After three days, the medium was replaced with DMEM containing 10% FBS, 1% penicillin/streptomycin, and 10 μg/mL of insulin. Thereafter, the medium was replaced with fresh DMEM containing 10% FBS, and 1% penicillin/streptomycin every two days[39]. We confirmed the differentiation into adipocytes using Oil Red O staining (data not shown).

On the tenth day after the induction of differentiation into adipocytes, the cells were incubated in FBS-free DMEM for 18 hours and then challenged with FBS-free DMEM containing S1P bound to various vehicles prepared as described below for 30 minutes, 1 hour, or 4 hours. With regard to the experiment utilizing HDL as a vehicle for S1P, we used HDL at the final concentration of 800 μg protein/mL. We considered that the innate S1P level on HDL could be negligible, since the S1P levels in HDL were much lower than 10 μM (75.9 nM in total HDL, 40.8 nM in HDL2, 73.5 nM in HDL3, and 16.4 nM in LDR). Then, the cellular protein and total RNA were extracted and analyzed. With regard to experiments with pharmacological inhibitors or antagonists, the cells were pre-incubated with FBS-free DMEM containing pharmacological inhibitors or receptor antagonists for 30 minutes prior to treatment with S1P.

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza Inc. (Walkersville, MD) and cultured in endothelial basal medium-2 (EBM-2) culture medium (CC-3156; Lonza Inc.) supplemented with endothelial growth medium-2 (EGM-2) SingleQuots and growth factors (CC-4176; Lonza Inc.). At around 70% confluence, the medium was replaced for growth factor−free medium for 4 hours and then challenged with S1P using albumin or recombinant ApoM as a vehicle. After another 1 hour, total RNA was extracted and analyzed.

Preparation of Vehicles Containing Various Concentrations of ApoM-Containing Lipoproteins and Albumin

We prepared vehicles containing various concentrations of apoM-containing lipoproteins and albumin as described in a previous study[40]. In brief, HepG2 cells purchased from the American Type Culture Col-

lection (ATCC, Manassas, VA) were cultured on 10-cm collagen-coated dishes. After the cells had reached around 70% confluence, they were infected with adenoviruses coding apoM (Ad-apoM)[41] or control blank adenovirus (Ad-null) at the MOI of 25 or were treated with siRNA against human albumin (si-Alb, sc-45606), siRNA against human apoM (si-ApoM, sc-61978), or control siRNA (si-Ctl, sc-37007) obtained from Santa Cruz Biotechnology. After 48 hours, the medium was replaced with 12 mL of FBS-free DMEM, the cells were incubated for another 24 hours, and the medium was collected and condensed to 500 μL using Amicon Ultra-15 (UFC901008; Millipore Co., Bedford, MA)[42]. To investigate the distribution of S1P bound to each vehicle between the HDL ($d<1.21$) and LDP fractions ($d>1.21$), we dissolved C_{17}S1P at the final concentration of 1 μM with each vehicle, separated these fractions with an ultracentrifugation method, and measured and calculated the C_{17}S1P concentration bound to each fraction in the media prior to separation.

Animal Experiments

Nine-week-old male C57BL6J mice were obtained from CLEA Japan (Tokyo, Japan). The mice were infected with Ad-apoM or Ad-null at a dose of 2.5×10^3 pfu/g body weight via the murine tail vein as described in a previous study[41]. Analyses were performed after five days. The levels of apoM and S1P in the murine plasma were determined with an HPLC method and human apoM ELISA, respectively, as described above.

The concentration of PAI-1 in the murine plasma was determined using a commercially available ELISA assay (1MPAIKT-TOT; Innovative Research, Novi, MI) according to the manufacturer's protocol.

All animal experiments were conducted in accordance with the guidelines for Animal Care and were approved by the animal committee of The University of Tokyo.

Real-Time PCR

Total RNA was extracted from 3T3L1 adipocytes or murine adipose tissues using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich Co.) and subjected to reverse transcription using ReverTra Ace qPCR RT Master Mix (TOYOBO Co., LTD, Osaka, Japan). Real-time quantitative PCR was performed using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) with hybridization probes and primers purchased from Applied Biosystems: Murine PAI-1 (Mm00435860_m1) or human PAI-1 (Hs01126606_m1) and murine 18S (Mm03928990_g1) or human 18S (Hs99999901_S1). The expression level of PAI-1 was adjusted using that of 18S as an endogenous control.

Western Blotting

All cellular proteins were extracted as described in previous studies[41, 42]. Western blotting was performed using 30 μg of cellular proteins according to the standard method. The following antibodies were used: Anti-human apoM antiserum (developed in a previous study[41]), anti-apoA-I antibody (AB740; Chemicon International Inc., Temecula, CA), anti-human albumin antibody (E80-129A; Bethyl Laboratories, Inc. Montgomery, TX), anti-phosphomoesin, anti-total moesin, (SAB4504260, 4501926; Sigma-Aldrich Co.), and anti-β-actin (PM053; MBL, Nagoya, Japan). Intensities of the bands were measured using Image J (from NIH).

Construction of Recombinant ApoM

Recombinant human apoM was constructed with the Brevibacillus Expression System (HB300, TaKaRa Bio Inc., Shiga, Japan) from the previously cloned human apoM cDNA[41]. The following primers were utilized: Forward primer, 5'gatgacgatgacaaaatgttccaccaaatttgg3'; and reverse primer, 5'catcctgttaagctttcagttattggacagctcac3'. The constructed human apoM recombinant was purified with HisTrap FF equipped with the AKTA system (17-5255-01, GE Healthcare Bio-Science AB, Uppsala, Sweden) and measured for the protein levels with DC Protein Assay (5000112JA, Biorad laboratories, Inc. Hercules, CA).

Statistical Analyses

All the data were statistically analyzed using SPSS (Chicago, IL). The results were expressed as the mean ± SD. In clinical studies, the values obtained from three groups were compared using the Kruskal–Wallis test followed by the Games–Howell test as a post-hoc test, since the normality or equality of variance had been rejected with the Kolmogorov–Smirnov test or the Levene test for most of the parameters. Correlations were sought using the Spearman correlation test. With regard to *in vitro* experiments, statistical significance between two groups was evaluated using the Student *t*-test, and differences between more than two groups were assessed using a one-way ANOVA followed by the Scheffe test as a post-hoc test. *P* values less than 0.05 were considered to be statistically significant.

