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Triterpenoids Isolated from *Zizyphus jujuba* Inhibit Foam Cell Formation in Macrophages

Yukio Fujiwara,[†] Akinori Hayashida,[‡] Keiichiro Tsurushima,[‡] Ryoji Nagai,[§] Makiko Yoshitomi,[‡] Norika Daiguji,[‡] Naomi Sakashita,[†] Motohiro Takeya,[†] Sachiko Tsukamoto,[‡] and Tsuyoshi Ikeda*[,‡]

[†]Department of Cell Pathology, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Kumamoto, Japan

[‡]Department of Natural Medicine, Graduate School of Pharmaceutical Sciences, Kumamoto University, Oe-honmachi 5-1, Kumamoto 862-0973, Kumamoto, Japan

[§]Department of Food and Nutrition, Laboratory of Nutritional Science and Biochemistry, Japan Women's University, 2-8-1 Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan

Supporting Information

ABSTRACT: Because foam cell formation in macrophages is believed to play an essential role in the progression of early atherosclerotic lesions in vivo, prevention of foam cell formation is considered to be one of the major targets for the treatment of atherosclerosis. The present study examined the inhibitory effect of 50 crude plant extracts on foam cell formation. Among those crude extracts, Zizyphi Fructus (ZF) and Zizyphi Semen (ZS) extracts significantly inhibited the foam cell formation induced by acetylated LDL. Furthermore, triterpenoids such as oleanonic acid, pomolic acid, and pomonic acid were the major active compounds, and triterpenoids containing a carboxylic acid at C-28 play an important role in the inhibitory effect on foam cell formation in human macrophages. These data suggest that triterpenoids in *Zizyphus jujuba*, the plant source of ZF and ZS, may therefore be useful for the prevention of atherosclerosis.

KEYWORDS: macrophage, foam cell, ACAT, atherosclerosis, *Zizyphus jujuba*

INTRODUCTION

The presence of a massive cluster of macrophage-derived foam cells in situ in the subendothelial spaces is one of the characteristic features of the early stages of atherosclerotic lesions.[1] Foam cells produce various bioactive molecules, such as cytokines, growth factors, and proteases, which play an important role in the development and progression of atherosclerotic lesions.[1] Macrophages take up chemically modified low-density lipoproteins (LDL), such as oxidized LDL (Ox-LDL) and acetylated LDL (acetyl-LDL) through their scavenger receptors.[2] Several scavenger receptors have been identified so far, including the class A scavenger receptor (SR-A),[3] class B scavenger receptor (CD36),[4] class B scavenger receptor type-I (SR-BI),[5] and lectin-like oxidized LDL receptor-1 (LOX-1).[6] Because free cholesterol, which is incorporated into the cells with modified LDL through the scavenger receptors, is toxic to the cells, it is esterified to the cholesterol ester (CE) by acyl coenzyme A:cholesterol acyltransferase (ACAT), an intracellular enzyme located in the rough endoplasmic reticulum.[7] These reactions change the macrophages to foam cells that are characterized by intracellular accumulation of CE. Foam cell formation by these mechanisms is believed to play an essential role in the progression of early atherosclerotic lesions in vivo, so prevention of foam cell formation is considered to be one of the major targets for the treatment of atherosclerosis.

From this point of view, many investigators have previously examined the usefulness of a number of antiatherosclerotic agents using various strategies, such as prevention of LDL oxidation,[8] inhibition of scavenger receptor expression,[9] and ACAT activity.[10] Because lifestyle-related diseases such as atherosclerosis and diabetes progress gradually due to unfavorable dietary habits, improvement of daily nutritional intake is thought to help prevent the pathogenesis of those diseases. For this reason, we tested the inhibitory effect of 50 crude plant extracts on foam cell formation in human monocyte-derived macrophages.

MATERIALS AND METHODS

Chemicals. Zizyphi Fructus (lot SUS312425) and Zizyphi Semen (lot 352513), inspected and certified by regulation of the Japanese Pharmacopeia, were purchased from Uchida Wakan-yaku Co. Ltd. (Tokyo, Japan). Triolein, cholesterol oleate, phenylmethanesulfonyl fluoride, cholesterol, L-α-phosphatidylcholine, taurocholic acid, and oleoyl coenzyme A were purchased from Sigma-Aldrich Japan (Osaka, Japan). Leupeptin and pepstatin A were purchased from the Peptide Institute (Osaka, Japan). Penicillin G, streptomycin sulfate, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Tokyo, Japan). Na[^{125}I] (17 Ci/mg), [9,10-^{3}H]oleate (4 Ci/mg), [^{14}C]oleoyl coenzyme A (50 μCi/mg), and [1α, 2α(n)-^{3}H]cholesteryl

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oleate (1 mCi/mg) were purchased from GE Healthcare (Tokyo, Japan). Other chemicals were of the best grade available from commercial sources.

General Experimental Procedures. Optical rotations and UV spectra were acquired on JASCO DIP-1000KUY and JASCO V-550 instruments, respectively. IR spectra were measured on a JASCO JIR-6500W instrument. FAB-MS was obtained on a JEOL JMS-DX303HF instrument. The ^{1}H and ^{13}C NMR spectra were recorded on a JEOLA δ-400 or α-500 spectrometer in pyridine-d_5. Chemical shifts were referenced to the residual solvent peaks (δ_H 7.24 and δ_C 149.8). Column chromatography was performed with Diaion HP-20 (Mitsubishi Kagaku, Tokyo, Japan), Sephadex LH-20 (GE Healthcare, Tokyo, Japan), Chromatorex ODS (Fuji Silysia Chemical Ltd., Aichi, Japan), or silica gel (Kanto Kagaku, Tokyo, Japan). HPLC was carried out using Cosmosil 5C18-AR-II (∅ 20 × 250 mm) (Nacalai Tesque, Kyoto, Japan) or Luna phenyl-hexyl (∅ 21.2 × 250 mm) (Shimadzu GLC, Tokyo, Japan) (2487 dual wavelength absorbance detector; 515 HPLC pump, Waters, Milford, MA).

