Esculeogenin A, a New Tomato Sapogenol, Ameliorates Hyperlipidemia and Atherosclerosis in ApoE-Deficient Mice by Inhibiting ACAT

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Objective—We recently identified esculeoside A, a new spirostanol-type glycoside, with a content in tomatoes that is 4-fold higher than that of lycopene. In the present study, we examined the effects of esculeoside A and esculeogenin A, a new aglycon of esculeoside A, on foam cell formation in vitro and atherogenesis in apoE-deficient mice.

Methods and Results—Esculeogenin A significantly inhibited the accumulation of cholesterol ester (CE) induced by acetylated low density lipoprotein (acetyl-LDL) in human monocyte-derived macrophages (HMDM) in a dose-dependent manner without inhibiting triglyceride accumulation, however, it did not inhibit the association of acetyl-LDL to the cells. Esculeogenin A also inhibited CE formation in Chinese hamster ovary cells overexpressing acyl-coenzymeA (CoA): cholesterol acyl-transferase (ACAT)-1 or ACAT-2, suggesting that esculeogenin A suppresses the activity of both ACAT-1 and ACAT-2. Furthermore, esculeogenin A prevented the expression of ACAT-1 protein, whereas that of SR-A and SR-BI was not suppressed. Oral administration of esculeoside A to apoE-deficient mice significantly reduced the levels of serum cholesterol, triglycerides, LDL-cholesterol, and the areas of atherosclerotic lesions without any detectable side effects.

Conclusions—Our study provides the first evidence that purified esculeoside A significantly suppresses the activity of ACAT protein and leads to reduction of atherogenesis. (Arterioscler Thromb Vasc Biol. 2007;27:2400-2406.)

Key Words: esculeogenin A ■ atherosclerosis ■ ACAT ■ human monocyte-derived macrophages ■ foam cells

The presence of a large cluster of macrophage-derived foam cells in situ in the subendothelial spaces is one of the characteristic features of early stage atherosclerotic lesions.1 Macrophages take up chemically-modified low-density lipoproteins (LDL), such as oxidized LDL (Ox-LDL) and acetylated LDL (acetyl-LDL) through the scavenger receptors2 such as class A scavenger receptor (SR-A),3 class B scavenger receptor (CD36),4 and class B scavenger receptor type-I (SR-BI).5 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-CoA: cholesterol acyl-transferase (ACAT)-1 or ACAT-2, suggesting that ACAT-1 plays a critical role in foam cell formation in macrophages; whereas ACAT-2 is responsible for the cholesterol absorption process in intestinal mucosal cells.6 Because foam cell formation by these mechanisms is believed to play an essential role in the progression of early atherosclerotic lesions in vivo, prevention of foam cell formation is considered as one of the major targets for the treatment of atherosclerosis. From this point of view, many investigators have examined the usefulness of a number of antiatherosclerotic agents using various strategies such as prevention of LDL oxidation,7 inhibition of scavenger receptor expression,8 and ACAT activity.9 We recently isolated esculeoside A,10 a new spirostanol type glycoside, from the fruit of tomatoes (Lycopersicon esculentum) such as the cherry tomato and the Momotaro tomato. In addition, we identified a new compound named esculeogenin A11 as aglycon of esculeoside A by hydrolysis of esculeoside A.

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Because the sugar chains of glycosides in natural products are degraded by the action of intestinal bacteria after oral administration, the aglycons act as physiologically active substances.\(^{15,16}\) Therefore, esculeoside A also changes into esculeogenin A in the intestine, followed by production of esculeogenin A in vivo.

Because lifestyle related diseases such as atherosclerosis and diabetes progress gradually because of unfavorable dietary habits, improvement of daily nutritional intake is thought to prevent the pathogenesis of these diseases. For this reason, we have prepared 50 crude extracts and 80 purified compounds from natural products, and measured their inhibitory effect on foam cell formation in human monocyte-derived macrophages (HMDM). The results showed that purified esculeogenin A significantly suppresses the activity of ACAT protein and leads to reduction of atherogenesis.

Methods

Preparation of Esculeoside A and Esculeogenin A

Esculeoside A and esculeogenin A were prepared as previously described\(^{13,14}\) (Please see supplemental Figure I, available online at http://atvb.ahajournals.org).