Results

Positive Correlation of Plasma PAI-1 Level with Plasma S1P Level, but not with ApoM Level, in ACS Subjects

First, we measured the concentrations of S1P,

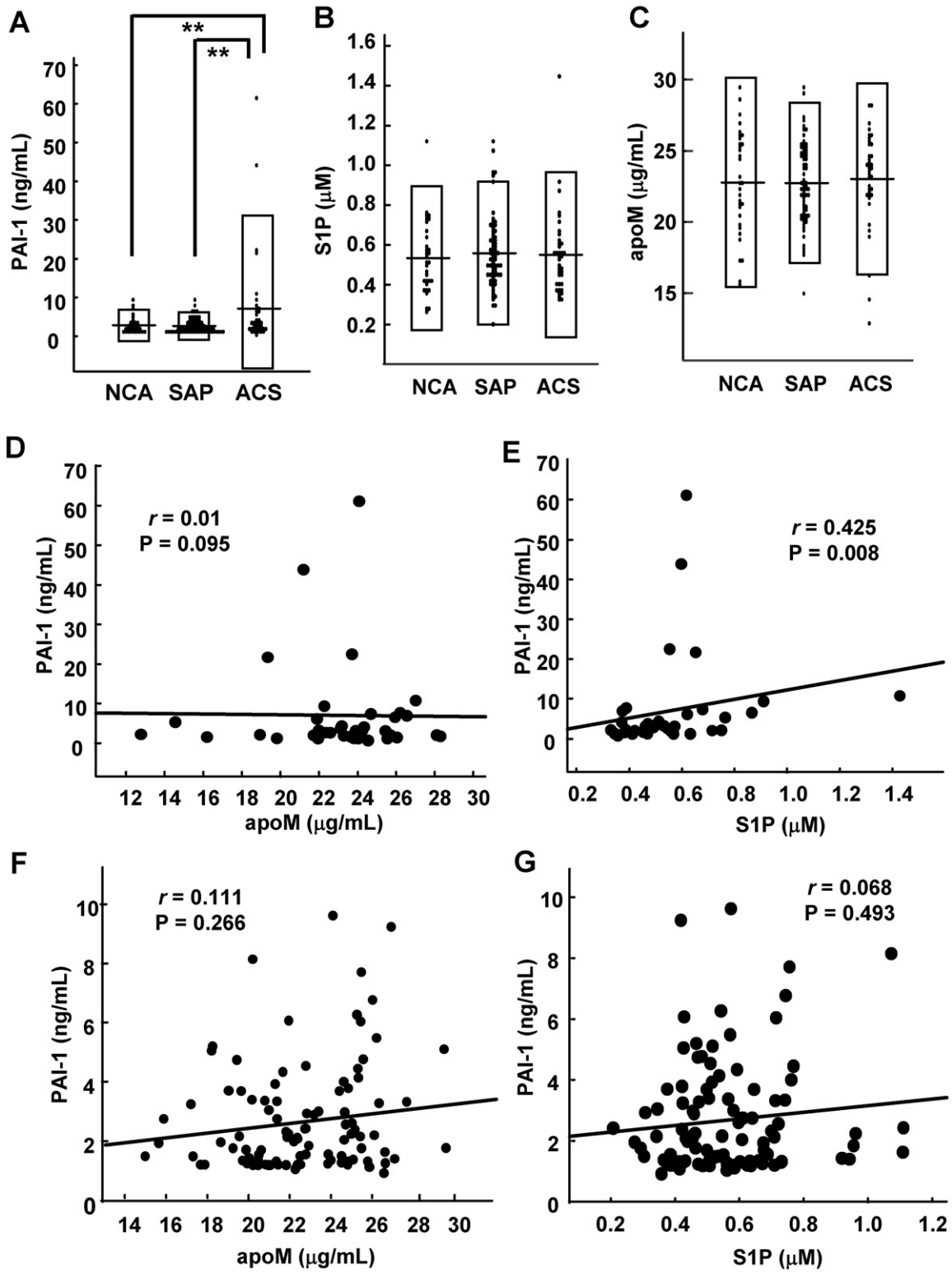

Fig. 1. Plasma PAI-1 concentrations were positively correlated with S1P levels, but not with apoM levels.

We measured the concentrations of plasminogen activator inhibitor 1 (PAI-1), sphingosine 1-phosphate (S1P), and apolipoprotein M (ApoM) in plasma samples obtained from normal coronary artery (NCA) ($n=32$), stable angina pectoris (SAP) ($n=71$), and acute coronary syndrome (ACS) subjects ($n=38$). (A−C) Plasma PAI-1 concentrations (A), plasma S1P concentrations (B), and plasma apoM concentrations (C) in the NCA, SAP, and ACS groups. (D−G) Correlations between the plasma PAI-1 levels and plasma apoM level (D, F) or the plasma S1P levels (E, G) in the ACS group (D, E) and the non-ACS group (F, G).