Preparation of Crude Plant Extracts. The following were used to make the 50 crude extracts: Astragali Radix, Epimedii Herba, Artemisiae Capillari Flos, Foeniculi Fructus, Scutellariae Radix, Phellodendori Cortex, Coptidis Rhizoma, Catalpae Fructus, Gambir, Saposhnikoviae Radix, Corydalis Tuber, Pogostemi Herba, Puerariae Radix, Zingiberis Processum Rhizoma, Aurantii Fructus, Immaturus, Armeniacae Semen, Sophorae Radix, Schizonepetae Spica, Cinnamomi Cortex, Magnoliae Flos, Schisandrae Fructus, Asiasari Radix, Rehmanniae Radix, Paeoniae Radix, Achyranthis Radix, Cnidii Monnieris Fructus, Atractylodis Lanceae Rhizoma, Zizyphi Fructus, Zizyphi Semen, Alismatis Rhizoma, Ophiopogonis Tuber, Atractylodis Rhizoma, Hoelen, Coicis Semen, Polygalae Radix, Glycyrrhizae Radix, Anemarrhenae Rhizoma, Zedoariae Rhizoma, Cassiae Semen, Pharbitidis Semen, Cyperi Rhizoma, Schisandrae Fructus, Phellodendri Cortex, Lithospermi Radix, Plantaginis Semen, Houttuyniae Herba, Nupharis Rhizoma, Polyporus, Aurantii Nobilis Pericarpium, Chrysanthemi Flos, and Angelicae Dahuricae Rhizoma. Each crude drug was extracted with MeOH (three times) by refluxing for 2 h. The extracts were concentrated in vacuo and the residues loaded onto a Diaion HP-20 column and eluted with H_2O and MeOH. The MeOH eluates were used for the inhibition test of the foam cell formation.

Extraction and Isolation of Triterpenoids from Zizyphi Fructus. Zizyphi Fructus (500 g) was extracted with MeOH (three times) by refluxing for 2 h, and the extract was concentrated in vacuo to afford residues (116 g). The residues were loaded onto a Diaion HP-20 column (80 × 300 mm) and eluted by a stepwise gradient of H_2O−MeOH (0−100% MeOH) to afford five fractions, 40% MeOH−H_2O (1.23 g), 60% MeOH−H_2O (0.50 g), 80% MeOH−H_2O (0.35 g), and 100% MeOH (1.98 g). Among them, the 100% MeOH fraction (0.40% from Zizyphi Fructus) showed the most potent inhibition of foam cell formation. The bioassay-guided purification of the fraction using silica gel, reversed-phase gel, and reversed-phase HPLC afforded four compounds, betulonic acid (12.5 mg, 0.0025%),[11] oleanonic acid (14.7 mg, 0.0029%),[12] pomonic acid (32.6 mg, 0.00 65%),[13] and alphitolic acid (27.1 mg, 0.0054%).[14] The structures were identified on the basis of their NMR spectra.[11−14]

Extraction and Isolation of Triterpenoids from Zizyphi Semen. Zizyphi Semen (2 kg) was extracted with MeOH (four times) at 80 °C for 1.5 h, and the extract was concentrated in vacuo to afford residues (122.7 g). This extract was partitioned between *n*-hexane and 90% MeOH−H_2O to afford an *n*-hexane fraction (29.7 g) and a 90% MeOH fraction (85.2 g). The 90% MeOH fraction was loaded onto a Diaion HP-20 column (∅ 60 × 300 mm) and eluted by a stepwise gradient of H_2O−MeOH (H_2O, 50% MeOH, 100% MeOH). The most potent fraction, the 100% MeOH eluate (0.30% from Zizyphi Semen), was purified by Sephadex LH-20, silica gel, reversed-phase gel, and reversed-phase HPLC to afford a new compound and nine known compounds. The known compounds were identified as betulinic acid (63.26 mg, 0.00316%),[15] betulin (20.08 mg, 0.00100%),[16] alphitolic acid (37.97 mg, 0.00190%),[14] pomolic acid (7.34 mg, 0.00037%),[17] pomolic acid 28-methyl ester (2.02 mg, 0.00010%),[18] pomonic acid (3.50 mg, 0.00018%),[13] ceanothic acid (21.33 mg, 0.00107%),[19] epiceanothic acid (1.73 mg, 0.00009%),[20] and ceanothic acid 28-methyl ester (1.41 mg, 0.00007%)[21] on the basis of their NMR spectra. The new compound showed a molecular formula of $C_{31}H_{48}O_5$ as determined by HR FAB MS (*m*/*z* 499.3439 [M − H]$^-$; calcd for $C_{31}H_{47}O_5$, 499.3423). The ^{1}H and ^{13}C NMR spectra of the compound were similar to those of ceanothic acid except for the presence of the signals of a methoxyl group. The HMBC experiment showed the correlation between methoxyl hydrogens (δ_H 3.57) and carboxyl carbon (δ_C 175.8, C-2), which indicated that the methoxy signal existed as a methyl ester at the C-2 position.

