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Tomatidine, a Tomato Sapogenol, Ameliorates Hyperlipidemia and Atherosclerosis in ApoE-Deficient Mice by Inhibiting Acyl-CoA:cholesterol Acyl-transferase (ACAT)

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Supporting Information

ABSTRACT: It was previously revealed that esculeoside A, a new glycoalkaloid, and esculeogenin A, a new aglycon of esculeoside A, contained in ripe tomato ameliorate atherosclerosis in apoE-deficent mice. This study examined whether tomatidine, the aglycone of tomatine, which is a major tomato glycoalkaloid, also shows similar inhibitory effects on cholesterol ester (CE) accumulation in human monocyte-derived macrophages (HMDM) and atherogenesis in apoE-deficient mice. Tomatidine significantly inhibited the CE accumulation induced by acetylated LDL in HMDM in a dose-dependent manner. Tomatidine also inhibited CE formation in Chinese hamster ovary cells overexpressing acyl-CoA:cholesterol acyl-transferase (ACAT)-1 or ACAT-2, suggesting that tomatidine suppresses both ACAT-1 and ACAT-2 activities. Furthermore, the oral administration of tomatidine to apoE-deficient mice significantly reduced levels of serum cholesterol, LDL-cholesterol, and areas of atherosclerotic lesions. The study provides the first evidence that tomatidine significantly suppresses the activity of ACAT and leads to reduction of atherogenesis.

KEYWORDS: *tomato, tomatidine, macrophage, acyl-CoA:cholesterol acyl-transferase, atherosclerosis*

INTRODUCTION

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] 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 scavenger receptors. The scavenger receptors identified to date include the class A scavenger receptor (SR-A), class B scavenger receptor (CD36), class B scavenger receptor type-I (SR-BI), and lectin-like oxidized LDL receptor-1 (LOX-1). 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 acyl-transferase (ACAT), an intracellular enzyme located in the rough endoplasmic reticulum.[2] These reactions change the macrophages to foam cells that are characterized by the intracellular accumulation of CE.

To date, two human ACAT isozymes (ACAT-1 and ACAT-2) have been identified. ACAT-1 is highly expressed by macrophage-derived foam cells in atherosclerotic lesions and is up-regulated during monocytic differentiation into macrophages.[3] In addition, ACAT-1 is located in the Kuppfer cells of the liver, kidneys, and adrenal cortical cells, whereas ACAT-2 is mainly located in hepatocytes and intestinal mucosal cells.[3] These findings are consistent with the notion 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.[3] 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 to be 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, inhibition of scavenger receptor expression, and inhibition of ACAT activity.

It has been demonstrated that tomatoes (*Solanum lycopersicon*) and tomato products prevent atherosclerosis, and numerous studies have been performed to clarify the role of

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lycopene, the major carotenoid in ripe tomatos (~90 mg/kg ripe tomato), and its mechanism of action. The potential antiatherogenic effects of lycopene have been ascribed mainly to its antioxidant capacity, which is related to the prevention of LDL oxidation.

We recently isolated esculeoside A,[4] a novel steroidal alkaloid glycoside, from the ripe fruit of tomatoes (~500 mg/kg ripe tomato) and identified a novel compound named esculeogenin A,[4] which is an aglycon of esculeoside A obtained by hydrolysis of esculeoside A. Esculeoside A and esculeogenin A ameliorated hyperlipidemia and atherosclerosis in apoE-deficent mice by inhibition of ACAT activity,[5] thus suggesting that steroidal alkaloid compounds such as esculeoside A and esculeogenin A have inhibitory effects on atherosclerosis, similar to lycopene.

On the other hand, tomatine, a major tomato steroidal alkaloid glycoside, is contained in the tomato plant,[6] including the unripe fruits (~500 mg/kg), leaves (~1000 mg/kg), stems (~900 mg/kg), and roots (~150 mg/kg), and is converted to esculeoside A upon ripening of the fruits.[7] Therefore, tomatine is primarily contained in the tomato plants, whereas lycopene and esculeside A are contained only in the fruit, thus making tomatine the most abundant compound present. Furthermore, tomatine is known to possess a variety of biological activities, including anticancer, antifungal, antibacterial, and anti-inflammatory activities.[8−11] Furthermore, it has been reported that tomatine reduces the plasma LDL cholesterol and triglyceride levels in hamsters[12,13] and that tomatine protects against DBP-induced liver and stomach tumors in rainbow trout.[14] On the other hand, little is known about the biological activities of tomatidine, an aglycon of tomatine. However, 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] suggesting that tomatine likely is converted into tomatidine in the intestines and that tomatidine may be the bioactive compound in vivo.