Isolation of Monocyte and Endocytic Uptake of Acetyl-LDL

Human peripheral mononuclear cells were isolated from the blood of healthy volunteers by Ficoll density gradient centrifugation (Ficoll-Paque from GE Healthcare Bio-Sciences). Acetyl-LDL was prepared by chemical modification of LDL with acetic anhydride as previously described.\(^{17}\) Acetyl-LDL was labeled with \(^{125}\)I as described by McFarlane.\(^{18}\) The differentiated human monocyte-derived macrophages (HMDM) were incubated at 37°C for 5 hour with 50 \(\mu\)g/mL of \(^{125}\)I-acetyl-LDL in the presence of the indicated concentration of esculeogenin A, then the cell associated radioactivity and cell degraded radioactivity were measured as described supplementary information I.

Assay for Foam Cell Formation (CE-Accumulation)

HMDM monolayers were incubated with 50 \(\mu\)g/mL acetyl-LDL for 24 hours in the presence of 0.1 mmol/L \(^{3}\)H)oleate conjugated with BSA, and cellular lipids were extracted for determination of radioactivity of cholesteryl-[\(^{3}\)H]oleate as described previously.\(^{19}\)

Assay for ACAT Activity and ACAT Expression

Microsomes prepared from HMDM were used as an enzyme source. Microsomes were incubated for 15 minutes with 250 \(\mu\)mol/L \(^{14}\)C]oleoyl-CoA in the presence or absence of esculeogenin A, and the formation of cholesteryl \(^{14}\)C]oleate was measured.

Expression level of ACAT-1 and scavenger receptors were measured by Western blotting analysis (please see supplementary information I).

In Vivo Antiatherosclerotic Activity

Apollipoprotein E (apoE)-deficient mice (C57BL/6.KOR-ApoE\(^{20}\)) were fed a normal rodent chow diet (Clea) and esculeoside A (50 and 100 mg/kg of body weight) containing diets and administered orally every day for 90 days. Total cholesterol, LDL cholesterol and triglyceride levels in serum were determined. Whole aorta were collected and stained with Sudan IV, and cross sections of proximal aorta were prepared and stained with oil red as described\(^{20}\) (please see supplementary information I).

For enhanced Materials and Methods of in vitro experiments used on this article, please see supplementary information I.
significant amounts of $^{125}$I-acetyl-LDL were associated with the cells (Figure 1C) and subjected to lysosomal degradation by the same cells (Figure 1C), whereas these cellular responses were not inhibited by esculeogenin A.

**Inhibitory Effect of Esculeogenin A on Acetyl-LDL–Induced CE Accumulation in Human ACAT Overexpressing CHO Cells**

We subsequently measured the involvement of ACAT in the esculeogenin A–induced reduction of CE accumulation in the HMDM. Incubation of CHO cells overexpressing human ACAT-1 (hACAT-1 CHO) and human ACAT-2 (hACAT-2 CHO) for 24 hours with medium containing $[^3]$H]oleate increased CE accumulation. Under these conditions, esculeogenin A inhibited CE accumulation in both hACAT-1 and hACAT-2 CHO cells in a dose-dependent manner (Figure 2A and 2B). Esculeogenin A caused no morphological changes or cytotoxic effects on both cells even at 100 μmol/L esculeogenin A (data not shown). Because esculeogenin A showed a significant inhibitory effect on CE accumulation in both HMDM and hACAT CHO cells in a similar fashion, esculeogenin A may serve as an inhibitor of cholesterol esterification, possibly by inhibiting ACAT activity and/or ACAT expression.

**Inhibitory Effects of Esculeogenin A on ACAT Activity in HMDM and hACAT CHO Cells**

As shown in Figure 2C, esculeogenin A inhibited ACAT activity in a dose-dependent manner. Similar results were observed by hACAT-1 and hACAT-2 CHO cells. Thus, formation of cholesterol $^{[14]}$C]oleate by microsomes prepared from hACAT-1 or hACAT-2 CHO cells was inhibited in the presence of esculeogenin A in a dose-dependent manner (Figure 2D and 2E). These data suggest that esculeogenin A has a significant inhibitory effect on foam cell formation from HMDM by inhibiting ACAT activity.