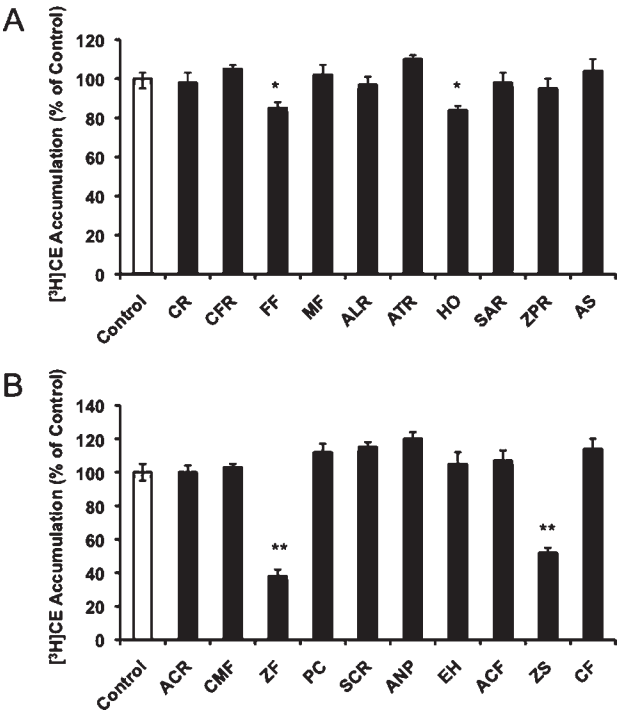

Figure 1. Inhibitory effect of crude plant extracts on CE accumulation due to acetyl-LDL uptake in HMDM. HMDM grown in 24-well plastic microplates were incubated with 50 μg/mL (acetyl-LDL) and 0.1 mM [^{3}H]oleate conjugated with BSA in the absence or presence of 100 μg/mL crude plant extracts (CR, Coptidis Rhizoma; CFR, Cimicifugae Rhizoma; FF, Foeniculi Fructus; MF, Magnoliae Flos; ALR, Atractylodis Lanceae Rhizoma; ATR, Atractylodis Rhizoma; HO, Hoelen; SAR, Saposhnikoviae Radix; ZPR, Zingiberis Processum Rhizoma; AS, Armeniacae Semen; ACR, Achyranthis Radix; CMF, Cnidii Monnieris Fructus; ZF, Zizyphi Fructus; PC, Phellodendri Cortex; SCR, Scutellariae Radix; ANP, Aurantii Nobilis Pericarpium; EH, Epimedii Herba; ACF, Artemisiae Capillari Flos; ZS, Zizyphi Semen; CF, Chrysanthemi Flos). After 24 h of incubation, the radioactivity of [^{3}H]CE separated by TLC was measured with a radioscanner, as described under Materials and Methods. The data are presented as the mean ± SD. *, $P < 0.05$, and **, $P < 0.001$, versus control.

Ceanothic Acid 2-Methyl Ester. The newly identified compound was a white amorphous powder with $[\alpha]_D(20)$ +27.6° (*c* 0.34, $CHCl_3$); UV λ_{max} (MeOH) nm (log e), 285 (3.68); IR (ZnSe) cm^{-1}, 2945, 2866, 1722, 1639, 1595, 1593, 1460, 1433, 1377, 1292, 1196, 1173, 1130, 1030, 1030, 1005, 756; HR-FAB-MS (negative), *m*/*z* 499.3439 [M − H]$^-$ (dalcd for $C_{31}H_{47}O_5$, 499.3423); ^{1}H NMR (in pyridine-d_5) δ 1.09 (3H, s, H_3-26), 1.12 (3H, s, H_3-27), 1.21 (1H, m, H-21), 1.21 (3H, s, H_3-24), 1.24 (1H, m, H-15), 1.29 (3H, s, H_3-25), 1.31 (1H, m, H-12), 1.32 (3H, s, H_3-23), 1.35 (1H, m, H-7), 1.42 (1H, m, H-7), 1.43 (2H, m, H_2-6), 1.45

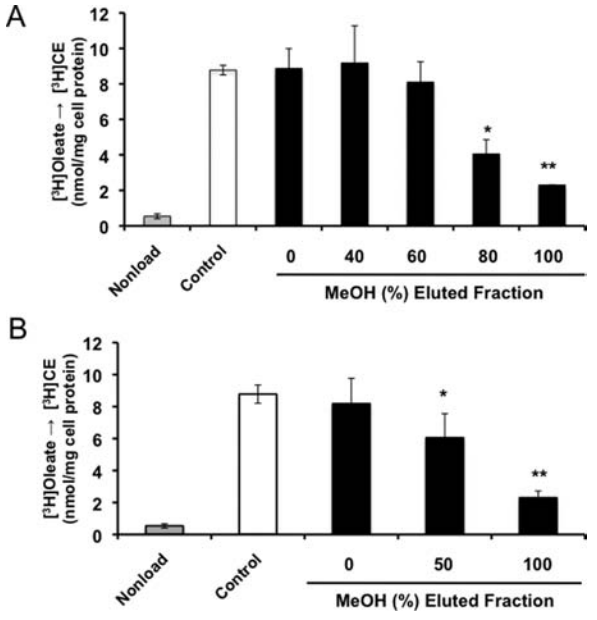

Figure 2. Inhibitory effect of the fractions purified by Diaion HP-20 column chromatography on CE accumulation due to acetylaed-LDL uptake in HMDM. HMDM grown in 24-well plastic microplates were incubated with 50 μg/mL acetyl-LDL and 0.1 mM [^{3}H]oleate conjugated with BSA in the absence or presence of the indicated MeOH eluted fractions (100 μg/mL) isolated from Zizyphi Fructus (A) and Zizyphi Semen (B). After a 24 h incubation, the radioactivity of [^{3}H]CE separated by TLC was measured with a radioscanner as described under Materials and Methods. The data are presented as the mean ± SD. *, $P < 0.05$, and **, $P < 0.001$, versus the control.

(1H, m, H-11), 1.52 (2H, m, H-16, H-22), 1.68 (1H, d, J = 11.4 Hz, H-11), 1.76 (1H, t, J = 11.4 Hz, H-18), 1.76 (3H, s, H_3-30), 1.83 (1H, dd, J = 12.8, 2.7 Hz, H-9) 1.89 (1H, dt, J = 13.7, 3.7 Hz, H-15), 1.98 (2H, m, H-5, H-12), 2.25 (2H, m, H-21, H-22), 2.61 (1H, br d, J = 12.9 Hz, H-16), 2.77 (1H, dt, J = 10.5, 3.0 Hz, H-13), 3.00 (1H, s, H-1), 3.57 (3H, s, OCH_3), 3.58 (1H, m, H-19), 4.59 (1H, s, H-3), 4.72 (1H, s, H-29), 4.93 (1H, s, H-29); ^{13}C NMR (in pyridine-d_5) δ 14.9 (q, C-27), 16.9 (q, C-26), 18.5 (q, C-25), 18.9 (t, C-6), 19.4 (q, C-30), 20.1 (q, C-24), 24.0 (t, C-11), 26.0 (t, C-12), 30.4 (t, C-15), 31.2 (t, C-21), 31.4 (q, C-23), 32.8 (t, C-16), 34.4 (t, C-7), 37.5 (t, C-22), 38.9 (d, C-13), 42.0 (s, C-14), 43.3 (s, C-8), 43.7 (s, C-4), 44.9 (d, C-9), 47.6 (d, C-19), 49.6 (d, C-18), 49.8 (s, C-10), 51.0 (OCH_3), 56.6 (s, C-17), 57.1 (d, C-5), 66.3 (d, C-1), 84.5 (d, C-1), 109.8 (t, C-29), 151.2 (s, C-20), 175.8 (s, C-2), 178.8 (s, C-28).