In this study, to clarify the in vivo bioactivity of tomatidine, we examined its effects on foam cell formation in human monocyte-derived macrophages (HMDM) and on atherogenesis in apoE-deficient mice.

MATERIALS AND METHODS

Chemicals and Materials. Oil Red O, 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 Peptide Institute (Osaka, Japan). Penicillin G, streptomycin sulfate, and RPMI 1640 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 oleate (1 mCi/mg) were purchased from GE Healthcare Bio-Sciences (Tokyo, Japan). Paraformaldehyde was purchased from Nacarai Tesque (Kyoto, Japan). Sudan IV was purchased from Kanto Chemical (Tokyo, Japan). G418 was purchased from Gibco (Grand Island, NY). All other chemicals were of the best grade available from commercial sources. Tomatine was isolated from tomato plants, and tomatidine was obtained by hydrolysis of tomatine as described previously.[17]

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.[18] Acetylated-LDL (acetyl-LDL)[18] was labeled with ^{125}I as described by McFarlane[19] to a specific radioactivity of 600 cpm/ng.

Cell Culture. Human monocytes were purified from the blood of healthy volunteers by Ficoll density gradient centrifugation (Ficoll-Paque from GE Healthcare Bio-Sciences). Purified monocytes were suspended in RPMI 1640 and seeded onto 24-well plates (4×10^5/well) or 6 cm dishes (2×10^6/dish). 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). The adherent monocytes were incubated for 7 days to induce their differentiation into macrophages. Under these conditions, the cells contained >95% macrophages and were >98% viable as determined by trypan blue staining. CHO cells stably overexpressing human ACAT-1 (hACAT-1 CHO cells) or human ACAT-2 (hACAT-2 CHO cells) were cultured in Nutrient mixture F-12 HAM medium (Sigma-Aldrich Japan) supplemented with 10% fetal calf serum, 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% CO_2 in air.

Endocytic Uptake of Acetyl-LDL. The differentiated HMDM seeded onto 24-well plates were washed with 1.0 mL of phosphate-buffered saline (PBS) and cultured in 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 ^{125}I-acetyl-LDL in the presence of the indicated concentrations of tomatidine, 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 resulting supernatant (0.25 mL) was used to determine the TCA-soluble radioactivity, which was considered to be an index of cellular degradation. Each well was washed with ice-cold PBS containing 1% BSA, and the cells were lysed with 0.1 N NaOH to determine the cell-associated radioactivity and cellular protein concentration.

Assay for Foam Cell Formation (CE Accumulation). HMDM monolayers were incubated with 50 μg/mL acetyl-LDL for 24 h in the presence of 0.1 mM [^{3}H]oleate conjugated with BSA, and cellular lipids were extracted for the determination of the radioactivity of cholesteryl-[^{3}H]oleate as described previously.[20]

Immunoblot Analyses. HMDM were solubilized with 1% Triton X-100, and the protein concentration was determined using the BCA protein assay reagent, followed by pretreatment by 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), and then 10 μg of protein was run on a 10% SDS−polyacrylamide gel and transferred to a polyvinylidine fluoride (PVDF) transfer membrane (Millipore, Bedford, MA). The membranes were exposed to an anti-human SR-A antibody (E5) and visualized using a horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody with the ECL Western blotting detection reagent (GE Healthcare Bio-Sciences). 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. SR-BI was detected by an anti-human SR-BI antibody (Novus Biologicals, Littleton, CO). 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. ACAT-1 was detected by an anti-human ACAT-1 antibody (Cayman Chemical Co., Ann Arbor, MI). The molecular size of ACAT-1 detected by this immunoblot was approximately 50 kDa. These membranes were reblotted with an anti-β-actin antibody as an internal calibration control. The density of the bands was measured with the Imaging Gauge software program in LAS 1000plus (Fujifilm, Tokyo, Japan).