**Inhibitory Effects of Esculeogenin A on SR-A, SR-BI, and ACAT-1 Expression in HMDM**

Incubation of HMDM for 24 hours in the presence of indicated concentration of esculeogenin A resulted in inhibition of ACAT-1 expression in a dose-dependent manner, whereas expression of SR-A and SR-BI remained unchanged compared with the control (supplementary Figure II in supplementary information I). These results suggest that esculeogenin A has a significant inhibitory effect on foam cell formation from HMDM through the inhibition of both the expression and activity of ACAT.

**Changes in Body Weight and Biochemical Data of Plasma Samples in ApoE-Deficient Mice**

We next administrated esculeoside A to apoE-deficient mice to examine effect on atherogenesis. As shown in Figure 3A, mean body weight gain was unchanged by administration of esculeoside A for 90 days. Total cholesterol levels showed a trend toward reductions after the oral administration of esculeoside A (50 mg/kg/d), and were significantly reduced by approximately 25% after administration of 100 mg/kg/d (Figure 3B). Furthermore, administration of esculeoside A (100 mg/kg/d) significantly reduced serum levels of LDL cholesterol (Figure 3C) and triglycerides (Figure 3D) by approximately 25% (Figure 3C) and 45% (Figure 3D), respectively, without changing TC/HDL ratio (data not shown) compared with the control group.

**Inhibition of ACAT Activity in ApoE-Deficient Mice by Esculeoside A Treatment**

Administration of esculeoside A (100 mg/kg/d) to apoE-deficient mice for 21 days significantly reduced the ACAT activity in the liver (supplemental Figure IIIA in supplementary information I) and peritoneal macrophages (supplemental Figure IIIB in supplementary information I), whereas intestinal ACAT activity (supplemental Figure IIIC in sup-
Esculeogenin A Inhibits Atherosclerosis

Inhibition of Atherogenesis in ApoE-Deficient Mice by Esculeoside A Treatment

Although surface atherosclerotic lesions were observed in both groups mainly in the arch region, total surface lesions in the esculeoside A (100 mg/kg/d)-treated group were significantly reduced compared with the control group (8.2% versus 13.8%, P<0.01; Figure 4A, 4B, and 4E). Cross sections of the aortic sinus showed a marked thickening of the intima filled with oil red O-positive foamy cells in the control mice (C), whereas such lesions were greatly reduced in esculeoside A-treated mice (D). The cross-sectional lesion area in the esculeoside A (100 mg/kg/d)-treated mice was significantly smaller (by 52%) than that of control mice (214,508 versus 444,555 μm², P<0.005; Figure 4C, 4D, and 4F). LC-MS/MS analyses demonstrated that esculeogenin A was detected from the aorta of esculeoside A-treated apoE-deficient mice (Supplementary Figure IV in supplementary information I), demonstrating that orally administrated esculeoside A is converted into esculeogenin A by intestinal bacteria.
Discussion

Because both of the ACAT isozymes are expressed in the liver and provide cholesteryl esters for VLDL, an ACAT-1 inhibitor, such as K-604, significantly decreases the serum cholesterol level. Furthermore, it is known that ACAT inhibitors block dietary cholesterol absorption in the intestines when animals are fed with high fat/high cholesterol diet. Because we conducted animal experiments using apoE-deficient mice administered a normal diet, it is reasonable that the administration of esculeoside A inhibited the ACAT activity in the liver and macrophages rather than in the intestine, and decrease endogenous cholesterol production. However, the mechanism by which esculeoside A ameliorates the level of triglycerides remains unclear. A similar question has arisen regarding some reported ACAT inhibitors. Thus, oral administration of ACAT inhibitors such as R-755, U-73482, and CI-976 to rat reduce the level of serum triglycerides, whereas its mechanism still remains poorly understood. Furthermore, although 3-hydroxy-3-methylglutaryl (HMG) Co-A reductase inhibitor, such as atorvastatin, decreases not only the serum level of cholesterol but also the triglycerides, little is known about its mechanism. Taken together, it is therefore likely that the administration of esculeoside A decreases endogenous cholesterol production by inhibiting the liver ACAT activity, followed by decreases in the serum triglycerides concentration like some reported ACAT inhibitors and atorvastatin. Field FJ et al demonstrated that although the inhibitory effect of β-sitosterol on ACAT activity is weak, it inhibits cholesterol absorption in the intestine because cholesterol in the mixed micelles is replaced by β-sitosterol because of its high hydrophobicity. Because esculeogenin A possesses 3 hydroxyl groups and shows higher hydrophilicity than betesitosterol, the replacement of esculeogenin A with cholesterol in the mixed micelles is negligible or unlikely to occur. Thus, the physicochemical property of esculeogenin A differs from β-sitosterol. Taken together, the present study indicates that the administration of esculeoside A may inhibit the development of atherosclerosis by decreasing the serum cholesterol level through the inhibition of ACAT in the liver and the suppression of foam cell formation by inhibiting ACAT activity in the macrophage.