Synthesis of Ursolic Acid Methyl Ester and Uvaol. A solution of (trimethylsilyl)diazomethane (2.0 M solution in n-hexane, 0.5 mL) was added to a mixture of MeOH−ether (1.5 mL, 2:1) containing ursolic acid (10 mg), and the reaction mixture was stirred at room temperature for 10 min to afford ursolic acid methyl ester in a quantitative yield. To the methanolic solution (1.0 mL) of the ursolic acid methyl ester (8 mg) was added $LiAlH_4$ (10 mg), and the mixture was kept at room temperature for 4 h. The reaction mixture was diluted with H_2O and adsorbed on MCI gel CHP-20P (2 mL). After being eluted with H_2O (10 mL) and MeOH (10 mL), the MeOH eluate was evaporated in vacuo, and the residue was purified chromatographically on silica gel column (hexane−acetone, 3:1) to give uvaol (6 mg).

Synthesis of Oleanonic Acid Methyl Ester and Pomonic Acid Methyl Ester. In the same manner as described above, oleanonic acid (10 mg) and pomonic acid (10 mg) were methylated with (trimethylsilyl)diazomethane (2.0 M solution in n-hexane, 0.5 mL) to generate oleanonic acid methyl ester (10 mg) and pomonic acid methyl ester (10 mg), respectively, in a quantitative yield.

Lipoproteins and Modifications. Human LDL (d = 1.019−1.063 g/mL) was isolated by sequential ultracentrifugation from the human plasma of consenting normolipidemic subjects after overnight fasting. LDL was dialyzed against 0.15 M NaCl and 1.0 μM EDTA (pH 7.4). Acetylated-LDL was prepared by chemical modification of LDL with acetic anhydride as described previously.[22]

Cell Culture. Human peripheral mononuclear cells were isolated from the blood of healthy volunteers by Ficoll density gradient centrifugation (Ficoll−Paque from GE Healthcare, Tokyo, Japan). Human monocytes were purified according to the modified method of Connor et al.[23] Purified monocytes were suspended in DMEM at 2 × 10^6 cells/mL and seeded onto 24-well plates (4 × 10^5/well) or 6 cm dishes (2 × 10^6/dish) (BD Biosciences PRIMARIA, Tokyo, Japan). After incubation for 1 h, the medium was replaced with DMEM supplemented with 10% human serum, streptomycin (0.1 mg/mL), and penicillin G (100 U/mL). Adherent monocytes were incubated for 7 days to induce differentiation into macrophages. These human monocyte-derived macrophages (HMDM) were used for all experiments.

Assay for Foam Cell Formation (CE Accumulation). HMDM were incubated for 24 h in the presence of 50 μg/mL acetylated-LDL and 0.1 mM [^{3}H]oleate conjugated with BSA, and cellular lipids were extracted to determine the radioactivity of cholesteryl-[^{3}H]oleate as described previously.[24]

Assay for ACAT Activity. HMDM were homogenized with buffer A (50 mmol/L Tris-HCl and 1 mmol/L EDTA at pH 7.8 with protease inhibitors). The enzyme activity was determined by the reconstituted assay as described previously.[24]

Immunoblot Analyses. To detect SR-A, the HMDM were solubilized with lysis buffer (1% Triton X-100 in PBS) containing protease inhibitors. These samples were incubated for 24 h at 37 °C with 3 units of N-glycosidase (Roche Applied Science, Tokyo, Japan), and then 10 μg of protein was separated by 10% SDS−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine fluoride (PVDF) transfer membranes (Millipore, Bedford, MA). The membranes were exposed to anti-human SR-A antibody (E5) and visualized by horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody with ECL Western blotting detection reagents (GE Healthcare, Tokyo, Japan). To detect ACAT-1, the HMDM were solubilized with 10% SDS. Ten micrograms of protein was used for 10% SDS-PAGE and transferred to PVDF transfer membranes. The membranes were exposed to anti-human ACAT-1 antibody (Cayman, Ann Arbor, MI) and visualized by HRP-conjugated anti-rabbit IgG antibody with ECL Western blotting detection reagents. To detect SR-BI and CD36, the HMDM were solubilized with lysis buffer (1% Triton X-100 in PBS) containing protease inhibitors. Fifty micrograms of protein was used for 10% SDS-PAGE and transferred to PVDF transfer membranes. The membranes were exposed to anti-human SR-BI antibody (Novus Biologicals, Littleton, CO) and anti-human CD36 antibody (Abcam, Tokyo, Japan) and visualized by HRP-conjugated anti-rabbit IgG antibody with ECL Western blotting detection reagents. To detect ABCA1, the HMDM were solubilized with lysis buffer (1 mM $MgCl_2$, 350 mM NaCl, 20 mM Hepes, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM $Na_4P_2O_7$, 1 mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 1 mM Na_3VO_4, 20% glycerol, and 1% NP40). Cell lysates were centrifuged for 10 min at 4 °C (10000g) to obtain the supernatants. Fifty micrograms of protein was used for 10% SDS-PAGE and transferred to PVDF transfer membranes. The membranes were exposed to anti-human ABCA1 antibody (Abcam) and visualized by HRP-conjugated anti-rabbit IgG antibody with ECL Western blotting detection reagents. These membranes were reblotted with anti-β-actin antibody as an internal calibration control. The density of bands was measured with the Imaging Gauge software program in LAS 4000plus (Fujifilm, Tokyo, Japan).