Assay for ACAT Activity. The cultured cells were homogenized with buffer A (50 mmol/L Tris-HCl and 1 mmol/L EDTA at pH 7.8 with protease inhibitors), and then the enzyme activity was determined by the reconstitution assay as previously described.[21] Briefly, homogenates obtained from cultured cells were mixed with 4 mol/L KCl and 20% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in buffer A to obtain final concentrations of 1 mol/L and 2%, respectively. These samples (80 μg in 20 μL) were

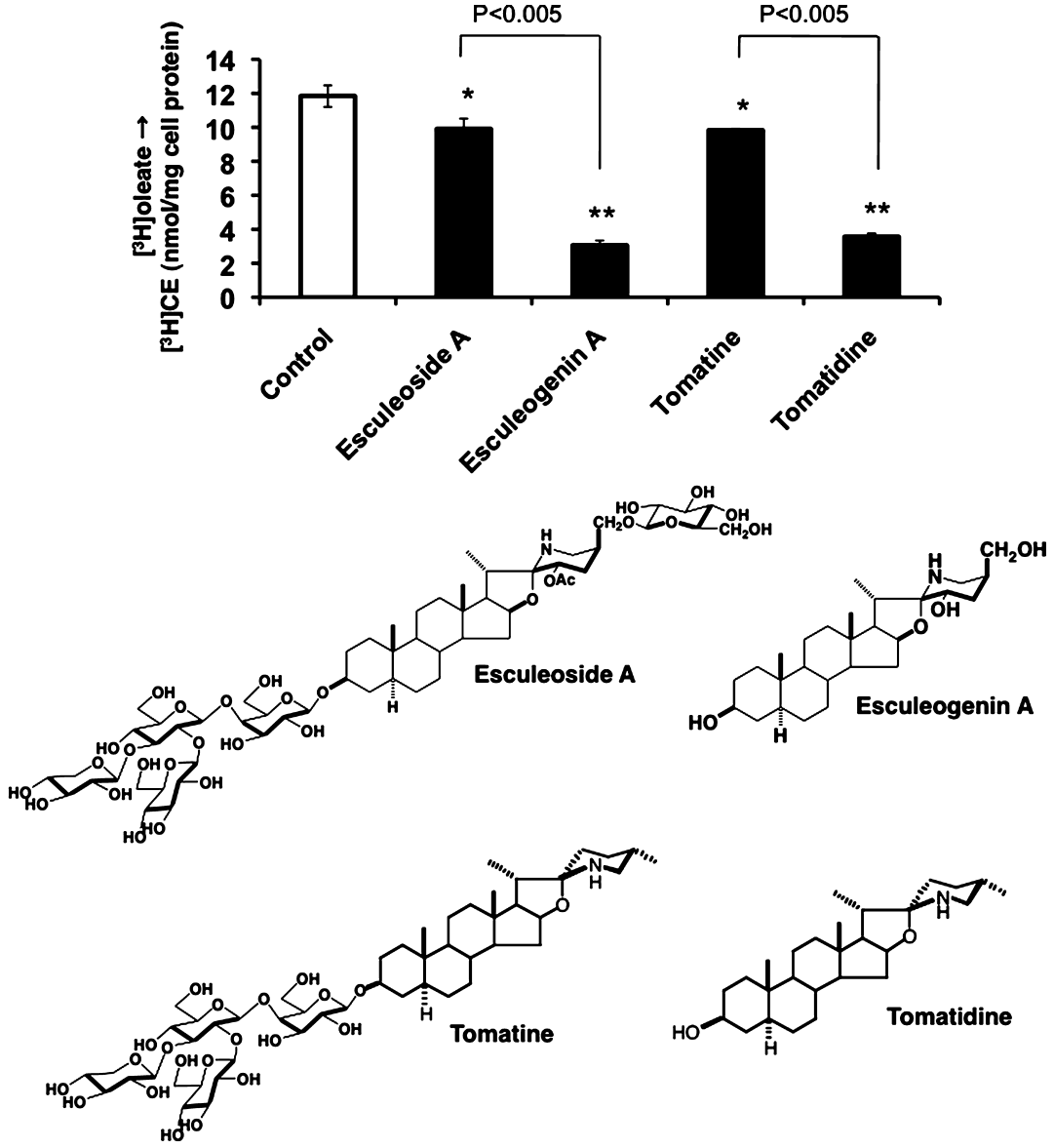

Figure 1. Inhibitory effects of tomato steroidal alkaloid compounds on CE accumulation by acetyl-LDL uptake in HMDM. HMDM were incubated with 50 μg/mL acetyl-LDL and 0.1 mM [^{3}H]oleate conjugated with BSA in the absence or presence of esculeoside A, esculeogenin A, tomatine, and tomatidine (30 μM). After a 24 h incubation, [^{3}H]CE was separated by TLC, and radioactivity was measured with a radioscanner as described under Materials and Methods. Experiments were repeated three times with almost identical results. The data are the mean ± SD. (*) $P < 0.01$, and (**) $P < 0.005$.

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}C]oleoyl-CoA (20 dpm/pmol) followed by incubation for 15 min at 37 °C . Lipids were then extracted, and the radioactive cholesteryl-[^{14}C]oleate was determined by thin-layer chromatography (TLC).

In Vivo Antiatherosclerotic Activity. All animal experiments were approved by the Ethics Committee for Animal Experiments of Kumamoto University and were performed in accordance with the Guideline for Animal Experiments of the laboratories. Six-week-old apolipoprotein E (apoE) deficient mice (C57BL/6.KOR-*Apoe*^{shl}) were purchased from SLC (Shizuoka, Japan). These mice were housed in a pathogen-free barrier facility (12 h/12 h 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 tomatidine (50 mg/kg of body weight)-containing diets, and these diets were administered orally every day for 70 days. We employed these concentrations because Kong et al. demonstrated that oral administration of 50 and 100 mg/kg/day of berberin, a compound isolated from a Chinese herb, to hamsters which were fed a high-fat and high-cholesterol diet significantly decreased their serum cholesterol and serum LDL cholesterol.