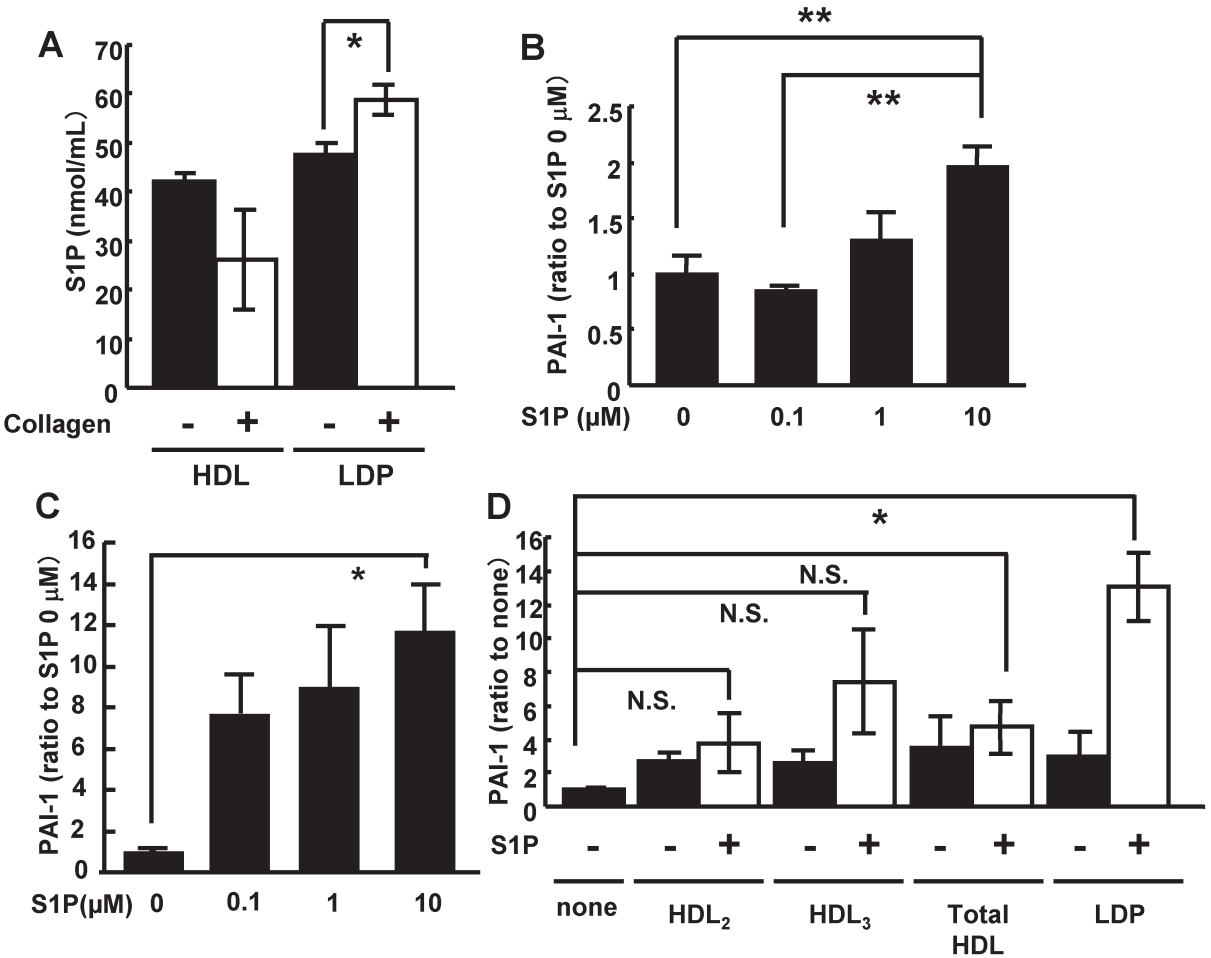

Fig. 2. S1P bound to HDL did not increase PAI-1 expression in 3T3L1 adipocytes.

(A) Blood was collected with 10% citrate from healthy donors and centrifuged to obtain platelet-rich plasma (PRP). PRP was stimulated with collagen, and HDLs and lipoprotein deplete plasma (LDP) were isolated and measured for sphingosine 1-phosphate (S1P) ($n=3$/each group). (B−D). 3T3L1 adipocytes were pre-incubated for 18 hours in FBS-free DMEM and the medium was exchanged for FBS-free DMEM with various concentrations of S1P (0, 0.1, 1, 10 μM) for 4 hours (B, D) or 1 hour (C). Then, the mRNA level of plasminogen activator inhibitor 1 (PAI-1) was analyzed using real-time PCR. 18S was utilized as an internal control. The effect of S1P bound to albumin ($n=5-6$/each group) (B, C) and the effect of S1P bound to HDLs, or LDP (D) on the PAI-1 mRNA level in 3T3L1 adipocytes ($n=3-4$/each group) are shown. $^{*}p<0.05$, $^{**}p<0.01$, N.S., not significant.

apoM, and PAI-1 in the plasma of NCA, SAP, and ACS subjects ($n=32$, 71, and 38, respectively), as described in a previous study[36], to investigate whether the association with the plasma PAI-1 level in human subjects differs between S1P bound to albumin and S1P bound to apoM. In subjects with ACS, the PAI-1 concentration was especially elevated, as compared with NCA and SAP groups ($P<0.01$) (**Fig. 1A**), while the plasma S1P and apoM levels did not differ significantly among these three groups (**Figs. 1B, 1C**). With regard to the correlation between S1P or apoM and PAI-1, the PAI-1 level was weakly, but significantly correlated with the S1P level ($r=0.425$, $P<0.01$), but not with the apoM level, in ACS subjects (**Figs. 1D, 1E**). In subjects with NCA and SAP, the PAI-1 level was not significantly correlated with either the apoM level or S1P level (**Figs. 1F, 1G**).

S1P Released from Activated Platelets is Preferably Fractionized to LDP Fraction, but not HDL Fraction

The result that different correlations with PAI-1 were observed for the plasma S1P and plasma apoM levels prompted us to investigate the correlation between the plasma PAI-1 level and the S1P level in the LDP fraction and that in the HDL fraction. Regrettably, however, all plasma samples had been frozen, and we were unable to isolate the plasma into

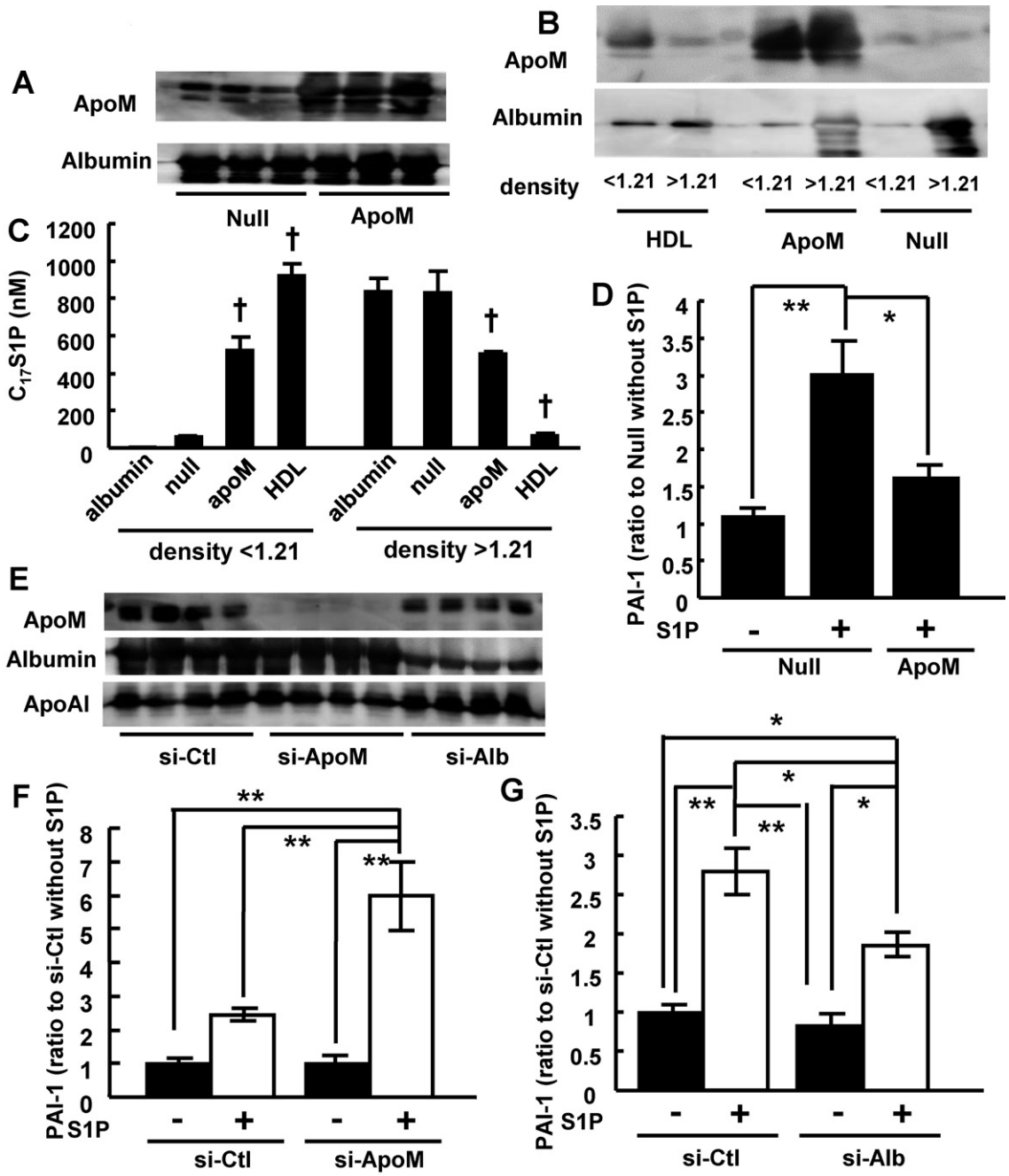

Fig. 3. S1P bound to apoM had weaker effect on the induction of PAI-1 in 3T3L1 adipocytes than S1P bound to albumin.