Many researchers have examined the usefulness of a number of antiatherosclerotic agents using as a strategy inhibition of ACAT activity. Mice lacking ACAT-2 exhibited a restricted capacity to absorb cholesterol and protection against diet-induced hypercholesterolemia and gallstone formation. Nonselective ACAT inhibition is known to reduce atherosclerosis in apoprotein E–deficient mice. NTE-122 and F-1394, a nonselective ACAT inhibitors, prevents the progression of atherosclerosis in cholesterol-fed rabbits. Moreover, the ACAT inhibitor avasimibe reduces macrophage and matrix metalloproteinase (MMP) expression in atherosclerotic lesions of hypercholesterolemic rabbits, and reduces atherosclerosis in addition to its cholesterol-lowering effect in apoE*3-Leiden mice. Sakashita et al demonstrated that ACAT-2 is also expressed in macrophage-derived foam cells in vitro and in vivo. Therefore, it is likely that esculeogenin A may have inhibited the activity of both ACAT-1 and ACAT-2 in the HMDM and showed a remarkable inhibitory effect of foam cell formation. However, Tardif et al reported that avasimibe, administered for 2 years to patients with atherosclerosis, did not reduce plaque volume. Furthermore, unfavorable aspects of ACAT-1–deficient mice is reported (please see Supplementary information II). However, Sahi et al reported that avasimibe increases CYP3A4 (Cytochrome P450; family 3, subfamily A, polypeptide 4) and multiple drug resistance protein 1 gene expression in vitro through activation of pregnane X receptor. Consequently, avasimibe causes clinically significant changes in the pharmacokinetics of the CYP3A4 substrate and P-glycoprotein substrate. The induction of CYP3A4 of P-glycoprotein may be the basis for the observed autoinduction of clearance for avasimibe itself. Therefore, it is speculated that avasimibe is degraded easily in the human body and does not function as an atheroprotective agent. Generally, reported ACAT inhibitors, such as CI976 and avasimibe, show high hydrophobicity attributable to their ring-shaped structure and the alkyl chain. However, because esculeogenin A has the hydroxyl and the amino group, but not the alkyl chain (please see supplementary figure I in supplementary information I), the hydrophobicity of esculeogenin A is considerably lower than that of cholesterol. Furthermore, although the reported ACAT inhibitors are divided roughly into urea compounds and amide compounds that contain an urea group and amide group, respectively, esculeogenin A does not belong to either of them.

We compared CI976, a synthetic ACAT inhibitor. CI976 inhibited cholesterol ester (CE) accumulation by 90% when human monocyte-derived macrophages were incubated with 5 μmol/L CI976, whereas 50 μmol/L esculeogenin A were required to show same activity. Moreover, although 5 μmol/L CI976 inhibited ACAT activity by 90%, 50 μmol/L esculeogenin A inhibited by 40% (data not shown), thus indicating that the inhibitory effect of esculeogenin A on foam cell formation and ACAT activity is lower than that of known ACAT inhibitor. However, it is known that preventive medicine is the most important approach to prevent the lifestyle related diseases such as atherosclerosis and type II diabetes, and improvement of daily nutritional intake is thought to prevent the pathogenesis of these diseases. Therefore, ACAT inhibitors which can be taken from normal daily meals are preferred to strong synthetic ACAT inhibitors. Because esculeoside A is the natural compound isolated from tomatoes, we can intake esculeoside A from tomato products at daily life and it would thus be a promising strategy for preventive medicine. Therefore, in spite of lower inhibiting activity compared with synthetic ACAT inhibitor, we believe that daily intake of esculeoside A can thus play a beneficial role in preventing the pathogenesis of atherosclerosis. In the present study, we administered 50 mg/kg BW and 100 mg/kg BW of esculeoside A. Based on the esculeoside A content of a tomato, this dosage corresponds to about 10 kg when the human body weight is estimated as 60 kg. As described in the Methods, this dosage was decided based on a previous study by Kong W et al. However, because human atherosclerosis progresses more mildly and takes
over the years, we speculate that atherosclerosis can be ameliorated even when taking only 1/20 or 1/50 of the mice dosage if tomatoes are eaten on a daily basis. We will need to conduct additional study to evaluate the inhibitory effect of esculeoside A on the pathogenesis of atherosclerosis in human. Furthermore, because the tomato contains not only esculeoside A but also lycopene which inhibits oxidation of circulating LDL,10 daily intake of tomatoes and tomato products may decrease the risk of cardiovascular diseases. Further studies will be required to elucidate the mechanism of esculeogenin A to inhibit activity and expression of ACAT.