[22] Twenty mice (10 for controls and 10 for 50 mg/kg/day tomatidine, respectively) were used for in vivo evaluation. Blood samples were collected from the abdominal aorta at 70 days after initiation of the treatment. The total cholesterol, LDL cholesterol, and triglyceride levels in serum were determined on an Olympus AU5200 automatic analyzer (Olympus, Tokyo) using standard enzymatic methods. For atherosclerotic lesion analyses, the mice were sacrificed after blood collection. Whole aortas were collected and stained with Sudan IV, and cross sections of the proximal aorta were prepared and stained with Oil Red O as described.[5] The luminal side of the stained aortas was photographed. Image capture and analysis were performed by using the IPAP-WIN software package (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% (w/v) 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, Japan). The sections were counterstained with Oil Red O and hematoxylin. Image capture and analysis were performed by using the IPAP-WIN software package (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, valve attachment sites and coronary ostia, but not the valve leaflets; second section, valve leaflets appear as small nodules in the valve attachment sites; third section, valves appear as complete and are joined to their attachment sites.

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

RESULTS AND DISCUSSION

We first measured the inhibitory effect of esculeoside A, esculegenin A, tomatine, and tomatidine on CE accumulation in HMDM. Incubation of HMDM for 24 h with 50 μg/mL acetyl-LDL increased their CE accumulation (Figure 1). Under the assay conditions used, esculeoside A and esculeogenin A significantly inhibited CE accumulation (Figure 1), and the inhibitory effects of esculeogenin A were stronger than those of esculeoside A, in agreement with previous results.[5] Similarly, tomatine and tomatidine also inhibited CE accumulation (Figure 1), and the inhibitory effect of tomatidine was stronger than that of tomatine. These data suggest that tomatidine has an inhibitory effect on CE accumulation and that the inhibitory effects of the aglycons of active tomato components (tomatidine and esculeogenin A) are stronger than those of the glycosides (tomatine and esculeside A). Therefore, we chose to further evaluate the antiatherogenic effects of tomatidine.