We prepared an apolipoprotein M-rich vehicle (ApoM) and a control vehicle (Null) or an apoM-depleted vehicle (si-ApoM), an albumin-depleted vehicle (si-Alb), and a control vehicle (si-Ctl), as described in the Materials and Methods section. 3T3L1 adipocytes were pre-incubated for 18 hours in FBS-free DMEM and the medium was exchanged for FBS-free DMEM with 10 μM of sphingosine 1-phosphate (S1P) bound to the various vehicles for 4 hours. Then, the mRNA level of PAI-1 was analyzed using real-time PCR. 18S was utilized as an internal control. (A, E) ApoM and albumin levels in each medium ($n=3-4$/group). (B, C) The distribution of ApoM (B) and C_{17}S1P (C, $n=3$/group) between HDL fraction ($d<1.21$) and lipoprotein-depleted plasma fraction ($d>1.21$), when 1 μM C_{17}S1P was bound to each vehicle. (D) Effects of S1P bound to apoM-rich vehicle and control vehicle on the PAI-1 mRNA level ($n=4$/group) (F, G) Effects of S1P bound to apoM-depleted vehicle, albumin-depleted vehicle, or control vehicle ($n=4-6$/group). $^{*}p<0.05$, $^{**}p<0.01$.

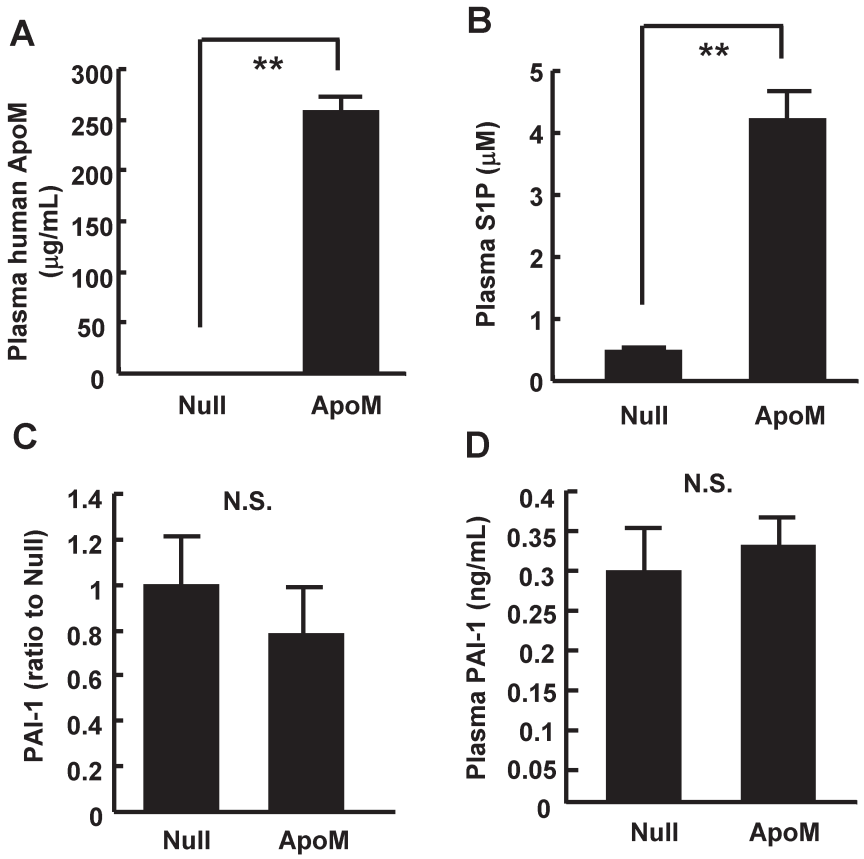

Fig. 4. Adenoviral overexpression of apoM in mice did not increase the PAI-1 levels in plasma and adipose tissue.

Nine-week-old male C57BL6J mice were infected with adenovirus coding human apolipoprotein M (ApoM) or control blank virus (Null). Analyses were performed after five days ($n=6$/group). (A) Plasma human apoM levels. (B) Plasma sphingosine 1-phosphate (S1P) levels. (C) PAI-1 mRNA level in murine adipose tissues. (D) Plasma plasminogen activator inhibitor 1 (PAI-1) level.

these fractions. Therefore, we activated the platelets, which are believed to be involved in the pathogenesis of ACS, and investigated to which fraction S1P was preferably distributed.

As shown in **Fig. 2A**, when we stimulated PRP, the S1P content was elevated in the LDP fraction, as compared with non-stimulated PRP ($P<0.05$), while that in the HDL fraction was not altered. Considering these results and the findings from the clinical study, S1P, which is released from platelets in ACS subjects, may preferably be fractionated to the LDP fraction and bound to albumin, while S1P bound to albumin, but not to apoM/HDL, induced PAI-1 expression.

S1P Bound to LDP, but not to HDL, Increased PAI-1 Expression Level in 3T3L1 Cells

We next investigated the effect of S1P bound to HDL and LDP on the PAI-1 expression level in 3T3L1 adipocytes. First, we treated 3T3L1 adipocytes with various concentrations of S1P bound to albumin in the same manner as that described in a previous study[34], and we confirmed that the expression of PAI-1 was increased by 10 μM of S1P in 3T3L1 adipocytes at 4 hour after administration, while the expression of PAI-1 tended to be increased by 0.1 or 1 μM of S1P at 1 hour after administration (**Figs. 2B, 2C**). Since innate concentrations of HDL and apoM-rich vehicle were 50 to 100 nM (as described in *Methods* section and previous study[31]), we investigated the effective properties of S1P on 3T3L1 adipocytes at the concentration of 10 μM of S1P and at 4 hour after administration in the following experiments utilizing these vehicles, to ignore the influences of innate S1P riding on the vehicles.