Endocytic Uptake of Acetylated-LDL. The HMDM seeded onto 24-well plates were washed with 1.0 mL of phosphate-buffered

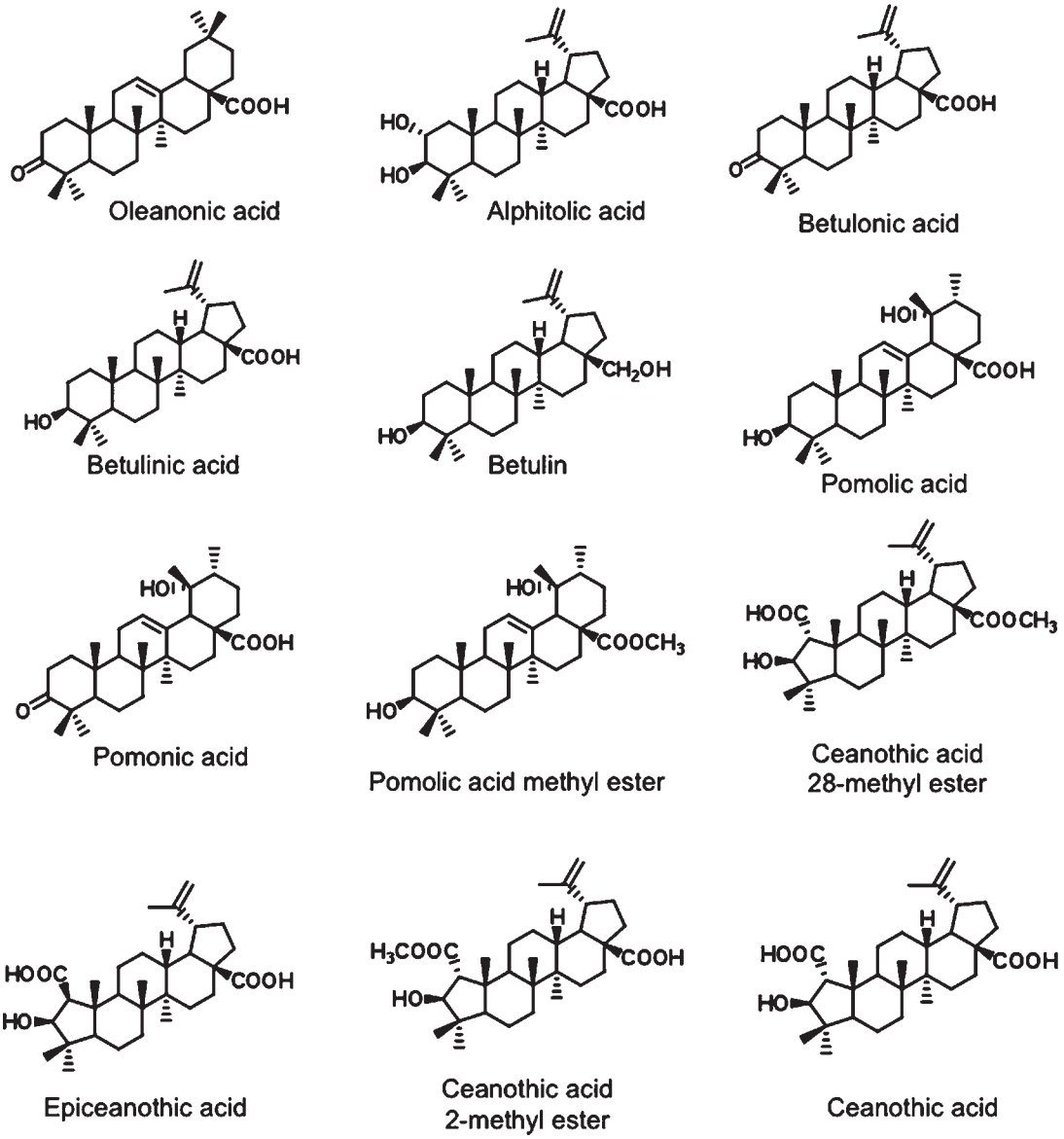

Figure 3. Triterpenoids isolated from *Zizyphus jujuba*.

saline (PBS) and replaced with DMEM containing 3% bovine serum albumin (BSA), 100 units/mL penicillin, and 100 μg/mL streptomycin (medium A). The cells in each well were incubated at 37 °C for 5 h in 0.5 mL of medium A with 50 μg/mL ^{125}I-acetylated-LDL in the presence of triterpenoids, and then 0.375 mL of the culture medium was taken from each well and mixed with 0.15 mL of 40% trichloroacetic acid (TCA) in a vortex mixer. To this solution we added 0.1 mL of 0.7 M $AgNO_3$, followed by centrifugation. The resultant supernatant (0.25 mL) was used to determine TCA-soluble radioactivity, which was considered as an index of cellular degradation. To measure the cell-associated radioactivity, each well was washed twice with 1.0 mL of ice-cold PBS containing 1% BSA and two more times with ice-cold PBS. The cells were lysed with 1.0 mL of 0.1 N NaOH for 1 h at 37 °C to determine the cell-associated radioactivity and cellular proteins.

Statistical Analysis. All data were expressed as the mean ± SD. Differences between the groups were examined to determine statistical significance using the Mann−Whitney U-test and the nonrepeated measures ANOVA. A p value of <0.05 denoted the presence of a statistically significant difference.

RESULTS AND DISCUSSION

We first tested the inhibitory effect of the crude plant extracts on CE accumulation in HMDM. Incubation of HMDM for 24 h with 50 μg/mL acetylated-LDL increased CE accumulation. Under the assay conditions employed, extracts of Foeniculi Fructus, Hoelen, Zizyphi Semen, and Zizyphi Fructus inhibited CE accumulation (Figure 1). The extracts of Zizyphi Semen and Zizyphi Fructus significantly inhibited CE accumulation, suggesting that the compound(s) contained in Zizyphi Semen (seeds of *Z. jujuba*) and Zizyphi Fructus (fruits of *Z. jujuba*) could inhibit CE accumulation. Next, the extracts of Zizyphi Semen and Zizyphi Fructus were separated by Diaion HP-20 column chromatography and eluted with 0−100% MeOH to give three or five fractions. As shown in Figure 2, each 100% MeOH fraction of Zizyphi Fructus and Zizyphi Semen significantly inhibited CE accumulation. Purification of the active fractions afforded triterpenoids (Figure 3), which inhibited CE accumulation (Figure 4). However, those triterpenoids caused no morphological changes