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Disclosures

None.

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Supplementary Information II

Unfavorable aspects of synthetic ACAT inhibitors and ACAT-1 deficient mice.

Atherothrombotic vascular disease occurs in two major stages. In the first stage, which evolves over years, atherogenic lipoproteins are internalized by arterial wall macrophages, leading to massive cholesteryl ester accumulation, foam cells formation. The process involves transport of lipoprotein-derived cholesterol to the endoplasmic reticulum, where it is esterified by ACAT. Early foam cell lesions are non-occlusive and are therefore largely asymptomatic. In the second stage, macrophage-rich plaques rupture or erode, which exposes circulating platelets to thrombogenic material in the lesions. This process leads within minutes to acute arterial occlusion and tissue infarction. A key event in plaque disruption is macrophage death, leading to generation of the necrotic or lipid core. Necrotic cores are thought to promote plaque disruption through release of proteases, inflammatory cytokines, and pro-coagulant/thrombotic molecules as well as through imparting physical stress on the arterial wall. Because late lesional macrophages accumulate large amounts unesterified, or free cholesterol, which is cytotoxic, one proposed inducer of late lesional macrophage death is free cholesterol loading. In fact, Sandoz 58-035, a synthetic ACAT inhibitor, promotes apoptosis in the macrophages by increasing intracellular free cholesterol \(^1\). Furthermore, mice lacking ACAT-1 exhibited toxic accumulation of unesterified cholesterol in the skin and brain in the setting of
hypercholesterolemia $^{2,3}$. The macrophage-specific ACAT-1 deficient mice had increased atherosclerotic lesions $^{4}$. These reports strongly demonstrate that the excessive administration of synthetic ACAT inhibitor and mice lacking ACAT thus shows a reverse effect.


Supplementary Information I

Enhanced Materials and Methods

Preparation of Esculeoside A and Esculeogenin A

Esculeoside A was isolated from ripe fruits of tomato as previously described \(^1\). Briefly, fresh ripe tomato fruits were smashed and then filtered. The supernatant was applied to Diaion HP-20 column chromatography and the methanol extract was passed through a reverse silica gel column chromatography. Then, the 60% methanol eluate gave esculeoside A (Supplementary Figure I). Esculeogenin A was obtained by acid hydrolysis of esculeoside A \(^2\) (Supplementary Figure I).

Lipoproteins and Their 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 agent 0.15 M NaCl and 1.0 µM EDTA (pH 7.4). Acetyl-LDL was prepared by chemical modification of LDL with acetic anhydride as previously described \(^3\). The levels of endotoxin associated with these lipoproteins were less than 1 pg/µg of protein, which were measured by a commercially available kit (Toxicolor system; Seikagaku Corp., Tokyo, Japan) \(^4\). Moreover, cell viability was not affected by endotoxin at concentrations less than
1.0 ng/mL in our experimental system. Acetyl-LDL was labeled with $^{125}\text{I}$ as described by McFarlane $^5$ to a specific radioactivity of 600 cpm/ng.