Next, we utilized isolated HDL_2, HDL_3, total HDL, or LDP as a vehicle and investigated whether the type of vehicle affected the properties of S1P during the induction of PAI-1. As shown in **Fig. 2C**, 10

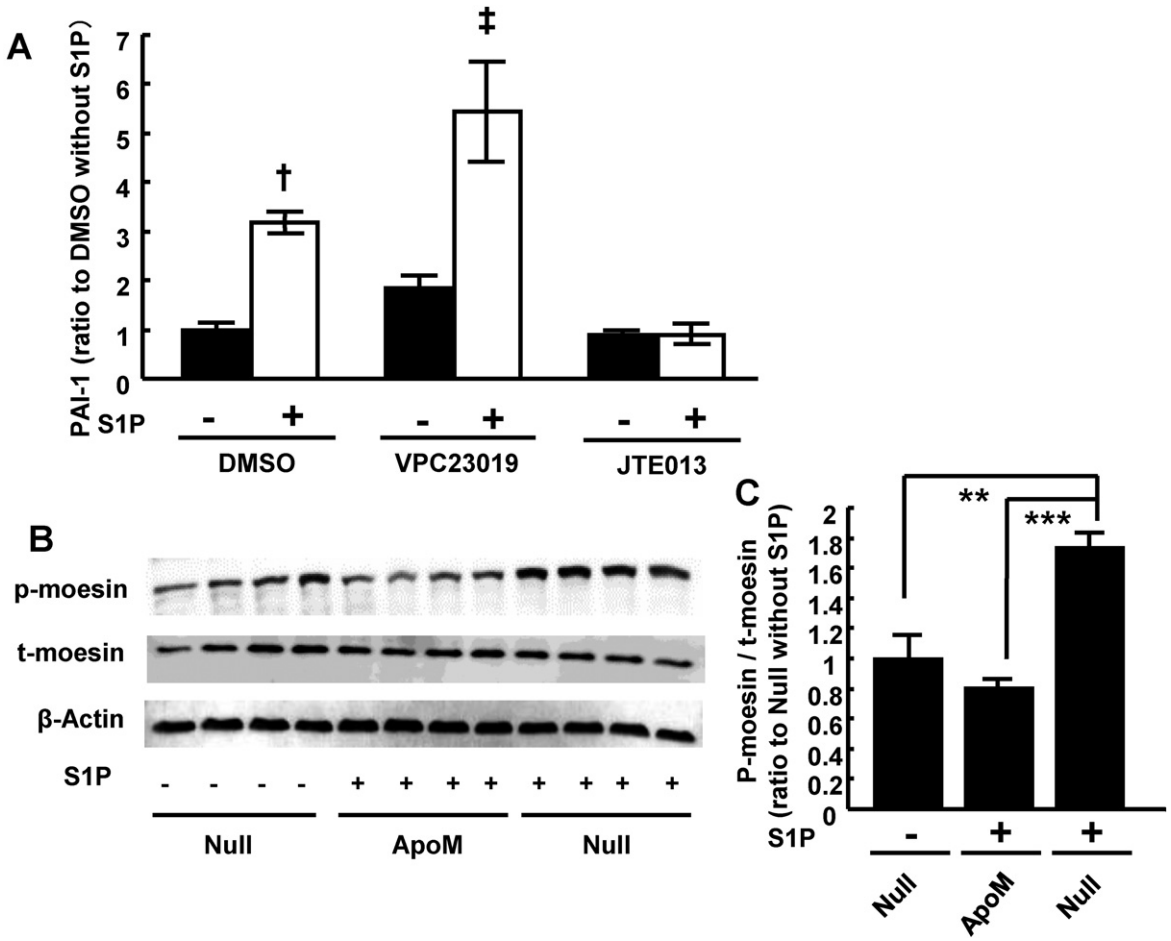

Fig. 5. S1P bound to albumin induced PAI-1 expression by activating S1PR2 pathway in 3T3L1 adipocytes.

(A) 3T3L1 adipocytes were pre-incubated for 18 hours in FBS-free DMEM and the medium was exchanged for FBS-free DMEM containing 20 μM of VPC23019, JTE013, or DMSO for 30 minutes. Then, the cells were challenged with a medium containing 10 μM of sphingosine 1-phosphate (S1P) with the corresponding inhibitors for 4 hours. The mRNA level of plasminogen activator inhibitor 1 (PAI-1) was analyzed using real-time PCR. 18S was utilized as an internal control ($n=4-6$/group). $^{\dagger}p<0.01$ vs. DMSO alone, YC1 alone, S1P + VPC, and JTE alone, and $p<0.05$ vs. S1P + JTE. $^{\ddagger}p<0.01$ vs. other groups. (B, C). 3T3L1 adipocytes were pre-incubated for 18 hours in FBS-free DMEM, and the medium was exchanged for FBS-free DMEM with 10 μM of S1P bound to apoM-rich vehicle (ApoM) or control vehicle (Null) for 4 hours. Then, the modulation of the phosphorylation of moesin in 3T3L1 cells was examined using western blotting ($n=6$/group). $^{**}p<0.01$, $^{***}p<0.001$.

μM of S1P bound to HDL_2, HDL_3, or total HDL did not increase the PAI-1 expression level, as compared with treatment with the medium alone (none), while treatment with 10 μM of S1P bound to LDP significantly increased the PAI-1 mRNA level ($P<0.05$). This result indicates that S1P bound to LDP (albumin) stimulates the PAI-1 expression level in adipocytes, while S1P bound to HDL (apoM) does not stimulate the expression level.

S1P Bound to Albumin Induced PAI-1 Expression to a Greater Extent than S1P Bound to ApoM

Since apoM is a vehicle of S1P on HDL, we prepared an apoM-rich vehicle or a control vehicle from the conditional medium of HepG2 cells infected with Ad-apoM or HepG2 cells infected with Ad-null (**Fig. 3A**), as described in the Methods section. The detail characteristics of these vehicles were investigated in a previous study[31]. With regard to the distribution of S1P bound to these vehicles between HDL ($d<1.21$ g/mL) and LDP fractions ($d>1.21$ g/mL), we observed that S1P and apoM were distributed to both HDL and LDP fractions in the apoM-rich vehicle, while S1P was distributed mainly to LDP fraction in the control vehicle (**Figs. 3B, 3C**). Then we examined the effect of S1P on PAI-1 expression when either the apoM-rich vehicle or control vehicle was used. Significantly, as shown in **Fig. 3D**, S1P did increase PAI-1