Next, we measured the concentration-dependent effects of tomatidine on CE accumulation in HMDM. As a result, tomatidine was observed to significantly inhibit CE accumulation in a dose-dependent manner (Figure 2A), whereas tomatidine did not show any inhibitory effect on triglyceride (TG) synthesis even at the highest dose (Figure 2B). These data suggested that tomatidine selectively inhibits CE synthesis in the HMDM.

When 50 μg/mL of ^{125}I-acetyl-LDL was incubated at 37 °C for 5 h with the cells, significant amounts of ^{125}I-acetyl-LDL were associated with the cells (Figure 2C) and were subjected to lysosomal degradation by these cells (Figure 2C), whereas these cellular responses were not inhibited by tomatidine.

On the basis of this finding, we subsequently measured the involvement of ACAT in the tomatidine-induced reduction of CE accumulation in the HMDM. The incubation of CHO cells overexpressing human ACAT-1 (hACAT-1 CHO) and human ACAT-2 (hACAT-2 CHO) for 24 h with medium containing 10% fetal calf serum in the presence of [^{3}H]oleate increased CE accumulation. Under these conditions, tomatidine inhibited CE accumulation in both hACAT-1 and hACAT-2 CHO cells in a dose-dependent manner (Figure 2D). Tomatidine caused no morphological changes or cytotoxic effects on the cells, even at the 100 μM concentration (data not shown). Because tomatidine showed a significant inhibitory effect on CE accumulation in both HMDM and hACAT CHO cells in a similar fashion, it appears that tomatidine may serve as an inhibitor of cholesterol esterification, possibly by inhibiting ACAT activity and/or ACAT expression.

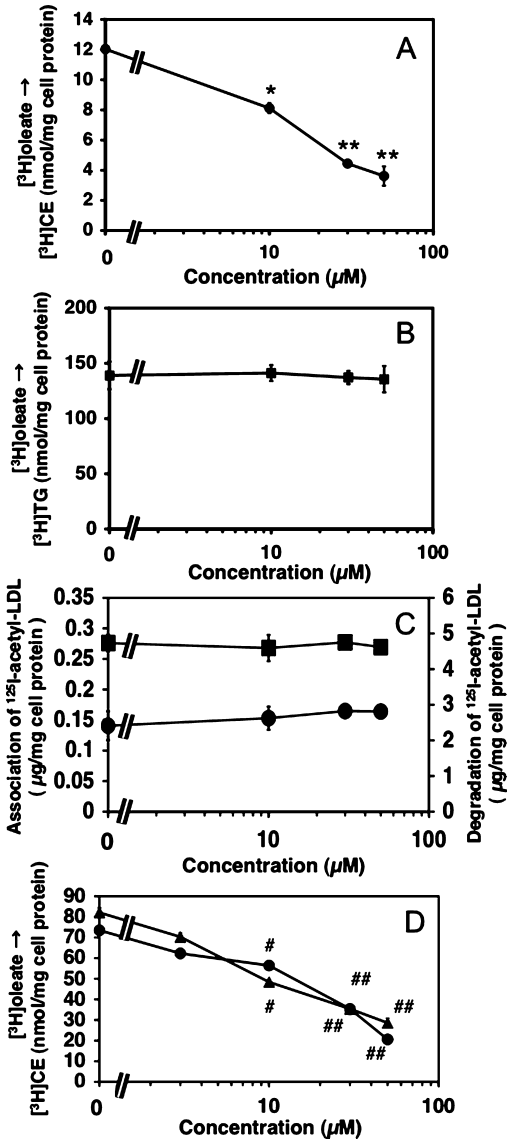

Figure 2. Concentration-dependent inhibitory effect of tomatidine on CE accumulation by acetyl-LDL uptake in HMDM and effects of tomatidine on endocytic uptake of ^{125}I-acetyl-LDL by HMDM and CE accumulation in CHO cells overexpressing human ACAT. HMDM were incubated with 50 μg/mL acetyl-LDL and 0.1 mM [^{3}H]oleate conjugated with BSA in the absence or presence of tomatidine (●). After a 24 h incubation, [^{3}H]CE (A) and [^{3}H]TG (B) were separated by TLC, and the radioactivity was measured with a radioscanner as described under Materials and Methods. HMDM were incubated for 5 h with the indicated concentrations of tomatidine and with 50 μg/mL ^{125}I-acetyl-LDL, followed by determination of cell association (●) and cell degradation (■) of ^{125}I-acetyl-LDL as described under Material Methods. (C) hACAT-1 CHO cells (●) and hACAT-2 CHO cells (▲) were incubated with medium containing 10% fetal calf serum in the presence of 0.1 mM [^{3}H]oleate-conjugated BSA and the indicated concentrations of tomatidine. Then, [^{3}H]CE was separated by TLC, and the radioactivity was measured (D). The experiments were repeated three times with almost identical results. The data are the mean ± SD. (*) $P < 0.01$, (**) $P < 0.005$, (#) $P < 0.001$, and (##) $P < 0.0001$.