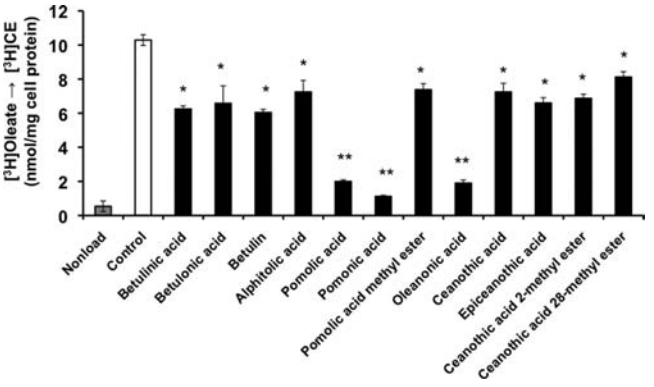

Figure 4. Inhibitory effect of isolated compounds from *Zizyphus jujuba* on CE accumulation by (acetyl-LDL) uptake in HMDM. HMDM grown in 24-well plastic microplates were incubated with 50 μg/mL (acetyl-LDL) and 0.1 mM [^{3}H]oleate conjugated with BSA in the absence or presence of isolated compounds from *Z. jujuba* (50 μM). After a 24 h incubation, the radioactivity of [^{3}H]CE separated by TLC was measured with a radioscanner as described under Materials and Methods. The data are presented as the mean ± SD. ∗, $P < 0.01$, and ∗∗, $P < 0.00001$, versus the control.

or cytotoxic effects on HMDM, even at 100 μM (Supporting Information).

Among them, oleanonic acid, pomolic acid, and pomonic acid significantly inhibited CE accumulation, suggesting that ursane-type triterpenoids (pomolic acid and pomonic acid) and oleanane-type triterpenoids (oleanonic acid) show more potent inhibition of CE accumulation than lupane-type triterpenoids (betlinic acid, betulin, and alphitolic acid) and ceanothane-type triterpenoids (epiceanothic acid, epiceanothic acid, ceanothic acid 28-methyl ester, and ceanothic acid 2-methyl ester) (Figure 4). Furthermore, the inhibitory effect of pomolic acid was more potent than that of pomolic acid 28-methyl ester (Figure 4), thus suggesting that the presence of a functional group at C-28 in the ursane-type and oleanane-type triterpenoids may correlate with their inhibitory effect on CE accumulation. To confirm this hypothesis, we changed the C-28 carboxylic acids of pomonic acid and oleanonic acid into their methyl esters and that of ursolic acid, a major ursane-type triterpenoid, into C-28 carboxylic methyl esters, C-28 methyl (α-amyrin) and C-28 hydroxymethyl (uvaol), and tested their inhibitory effects on CE accumulation. As shown in Figure 5, pomolic acid, pomonic acid, oleanonic acid, and ursolic acid significantly inhibited CE accumulation. On the other hand, the inhibitory effects of the derivatives were significantly decreased. These data strongly indicate that the C-28 carboxylic acid of ursane-type and oleanane-type triterpenoids plays an important role in their inhibitory effect on CE accumulation in human macrophages.

It was recently reported that oleanolic acid, betulonic acid, and ursolic acid can inhibit the enzymatic activity of ACAT, an intracellular enzyme located in the rough endoplasmic reticulum that changes free cholesterol into cholesterol ester.[25] This finding suggests that pomolic acid, pomonic acid, and oleanonic acid also may suppress ACAT activity. To test this possibility, the inhibitory effects of the triterpenoids on CE formation were tested in a cell-free system. Microsomes prepared from HMDM were used as an enzyme source. Microsomes were incubated for 15 min with 250 μmol/L [^{14}C]oleoyl-CoA in the presence or absence of the triterpenoids, and the formation of cholesteryl [^{14}C]oleate was measured. As shown in Figure 6, pomolic acid, pomonic acid, and oleanolic acid significantly suppressed the ACAT activity, whereas the derivatives of the triterpenoids were inactive. However, those triterpenoids did not change the expression of ACAT, scavenger receptors (SR-A, CD36, and SR-BI), or ABCA-1 (Figure 7A). Furthermore, those triterpenoids did not affect the endocytic uptake of ^{125}I-acetylated-LDL (Figure 7B). These data demonstrate that the major effective triterpenoids isolated from *Z. jujuba* inhibit foam cell formation by suppression of ACAT activity. Furthermore, the C-28 carboxylic acid of the most effective triterpenoids plays an important role in their inhibitory effect on ACAT activity.

Acute coronary syndromes (ACS), including unstable angina, myocardial infarction, and sudden coronary death, are the major causes of cardiovascular diseases. Clinical studies demonstrate that the occurrence of ACS always correlates with the presence of vulnerable atherosclerotic plaques and subsequent thrombosis. In the early stage of atherosclerogenesis, macrophages penetrate into the intima, efficiently take up modified LDL, and store cholesterol and fatty acids as forms of neutral lipids in the cytosolic lipid droplets. Free cholesterol is esterified to CE by ACAT, thus leading to the formation of macrophage-derived foam cells in atherosclerotic lesions.[26] In the present study, we first screened 50 crude plant extracts for inhibitory effects on CE accumulation in HMDM. Among the extracts, those from Zizyphi Fructus and Zizyphi Semen significantly inhibited CE accumulation (Figure 1). Zizyphi Fructus and Zizyphi Semen are the fruit and seeds of *Z. jujuba*, respectively. Zizyphi Fructus has been widely used in folk medicine as a tonic, an ingredient of many preparations, and as a health food in Asia. On the other hand, Zizyphi Semen has been used as a tranquilizer, an analgesic, and an anticonvulsant in oriental countries and has also been prescribed for the treatment of insomnia and anxiety in Asia. The current study demonstrated that triterpenoids (Figure 3) isolated from *Z. jujuba* inhibited foam cell formation (Figure 4). Among the various triterpenoids present in the extracts, oleanonic acid, pomolic acid, and pomonic acid significantly inhibited CE accumulation, suggesting that ursane-type (pomolic acid and pomonic acid) and oleanane-type (oleanonic acid) triterpenoids show more potent inhibition of CE accumulation than lupane-type (betlinic acid, betulin, and alphitolic acid) and ceanothane-type (epiceanothic acid, epiceanothic acid, ceanothic acid 28-methyl ester, and ceanothic acid 2-methyl ester) triterpenoids (Figure 4). Furthermore, our findings revealed that the C-28 carboxylic acid of ursane-type and oleanane-type triterpenoids plays an important role in their inhibitory effect on CE accumulation in human macrophages (Figure 5). It is known that pomolic acid enhances glucose uptake in L6 rat skeletal muscle cells, inhibits the proliferation of K562 chronic myeloid leukemia cells, and has antifibrotic and antiinflammatory activity.[27−30] Oleanonic acid exerts an inhibitory effect on the proliferation of human nasopharyngeal carcinoma HONE-1 and oral epidermoid carcinoma KB cells and has an anti-inflammatory activity.[31,32] However, the effects of oleanonic acid, pomolic acid, and pomonic acid on macrophages is not clear. Therefore, this is the first evidence that oleanonic acid, pomolic acid, and pomonic acid have a strong inhibitory effect on foam cell formation in macrophages.