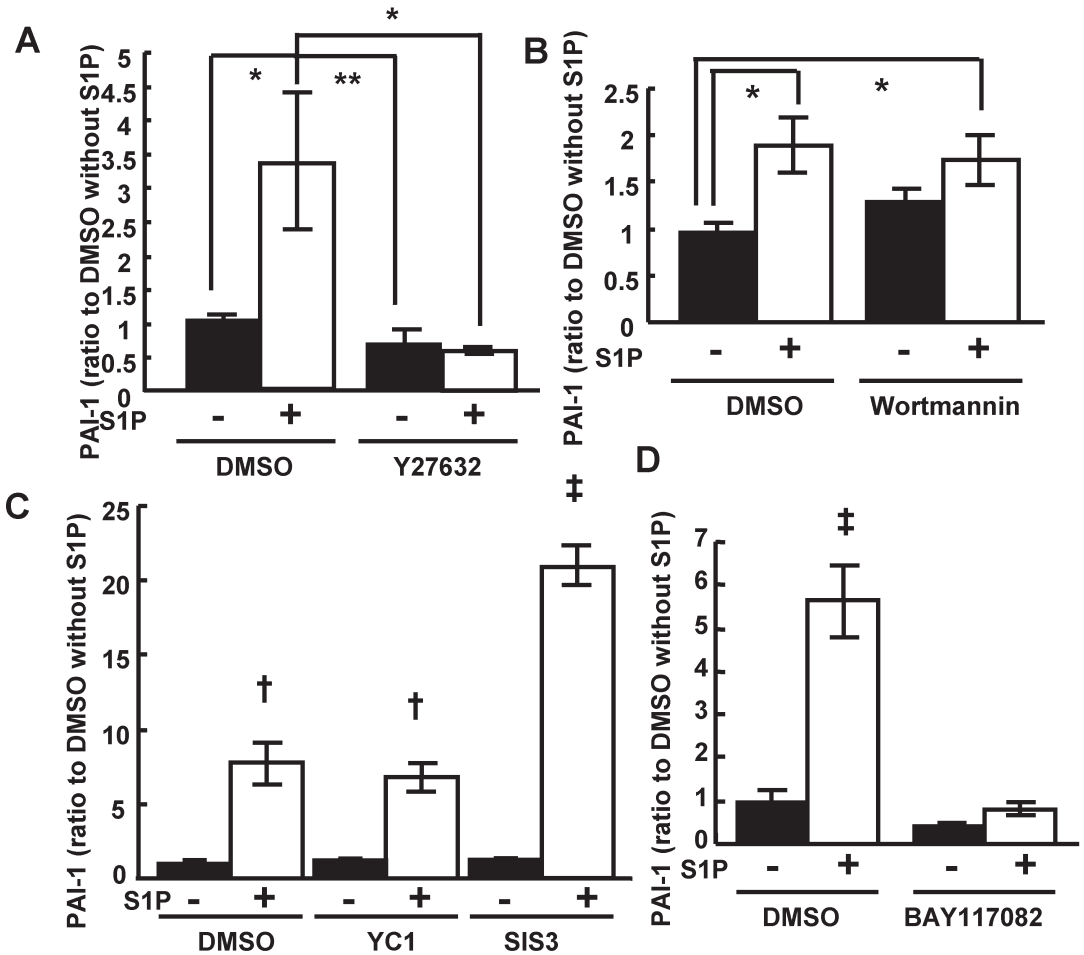

Fig. 6. S1P bound to albumin induced PAI-1 expression through S1PR2-Rho/ROCK-NFκB pathway in 3T3L1 adipocytes.

(A–D). Modulation of the plasminogen activator inhibitor 1 (PAI-1) expression levels induced by sphingosine 1-phosphate (S1P) bound to albumin by various pharmacological inhibitors: Y23632 ($n=4-5$/group) (A), wortmannin ($n=6$/group) (B), YC1 or SIS3 ($n=5-6$/group) (C), and BAY117082 ($n=6$/group) (D) or DMSO as a vehicle. 3T3L1 adipocytes were pre-incubated for 18 hours in FBS-free DMEM and the medium was exchanged for FBS-free DMEM containing the various inhibitors for 30 minutes. Then, they were challenged with the medium containing 10 μM of S1P with the corresponding inhibitors for 4 hours. The mRNA level of PAI-1 was analyzed using real-time PCR. 18S was utilized as an internal control. $^{*}p<0.05$, $^{**}p<0.01$, $^{\dagger}p<0.05$ vs. DMSO alone, YC1 alone, and S1S3 alone, $^{\ddagger}p<0.01$ vs. other groups.

expression, but to a much lesser extent, when the apoM-rich vehicle was utilized, as compared with the control vehicle.

Furthermore, we prepared an apoM-deleted vehicle, an albumin-deleted vehicle, and a control vehicle with siRNA against apoM (si-ApoM), albumin (si-Alb), and control siRNA (si-Ctl), respectively (**Fig. 3E**), and compared the effects of S1P on the induction of PAI-1 expression. We found that 10 μM of S1P dissolved in the apoM-deleted vehicle increased the PAI-1 mRNA level to a greater extent than that in the control vehicle, while 10 μM of S1P dissolved in the albumin-depleted vehicle did increase PAI-1 expression, but to a lesser extent than that in the control vehicle (**Figs. 3F, 3G**). These results also suggested that S1P bound to albumin may stimulate PAI-1 expression in 3T3L1 adipocytes more strongly than S1P bound to apoM.

Adenoviral ApoM Overexpression in Mice did not Increase Plasma PAI-1 Concentration or PAI-1 Expression Level in Adipose Tissue

To confirm the difference between S1P bound to albumin and S1P bound to apoM *in vivo*, we examined the effect of apoM on PAI-1 expression in murine adipose tissues and the plasma PAI-1 protein level by administering Ad-apoM, which were confirmed to

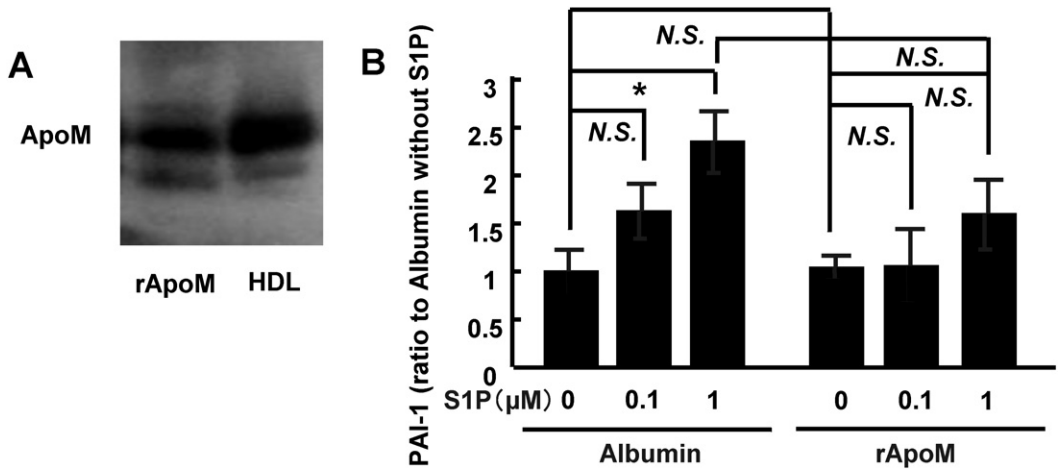

Fig. 7. S1P bound to recombinant apoM did not induce PAI-1 expression.

3T3L1 adipocytes were pre-incubated for 18 hours in FBS-free DMEM and the medium was exchanged for FBS-free DMEM with 0, 0.1, and 1 μM of sphingosine 1-phosphate (S1P) bound to albumin or recombinant apoM (rapoM) (A) for 1 hour. Then, the mRNA level of PAI-1 was analyzed using real-time PCR (B). 18S was utilized as an internal control ($n = 3$/group).

increase the plasma human apoM and S1P levels (**Figs. 4A, 4B**), or Ad-null.