Microsomes prepared from HMDM were used as an enzyme source. The microsomes were incubated for 15 min with 250 μmol/L [^{14}C]oleoyl-CoA in the presence or absence of tomatidine, and the formation of cholesteryl [^{14}C]oleate was

measured. As shown in Figure 3A, tomatidine inhibited ACAT activity in a dose-dependent manner. Similar results were

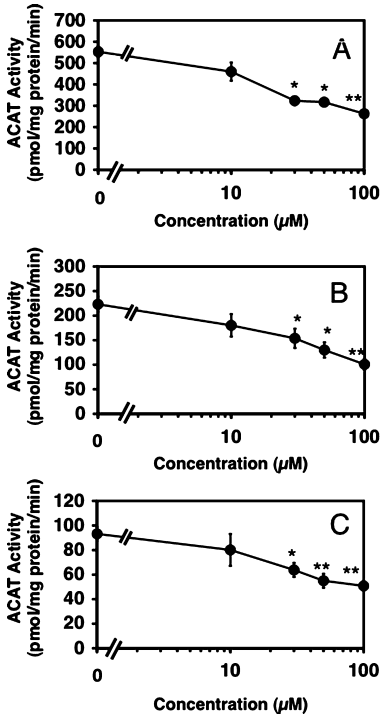

Figure 3. Inhibitory effects of tomatidine on ACAT activity in HMDM and hACAT CHO cells. HMDM (A), hACAT-1 CHO cells (B), or hACAT-2 CHO cells (C) homogenized with buffer A and reconstituted with sodium PC mixed micelles together with tomatidine (●). The ACAT activity was measured as described under Materials and Methods. All experiments were repeated three times with almost identical results. The data are the mean ± SD. (*) $P < 0.005$, and (**) $P < 0.001$.

observed in hACAT-1 and hACAT-2 CHO cells. Therefore, the formation of cholesteryl [^{14}C]oleate by microsomes prepared from both hACAT-1 or hACAT-2 CHO cells was inhibited in the presence of tomatidine in a dose-dependent manner (Figure 3B,C). These data suggest that tomatidine has a significant inhibitory effect on foam cell formation of HMDM by inhibiting the activity of both ACAT-1 and -2.

Incubation of HMDM for 24 h in the presence of the indicated concentrations of tomatidine resulted in no changes in the expression of ACAT-1, SR-A, or SR-BI compared to the control (Figure 4). These results suggest that tomatidine has a significant inhibitory effect on foam cell formation in HMDM by directly inhibiting ACAT activity, rather than affecting the expression levels of the enzyme or of other mediators of foam cell formation. Furthermore, tomatidine significantly inhibited CE accumulation and ACAT activity in apoE-deficient mouse peritoneal macrophages, whereas it did not affect the expression of ACAT and scavenger receptors (supplementary Figure 2 in the Supporting Information). These results suggest that tomatidine significantly inhibited both CE accumulation and ACAT activity not only in HMDM but also in mouse peritoneal macrophages.

Because tomatidine suppressed foam cell formation by inhibition of ACAT activity, we next administered tomatidine to apoE-deficient mice to examine its effects on atherogenesis. As shown in Figure 5A, the mean body weight gain was

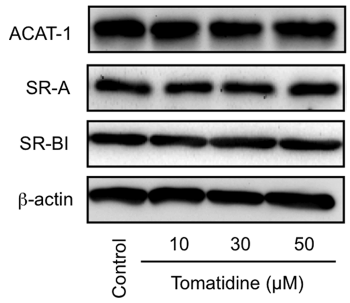

Figure 4. Inhibitory effects of tomatidine on SR-A, SR-BI, and ACAT-1 expression in HMDM. HMDM were incubated with tomatidine for 24 h, and then cells were harvested and subjected to an immunoblot analysis using antibodies against human ACAT-1, human SR-A, human SR-BI, and human β-actin as described under Materials and Methods.