Several types of inhibitors of the accumulation of lipid droplets in macrophages have been reported.[33−37] Sterol derivatives such

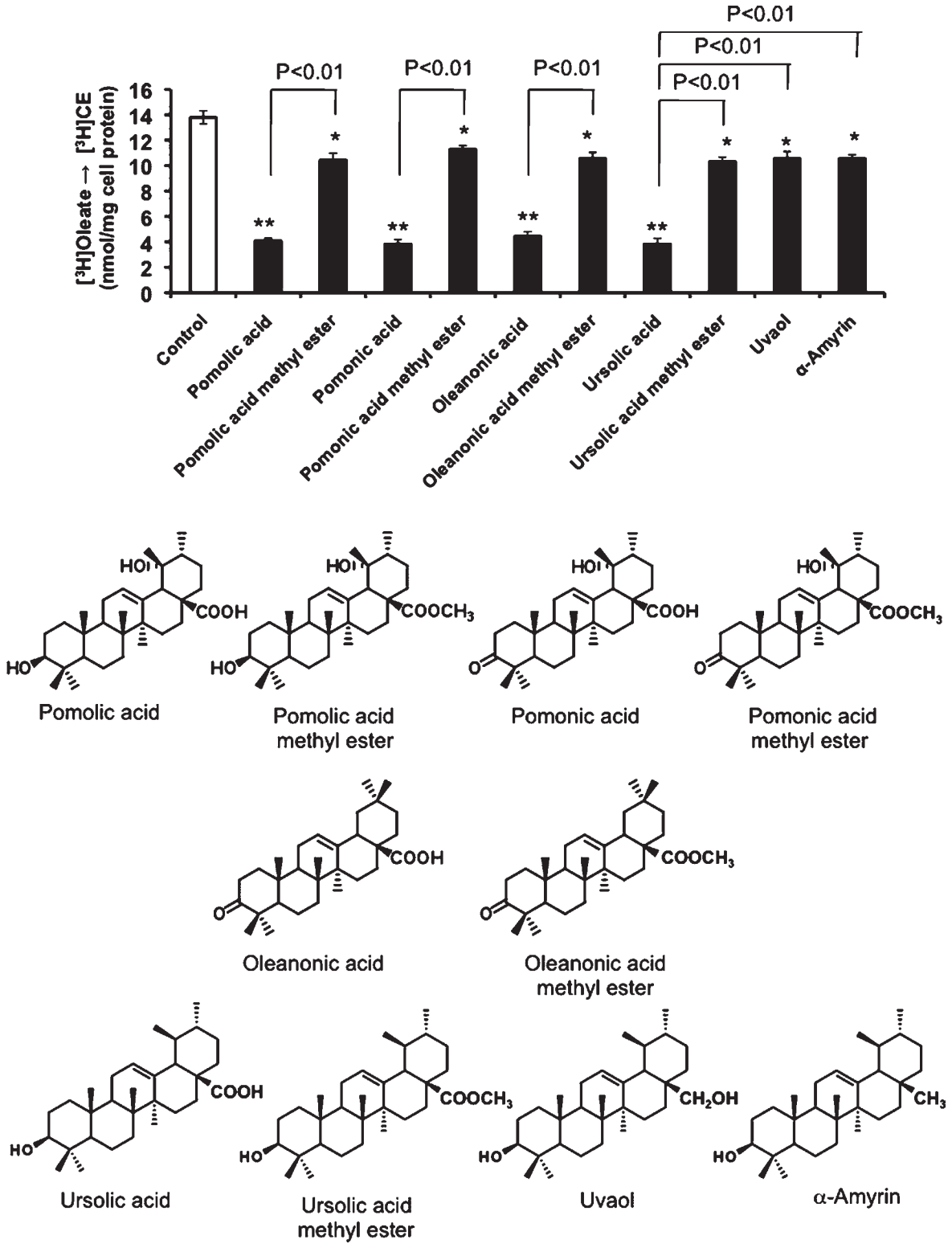

Figure 5. Inhibitory effect of triterpenoid derivatives on CE accumulation by acetyl-LDL uptake in HMDM. HMDM grown in 24-well plastic microplates were incubated with 50 μg/mL (acetyl-LDL) and 0.1 mM [^{3}H]oleate conjugated with BSA in the absence or presence of triterpenoids and their derivatives (50 μM). After a 24 h incubation, the radioactivity of [^{3}H]CE separated by TLC was measured with a radioscanner as described under Materials and Methods. The data are presented as the mean ± SD. *, $P < 0.01$; **, $P < 0.00001$, versus the control.

as U18666A,[33] progesterone, and pregnenolone [34] inhibit the movement of cholesterol out of the lysosome and the activity of multidrug-resistant P-glycoproteins in the plasma membrane,[35] and a large number of ACAT inhibitors block cholesterol

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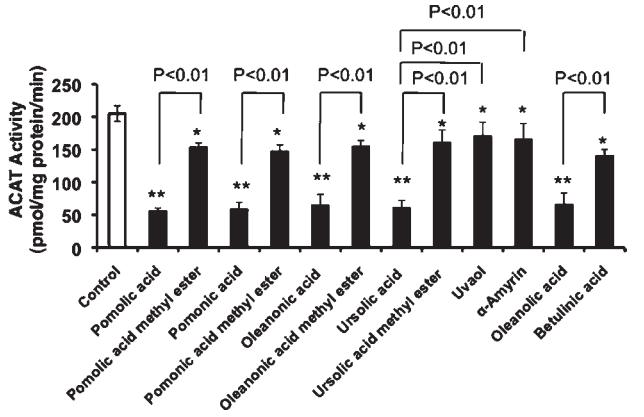