**Cell Culture**

Human peripheral mononuclear cells were isolated from the blood of healthy volunteers by Ficoll density gradient centrifugation (Ficoll-Paque from GE Healthcare Bio-Sciences). Human monocytes were purified according to the modified method of Connor et al. $^6$. Purified monocytes were suspended in RPMI 1640 at 2 x 10$^6$ cells/mL and seeded onto 24-well plates (4 x 10$^5$/well) or 6-cm dishes (2 x 10$^6$/dish) (BD Biosciences PRIMARIA, Tokyo). After a 1-h incubation for adherence, the medium was replaced by RPMI 1640 supplemented with 10% pooled human serum, streptomycin (0.1 mg/mL), and penicillin G (100 U/mL). Adhered monocytes were incubated for 7 days to induce differentiation into macrophages (the medium was replaced every 3 days). Differentiation of monocytes into macrophages (human monocyte-derived macrophages) was assessed after 7 days of incubation by three categories: adherence to culture plates, morphological features such as presence of mononuclear cells after Giemsa staining, and the capacity to take up carbon particles $^7$. Under these conditions, the cells contained >95% macrophages and were >98% viable as determined by trypan blue staining. These human monocyte-derived macrophages were used for each experiment. CHO cells stably overexpressing human ACAT-1 (hACAT-1 CHO cells) or human ACAT-2 (hACAT-2 CHO
cells) were kindly gifted from Dr Chang, department of biochemistry, Dartmouth medical school. These cells were cultured in Nutrient mixture F-12 HAM medium (Sigma-Aldrich Japan) supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 50 units/mL penicillin G, 50 µg/mL streptomycin and 800 µg/mL G418. All cellular experiments were performed at 37°C in a humidified atmosphere of 5% CO₂ in air.

**Endocytic uptake of acetyl-LDL**

The differentiated human monocyte-derived macrophages seeded onto 24-well plates were washed with 1.0 mL of phosphate buffered saline (PBS) and replaced with Dulbecco's modified Eagle's medium (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 ¹²⁵I-acetyl-LDL in the presence of the indicated concentration of esculeogenin A, 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 AgNO₃, 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. The protein concentration was measured by bicinchoninic acid (BCA) protein assay reagent (Pierce, IL).

**Assay for foam cell formation (CE-accumulation)**

Human monocyte derived macrophages monolayers were incubated with 50 µg/mL acetyl-LDL for 24 h in the presence of 0.1 mM [³H]oleate conjugated with BSA, and cellular lipids were extracted for determination of radioactivity of cholesteryl-[³H]oleate as described previously ⁹.

**Immunoblot analyses**

Differentiated macrophages were solubilized with 1% Triton X-100, and the protein concentration determined using the BCA protein assay reagent, followed by pretreatment with boiling for 3 min in 2% SDS and 2-mercaptoethanol. These samples were incubated for 24 h at 37°C with 3 units of N-glycosidase (Roche Applied Science), then 10 µg of protein was run on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) transfer membrane (Millipore, Bedford, MA). The membranes were exposed to an anti-human SR-A antibody (E5) ¹⁰ and visualized by horseradish peroxidase-conjugated anti-mouse IgG antibody with the ECL western blotting detection reagent (GE Healthcare Bio-Sciences) ¹⁰. The molecular size of SR-A detected by this immunoblot was approximately 50 kDa, representing a deglycosylated form of monomeric SR-A of 70 kDa.
To detect SR-BI, differentiated macrophages were solubilized with 1% Triton X-100, and the protein concentration determined followed by pretreatment with boiling for 3 min in 2% SDS and 2-mercaptoethanol. The protein (10 µg) was run on a 10% SDS-polyacrylamide gel and transferred to a PVDF transfer membrane. The membranes were exposed to an anti-human SR-BI antibody (Novus Biologicals, Littleton, CO) and visualized by a horseradish peroxidase-conjugated anti-rabbit IgG antibody. The molecular size of SR-BI detected by this immunoblot was approximately 57 kDa, representing a deglycosylated form of SR-BI of approximately 82 kDa.