As shown in **Figs. 4C, 4D**, we observed that neither the PAI-1 mRNA expression in murine adipose tissues nor the PAI-1 protein concentration in murine plasma was altered by the overexpression of apoM. Therefore, it is likely that the S1P increase by apoM overexpression fails to increase the PAI-1 level *in vivo*.

S1P Bound to Albumin, but not Bound to ApoM, Increased PAI-1 Expression Level by Activating S1PR2

At present, five types of S1P receptors (S1PR1-5) have been identified as specific receptors for S1P. In adipocytes, S1PR1-3 were found to be expressed[34]. Therefore, we next investigated the possible involvement of these receptors in the induction of PAI-1. We treated 3T3L1 adipocytes with an S1PR2 antagonist (JTE013) and S1PR1/3 antagonist (VPC23019) and analyzed the modulation of S1P-induced PAI-1 expression by each S1P antagonist. As shown in **Fig. 5A**, we found that JTE013 inhibited the effect of S1P bound to albumin, while VPC23019 did not inhibit the effect. Interestingly, we observed that treatment with VPC23019 alone tended to increase the PAI-1 mRNA level significantly. These results suggested that S1P bound to albumin, but not bound to apoM, induces PAI-1 via the S1PR2 pathway. In fact, we observed that the phosphorylation of moesin, which is reportedly under the control of the S1PR2 signal, was induced by S1P only when we utilized the control vehicle, and not when we used the apoM-rich vehicle (**Figs. 5B, 5C**).

S1P Bound to Albumin Induced PAI-1 Expression by Activating NF-κB

To examine which downstream signaling pathways are deemed to be under the control of S1PR2 and to be involved in the induction of PAI-1, we first treated 3T3L1 adipocytes with a Rho/ROCK inhibitor (Y27632), since the Rho/ROCK pathway has been shown to exist under the control of S1PR2[43]. We found that Y27632 completely inhibited the effects of S1P on the PAI-1 expression level (**Fig. 6A**), while wortmannin, which inhibits the PI3K-Akt signal and is proposed to be mainly under the control of the S1PR1 pathway (rather than S1PR2 pathway), did not inhibit the effect of S1P bound to albumin (**Fig. 6B**). We next examined the signal pathways demonstrated to exist under the control of Rho/ROCK by utilizing a HIF1α inhibitor (YC1), a Smad3 inhibitor (SIS3), and an NF-κB inhibitor (BAY11-7082). As shown in **Figs. 6C** and **6D**, neither YC1 nor SIS3 inhibited the effect of S1P bound to albumin on the induction of PAI-1 expression, while BAY11-7082 did inhibit this effect. These results suggest that S1P bound to albumin may induce PAI-1 expression through the S1PR2-Rho/ROCK -NFκB pathway.

S1P Bound to Recombinant ApoM did not Induce PAI-1 Expression

We also investigated whether S1P bound to recombinant apoM (**Fig. 7A**) increased PAI-1 expression. As shown in **Fig. 7B**, S1P bound to recombinant apoM

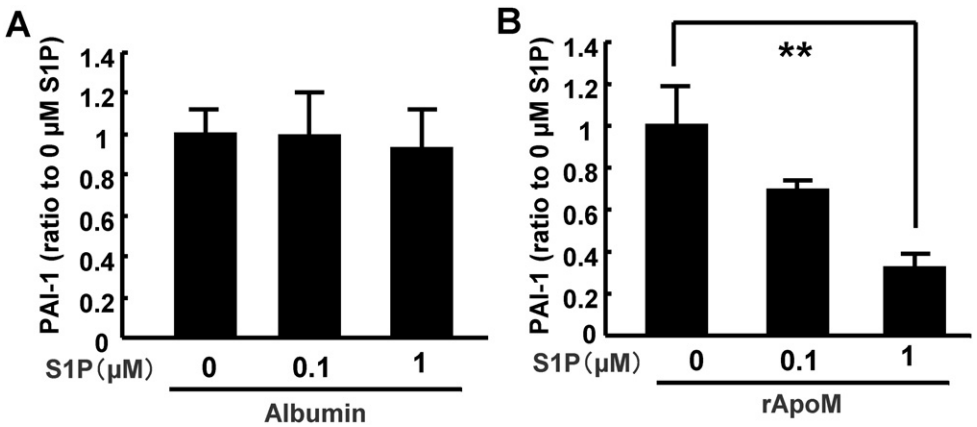

Fig. 8. S1P bound to recombinant apoM decreased PAI-1 expression in HUVECs, while S1P bound to albumin did not decrease it.

HUVECs were challenged with S1P using albumin (A) or recombinant ApoM (B) as a vehicle after cultured in growth factor−free medium for 4 hours. After another 1 hour, total RNA were extracted and analyzed. 18S was utilized as an internal control ($n=4-5$/group).

did not increase PAI-1 expression significantly, while S1P bound to albumin did increase the expression. This result suggests that S1P bound to apoM may have a lesser potency in the upregulation of PAI-1.

S1P Bound to Recombinant ApoM Suppressed PAI-1 Expression in HUVECs, but S1P Bound to Albumin did not Suppress PAI-1 Expression

At last, we compared the effects of S1P on PAI-1 expression on other PAI-1-producing cells, such as HUVECs, between S1P bound to albumin and recombinant apoM. In HUVECs, albumin-bound S1P failed to affect PAI-1 expression (**Fig. 8A**), while apoM-bound S1P decreased the PAI-1 expression level in a dose-dependent manner **(Fig. 8B)**. These results suggest that the possible difference in the modulation of PAI-1 level between S1P bound to albumin and apoM may also exist in cells other than adipocytes.

Discussion

S1P is a bioactive lipid mediator that is believed to possess anti-atherosclerosis properties. Contrary to these possible anti-atherosclerosis properties, some basic studies have demonstrated that S1P induces the expression of PAI-1, which is an important prothrombotic factor. This property of S1P seems somehow inconsistent with the results of a clinical study, since HDL, on which two-thirds of the S1P in plasma is carried, was negatively correlated with PAI-1 expression in human subjects[29]. In recent years, an important finding was established with regard to S1P biology: apoM, a minor apolipoprotein riding on HDL, was elucidated to be a S1P vehicle[25]. In this study, we hypothesized that S1P bound to albumin and S1P bound to apoM may exert different effects on the induction of PAI-1. In reality, the plasma S1P level was significantly correlated with the plasma PAI-1 level in ACS subjects, while the plasma apoM level was not correlated (**Fig. 1**). The limitation of the clinical study was that we measured the active PAI-1 antigen, but not total PAI-1 antigen. Considering that S1P affects PAI-1 expression in adipocytes, it may be more desirable to measure the total PAI-1 antigen level to investigate the association between S1P and PAI-1; however, an active PAI-1 antigen directly reflects the anti-fibrinolytic effect of PAI-1 since total PAI-1 includes three interconvertible conformations, that is, active, latent, and substrate forms[44]. Therefore, this clinical study suggested that S1P not only affected the plasma PAI-1 level but also actually contributed to the pro-thrombotic state *in vivo* through increasing an active PAI-1 antigen level. Another limitation in this clinical study is that we could not investigate the distribution of S1P to albumin versus HDL since we had frozen the samples before separating lipoproteins. Instead, we investigated to which fraction S1P, secreted from activated platelets, was preferably distributed, and observed that S1P from platelets was more predominantly distributed to the lipoprotein-depleted fraction (**Fig. 2A**). Although the mechanism for this deviated distribution of S1P secreted from platelets between albumin and HDL is unknown at present, similar results have been reported in a previous study[45]. Moreover, albumin reportedly enhances the efflux of S1P[46, 47], suggesting the existence of unknown mechanisms for albumin to preferably receive S1P secreted from platelets.