Figure 6. Effect of triterpenoids on ACAT activity. HMDM were homogenized in buffer A. The homogenates obtained from cultured cells were mixed with 4 mol/L KCl and 20% CHAPS in buffer A to obtain final concentrations of 1 mol/L and 2%, respectively. These samples were reconstituted with sodium PC mixed micelles together with triterpenoids and their derivatives (50 μM). The enzyme reaction was initiated by adding [^{14}C]oleoyl-CoA followed by incubation for 15 min at 37 °C. Lipids were then extracted, the radioactive cholesteryl-[^{14}C]oleate was determined by TLC, and radioactivity was measured as described under Materials and Methods. The data are presented as the mean ± SD. *, $P < 0.01$, and **, $P < 0.0001$, versus the control.

esterification in the endoplasmic reticulum.[37] Oleanonic acid, pomolic acid, and pomonic acid also inhibit CE accumulation in macrophages by suppression of ACAT activity (Figure 6). Furthermore, we revealed that the C-28 carboxylic acid of those triterpenoids plays an important role in their inhibition of ACAT activity (Figure 6).

ACAT plays a significant role in intracellular cholesterol storage, lipoprotein assembly, steroid hormone production, and dietary cholesterol absorption. Two human ACAT isozymes, ACAT-1 and ACAT-2, have been identified so far.[37] ACAT-1 is highly expressed by macrophage-derived foam cells in atherosclerotic lesions and is up-regulated during monocytic differentiation into macrophages.[38,39] In addition, ACAT-1 is located in the Kupper cells of the liver, kidneys, and adrenal cortical cells, whereas ACAT-2 is mainly located in hepatocytes and intestinal mucosal cells.[40,41] Therefore, many researchers have examined numerous antiatherosclerotic agents for their potential to inhibit ACAT activity. Nonselective ACAT inhibitors are known to reduce atherosclerosis in apoE-deficient mice.[42] NTE-122, a nonselective ACAT inhibitor, prevents the progression of atherosclerosis in cholesterol-fed rabbits.[43] F-1394, another nonselective ACAT inhibitor, also prevents the progression of atherosclerosis in cholesterol-fed rabbits.[44] Furthermore, our previous study clearly demonstrated that esculeogenin A, isolated from tomatoes, significantly decreased CE accumulation in HMDM by inhibiting ACAT activity and also reduced the serum cholesterol level and atherosclerosis in apoE-deficient mice.[24] Therefore, the present study strongly indicates that oleanonic acid, pomolic acid, and pomonic acid may be able to prevent atherosclerosis by inhibiting ACAT. Taken together, it is likely that *Z. jujuba* contains several triterpenoids that inhibit foam cell formation, and daily intake of *Z. jujuba* may decrease the risk of cardiovascular diseases.

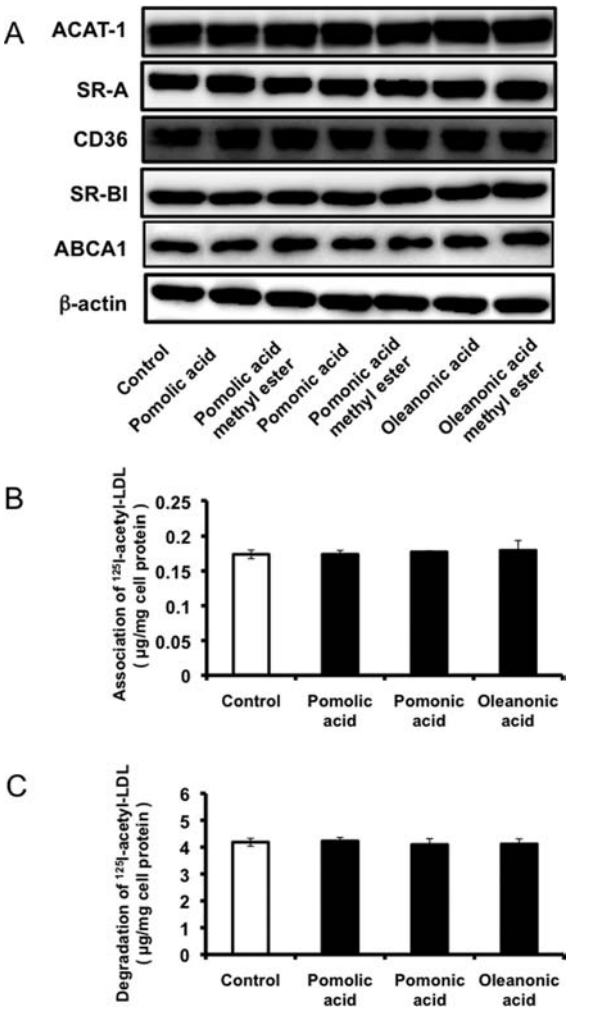

Figure 7. Effect of triterpenoids on scavenger receptors and ABCA1 expression and ^{125}I-(acetyl-LDL) endocytic uptake in HMDM. HMDM were incubated with triterpenoids and their derivatives (50 μM) for 24 h. The cells were harvested and subjected to immunoblot analyses using antibodies against human ACAT-1, human SR-A, human CD36, human SR-BI, and human ABCA1 as described under Materials and Methods (A). HMDM were incubated for 5 h with triterpenoids and with 50 μg/mL ^{125}I-(acetyl-LDL), followed by determination of cell association (B) and cell degradation (C) of ^{125}I-(acetyl-LDL) as described under Materials and Methods.

■ ASSOCIATED CONTENT

Supporting Information. Effect of triterpenoids on cell cytotoxity in HMDM. HMDM were incubated with triterpenoids (100 μM) for 24 h, followed by the determination of cell viability by WST-8 assay. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +81-96-371-4738. Fax: +81-96-362-7799. E-mail: tikeda@kumamoto-u.ac.jp.

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