To detect ACAT-1, differentiated macrophages were solubilized with 10% SDS, and the protein concentration was determined, followed by pretreatment with boiling for 3 min in 2% SDS and 2-mercaptoethanol. The protein (10 µg) was run on a 10% SDS-polyacrylamide gel and transferred to a PVDF transfer membrane. The membranes were exposed to an anti-human ACAT-1 antibody (Cayman Chemical Company, Ann Arbor, MI) and visualized by a horseradish peroxidase-conjugated anti-rabbit IgG antibody with the ECL western blotting detection reagent. The molecular size of ACAT-1 detected by this immunoblot was approximately 50 kDa. These membranes were re-blotted with an anti-β-actin antibody as an internal calibration control. The density of the bands was measured with Imaging Gauge software in LAS 1000plus (Fujifilm, Tokyo).

**Assay for ACAT Activity**
The cultured cells, peritoneal macrophage, liver and small intestine 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 previously described\textsuperscript{13,14}. Briefly, homogenates obtained from cultured cells were mixed with 4 mol/L KCl and 20% 3-[(3-cholamidopropyl)-dimethyl-ammonil]-1-propanesulfonate (CHAPS) in buffer A to obtain the final concentration of 1 mol/L and 2%, respectively. These samples (80 µg in 20 µL) were reconstituted with 140 µL of sodium taurocholate-cholesterol-phosphatidylcholine (PC) mixed micelles (0.2 as cholesterol/PC molar ratio). The enzyme reaction was initiated by adding 20 µL of 250 µmol/L of $[^{14}\text{C}]$oleoyl-CoA (20 dpm/pmol) followed by incubation for 15 min at 37°C. Lipids were then extracted and the radioactive cholesteryl-$[^{14}\text{C}]$oleate was determined by thin-layer chromatography (TLC).

\textit{In vivo Anti-atherosclerotic Activity}

The experimental protocol was approved by the local ethics review committee for animal experimentation. Six-week-old apolipoprotein E (apoE) deficient mice (C57BL/6.KOR-Apoeshl\textsuperscript{4}) were purchased from SLC (Shizuoka, Japan). These mice were housed in a pathogen-free barrier facility (12 h/12h light/dark cycle) and were fed a normal rodent chow diet (Clea, Japan) for 1 week after purchase. At this time the diets were changed to esculeoside A (50 and 100 mg/kg of body weight) containing diets and administered orally every day for 90 days. Powder
normal rodent chow was kneaded with 20% water in the presence or absence of esculeoside A, then cut and shaped chow were baked with 70°C for 3 h. Esculeoside A content in the chow was adjusted based on food intake a day and measuring their body weight every 2 weeks. For instance, 30 g of mouse ate 4 g of chow per day, we prepared baked chow containing 0.0375% and 0.075% esculeoside A, respectively. We employed these concentrations because Kong et al. demonstrated that oral administration of 50 and 100 mg/kg/day berberin, a compound isolated from a Chinese herb, to hamsters which were fed a high-fat and high-cholesterol diet significantly decreased serum cholesterol and serum LDL cholesterol 15. Furthermore, Nametame et al. 16 also demonstrated that oral administration of 50 mg/kg/day beauveriolide I and III, compounds isolated from the culture broth of fungal Beauveria sp. FO-6979, to apoE knockout mice decreased the pathogenesis of atherosclerosis. Thirty mice (10 for controls, 10 for 50 mg/kg/day and 10 for 100 mg/kg/day esculeoside A, respectively) were used for in vivo evaluation. Blood samples were collected from the abdominal aorta at 90 days. Total cholesterol, LDL cholesterol and triglyceride levels in serum were determined on Olympus AU5200 automatic analyzer (Olympus, Tokyo) using standard enzymatic methods. For atherosclerotic lesion analyses, mice were sacrificed after blood collection. Whole aorta were collected and stained with Sudan IV, and cross sections of proximal aorta were prepared and stained with oil red as described 17. The luminal side of the stained aortas was photographed. Image capture and analysis were performed by using IPAP-WIN (Sumika...
Technoservice, Hyogo, Japan). The extent of atherosclerosis was expressed as the percent of the lesion area of the entire aorta. The hearts were perfused with PBS containing 4% (wt/vol) paraformaldehyde, embedded in the optimal cutting temperature (OCT) compound (Sakura Tissue-Tek, Tokyo), and 6-µm-thick serial sections were cut by using a Cryostat (Leica, Tokyo). The sections were counterstained with oil red O and hematoxylin. Image capture and analysis were performed by using IPAP-WIN (Sumika Technoservice). The average size of the lesions in the following three sections was used to represent the lesion size for each mouse. First section: the valve attachment sites and the coronary ostia, but not the valve leaflets. Second section: the valve leaflets appear as small nodules in the valve attachment sites. Third section: the valves appear complete and are joined to their attachment sites. Furthermore, twenty mice (10 for controls and 10 for 100 mg/kg/day esculeoside A, respectively) were fed for 21 days to measure ACAT activity in the liver, intestine and peritoneal macrophages.