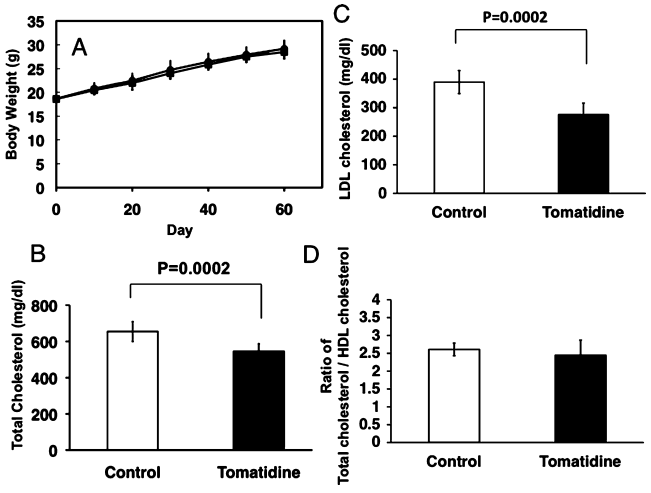

Figure 5. Changes in body weight and biochemical data of plasma samples in apoE-deficient mice. ApoE-deficient mice were fed diets with or without tomatidine (50 mg/kg/day) for 70 days ($n = 10$, each group), and body weight (A), plasma total cholesterol (B), LDL cholesterol (C), and the ratio of HDL cholesterol/total cholesterol (D) were measured.

unchanged by administration of tomatidine for 70 days. However, the total cholesterol levels significantly decreased by approximately 20% after the administration of 50 mg/kg/day tomatidine (Figure 5B). Furthermore, administration of tomatidine (50 mg/kg/day) significantly reduced the serum levels of LDL cholesterol (Figure 5C) by approximately 25%, without changing the TC/HDL ratio (Figure 5D) in comparison to the control group.

Tomatidine treatment reduced the atherosclerotic lesions in apoE-deficient mice. 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 (Figure 6A), whereas such lesions were greatly reduced in tomatidine-treated mice (Figure 6B). The cross-sectional lesion area in the tomatidine-treated (50 mg/kg/day) mice was significantly smaller than that of control mice (Figure 6C).

In the early stage of atherosclerogenesis, macrophages penetrate the intima, efficiently take up modified LDL, and store cholesterol and fatty acids as a form of neutral lipids in their cytosolic lipid droplets.[1] Free cholesterol is esterified to the CE by ACAT, leading to the formation of macrophage-derived foam cells in atherosclerotic lesions.[2] Therefore, the

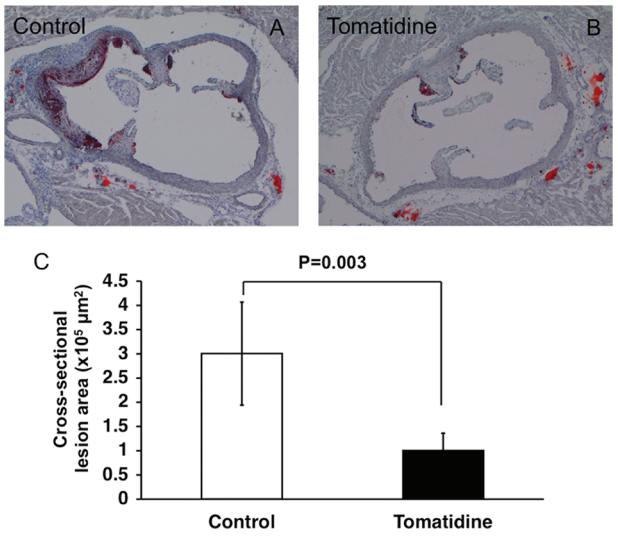

Figure 6. Inhibition of atherogenesis in apoE-deficient mice by tomatidine. Tomatidine treatment reduced the atherosclerotic lesions in apoE-deficient mice. Representative sections of aortic sinus were stained with Oil Red O in apoE-deficient mice without tomatidine treatment (A) and with tomatidine (50 mg/kg/day) treatment (B), magnification (×200). Quantitative evaluations of cross-sectional lesions of the aortic sinus (C).

prevention of foam cell formation is considered to be one of the major targets for the treatment of atherosclerosis.