First, we investigated whether different types of vehicles affect the PAI-1-inducing property of S1P. When we compared PAI-1-inducing effects using HDLs or LDP as a vehicle, we observed that S1P bound to LDP increased PAI-1 expression, while S1P bound to HDLs did not (**Fig. 2D**). We also found that S1P carried on the apoM-rich vehicle increased PAI-1 expression to a lesser extent than S1P bound to the control vehicle (**Fig. 3D**) and that S1P carried on the apoM-depleted vehicle increased PAI-1 expression to a greater extent, while the albumin-depleted vehicle increased PAI-1 expression to a lesser extent, relative to the control vehicle (**Fig. 3F, 3G**). These results support our hypothesis that S1P bound to apoM exhibits a different biological property from S1P bound to albumin. Although we cannot exclude the possibility that other factors in the apoM-rich vehicle may mediate these effects, considering the experiments with recombinant apoM (**Fig. 7**), we assume that the effects observed in the apoM-rich vehicle can be attributed to apoM.

In recent years, several studies have demonstrated the difference between S1P bound to apoM/HDL and S1P bound to albumin: S1P bound to HDL/apoM had a greater effect on the maintenance of the endothelial barrier[30] and the secretion of insulin from pancreas β-cells[31], while S1P bound to apoM had a suppressive effect on the proliferation of lymphocytes[32]. Interestingly, these effects are derived from the biological properties of S1P through S1PR1 or S1PR3. In fact, Galvani *et al.* very recently reported that S1P bound to apoM acts as a biased agonist toward S1PR1[33]. In this study, using pharmacological inhibitors we demonstrated that the S1PR2-Rho/ROCK-NFκB pathway may be involved in the induction of PAI-1 in 3T3L1 adipocytes (**Fig. 6**). These results suggested that apoM may not only strengthen the potency of S1P as an agonist toward S1PR1 but also weaken the activation of the S1PR2-Rho/ROCK-NFκB pathway. S1PR2 is one of the S1P receptors that was identified in 1993[48]. Although four other receptors have been demonstrated to be mainly coupled with the Gi-PI3K-Akt pathway and have beneficial properties in several fields, such as vascular biology and diabetes, S1PR2 is reportedly coupled with $G_{12/13}$ and G_q, but not G_i. S1PR2 has been demonstrated to exert harmful effects in the field of retinopathy[49], kidney diseases[50], and atherosclerosis[51]. Although the reason why S1P bound to apoM has a weaker effect on S1PR2 remains to be elucidated, the results of this study suggest the possible clinical use of apoM in atherosclerotic diseases: apoM may augment the beneficial properties of S1P, such as anti-apoptosis and the maintenance of the endothelial barrier (S1PR1 pathway), while apoM also attenuates the harmful effects of S1P, such as the induction of PAI-1 (resulting from S1PR2 pathway).

Another interesting observation of this study was that treatment with VPC23019 alone tended to increase the PAI-1 mRNA level (**Fig. 5A**). A previous study also indicated that VPC23019 itself tended to increase PAI-1 expression[34]. Although the reason why VPC23019 alone tends to increase PAI-1 expression remains to be elucidated, the potency of S1PR1 may suppress PAI-1 expression, which seems possible based on our results. Considering the crystal structure of S1PR1, ligand access to the binding pocket of S1PR1[52], which is located in the transmembrane region, can be gained laterally. Therefore, endogenous, especially membranous, S1P could somehow activate S1PR1, while treatment with VPC23019 could block this endogenous activity of S1PR1, resulting in the induction of PAI-1 expression. Further studies are necessary to elucidate the involvement of S1PR1 and endogenous S1P in the expression of PAI-1.

In addition to adipocytes, several cells have been proposed to express PAI-1, such as endothelium[53, 54]. We found the possible difference in PAI-1 induction or secretion between S1P bound to albumin and recombinant apoM. In HUVECs, S1P bound to albumin did not alter the PAI-1 levels, while S1P bound to recombinant apoM decreased PAI-1 expression (**Fig. 8**). Therefore, if S1P is truly involved in the elevation of PAI-1 in ACS subjects, adipocytes, rather than endothelial cells, may be a candidate for the source of PAI-1. However, although the response to S1P may vary between 3T3L1 adipocytes and other cells, it seems likely that apoM works to lower the PAI-1 level. Further studies are necessary to elucidate these differences.

A limitation of this study is that we did not utilize purified apoM-present or apoM-absent HDL in this study, since about 5% of HDL reportedly contains apoM and the remaining does not contain it[55]. Another limitation is that we have not examined the underlying mechanism for the possible selectivity of apoM-bound S1P, especially the receptor-ligand kinetics of S1P to each S1P receptor. One possible explanation is that the physical properties of S1P may differ when it is bound to apoM and when it is bound to albumin. According to a previous study[25], S1P is considered to be caged within apoM, which may make it easy for S1P to access S1PR1 but difficult to bind S1PR2. Another explanation is the possible difference in the distributions of S1PR1 and S1PR2 on plasma membrane rafts. Since apoM-bound S1P can selectively bind receptors for lipoprotein, such as SR-BI, apoM-bound S1P could theoretically attach to the cell

membrane in a different manner from albumin-bound S1P. Further studies including an examination of the influence of apoM on the biological properties of S1P in other types of cells or diseases are necessary. Furthermore, in this study, we could not demonstrate the activation of the promoter of PAI-1 by S1P bound to albumin. However, considering that several studies have demonstrated that Rho/ROCK-NFκB pathway positively regulates PAI-1 expression[56-58], this pathway, which can be activated by S1P bound to albumin but not S1P bound to apoM, may be important in the regulation of PAI-1, although further experiments, such as a luciferase assay, are needed to prove this hypothesis.

In summary, this study demonstrated that apoM-bound S1P has a weaker effect on the induction of PAI-1 in 3T3L1 adipocytes, possibly because of its failure to activate the S1PR2 pathway.

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None.

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