Detection of Esculeogenin A by nanoscale liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Esculeogenin A was extracted with 1ml 90% acetone at room temperature for 1 h from minced aortas (~ 25 mg) of normal diet- and esculeoside A-treated apoE-deficient mice. Those extracts were dried up by nitrogen gas. Those extracts in each sample was resolubilized with methanol at concentration of 2.5 mg/mL. After mixing by vortex and centrifuge, the extract solution was
filtered to PVDF membrane, 0.2 µm (Whatman, Brentford, UK), and its filtrate was used for LC-MS/MS analysis. Agilent 1100 system (Agilent 1100 series; Agilent, Palo Alto, CA, USA) coupled with Finnigan LTQ (Thermo Fisher, Waltham, MA, USA) was used for LC-MS analysis. Samples were applied to TSKgel ODS-80Tm (4.6 x 250 mm, 5 µm, TOSOH, Tokyo, Japan) column at 30°C of column oven temperature. Water (HPLC grade; solvent A) and acetonitrile (HPLC grade; solvent B) were used as mobile phase with gradient condition. Formic acid was added to both solvent at concentration of 0.1 % (v/v). The gradient program was as follows; 10% to 95% B (50 min), 95% B (5min), and 10% B (10min). Flow rate was set to 0.5 mL/min, and 10 µl of each sample was injected. MS data were obtained by positive-ion electrospray ionization (ESI) mode. Spray voltage and capillary temperature were set at 4.0 kV, and 330 °C, respectively. Nitrogen sheath gas and auxiliary gas were set at 30 and 10 arbitrary units. MS/MS normalized collision energy was set at 35%. The parent and daughter ion pairs of esculeogenin A was m/z 448 (parent; [M+H]⁺) > 430 (daughter; MS/MS). Esculeogenin A in each sample was identified by comparison of retention time, m/z and MS/MS pattern with those of authentic standard. The data was acquired and analyzed with Xcalibur 2.0 software (Thermo Fisher).

Statistical Analysis

All experimental data are expressed as mean ± SD. Differences between groups...
were examined for statistical significance using the Student’s $t$-test or one-way analysis of variance (ANOVA) with Newman-Keuls *post-hoc* test. A $P$ value less than 0.05 denoted the presence of a statistically significant difference.
Supplementary Figure I. Structures of Esculeoside A and Esculeogenin A.
Supplementary Figure II. Inhibitory effect of esculeogenin A on SR-A, SR-BI and ACAT-1 expression in HMDM. HMDM were incubated with esculeogenin A for 24 h. (A) The cells were harvested and subjected to immunoblot analyses using antibodies against human ACAT-1, human SR-A and human SR-BI. (B) Densitometric analysis of ACAT-1, SR-A and SR-BI immunoblot, which was normalized by β-actin as described in enhanced materials methods.
**Supplementary Figure III.** Inhibition of ACAT activity in apoE-deficient mice by esculeoside A treatment. ApoE deficient mice were fed diets with or without esculeoside A (EsA) (100 mg/kg/day) for 21 days (n=10, each group) and ACAT activity in the liver (A), peritoneal macrophages (B) and intestine (C) was measured as described in enhanced materials methods. Data are mean±SD. *, $P < 0.05$; **, $P < 0.005$. 
Supplementary Figure IV. Detection of esculeogenin A in mice aorta by LC-MS/MS analysis. ApoE deficient mice were fed diets with or without esculeoside A for 90 days and whole aorta were collected. Esculeogenin A was extracted with 90% acetone. The parent and daughter ion pairs of esculeogenin A was m/z 448 (parent; [M+H]) > 430 (daughter; MS/MS). Esculeogenin A in each sample was identified by comparison of retention time, m/z and MS/MS pattern with those of authentic standard as described as enhanced materials and methods.
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