Lycopene, a carotenoid without provitamin A activity, is one of the most notable compounds in the tomato. Daily intake of tomatoes and tomato products was shown to be associated with a decreased risk of chronic diseases such as cancer and cardiovascular diseases in several recent studies.[23] Lycopene is well-known to have antiatherogenic and anticancer effects, thus suggesting that lycopene is a major active compound related to the beneficial effects of consuming tomatoes and tomato products. However, we recently isolated a new compound called esculeoside A from fresh tomato fruits and revealed that the content of esculeoside A (~500 mg/kg) is higher than that of lycopene (~90 mg/kg).[4] Furthermore, we recently identified another new compound called esculeogenin A,[4] an aglycon of esculeoside A obtained by hydrolysis of esculeoside A. Esculeoside A and esculeogenin A both ameliorated hyperlipidemia and atherosclerosis in apoE-deficent mice by inhibition of ACAT activity,[5] indicating that not only lycopene but also esculeogenin A and esculeoside A are major active compounds related to the beneficial effects of tomatoes and tomato products.

Tomatine is another major tomato steroidal alkaloid glycoside and is contained in various parts of the tomato plant, including the unripe fruits (~500 mg/kg), leaves (~1000 mg/kg), stems (~900 mg/kg), and roots (~150 mg/kg). Furthermore, it was revealed that tomatine is converted to esculeoside A upon ripening of the fruits.[7] Given its prevalence, tomatine is the most abundant compound in the tomato plant. Furthermore, tomatine is known to possess a variety of biological activities, including anticancer, antifungal, antibacterial, and anti-inflammatory activities.[8−11] On the other hand, little is known about the biological activities of tomatidine. However, because the sugar chains of glycosides in natural products are degraded by the actions of intestinal bacteria after oral administration, the aglycons generally act as the physiologically active substances.[15,16] Moreover, we previously detected esculeogenin A, an aglycon of esculeoside A, which is a tomato glycoalkaloid (like tomatine), in the serum on apoE-deficient mice fed esculeoside A, which has the same glycoside chain, a lycotetraose, as tomatine.[5] This suggests that tomatine is likely converted into tomatidine in the intestines and performs as a bioactive compound in vivo. In addition, esculeogenin A significantly inhibited CE accumulation in macrophages. Therefore, we speculated that tomatidine may also inhibit CE accumulation in macrophages.

In the present study, tomatidine showed a significant inhibitory effect on CE accumulation in a dose-dependent manner, similar to that observed for esculeogenin A, whereas tomatine showed little inhibitory effect. We then determined the inhibitory mechanism underlying the effects of tomatidine on CE accumulation in HMDM. Tomatidine did not show any inhibitory effect on the endocytic uptake of ^{125}I-acetyl-LDL or the expression levels of scavenger receptors such as SR-A, SR-BI, or ACAT-1, whereas it significantly inhibited the activity of both ACAT-1 and ACAT-2. These data demonstrate that tomatidine serves as an ACAT inhibitor by suppressing the activity of ACAT, resulting in subsequent inhibition of CE accumulation in macrophages. We next conducted animal experiments using apoE-deficient mice. Oral administration of tomatidine significantly decreased not only the serum total cholesterol but also the area of atherosclerotic lesions in apoE-deficient mice. These results demonstrate that oral administration of tomatidine prevents the pathogenesis of atherosclerosis in vivo.

Many researchers have examined the usefulness of a number of antiatherosclerotic agents intended to inhibit ACAT activity. Mice lacking ACAT-2 exhibited a restricted capacity to absorb cholesterol and protection against diet-induced hypercholesterolemia and gallstone formation.[24] Nonselective ACAT inhibition is known to reduce atherosclerosis in apoprotein E-deficient mice.[25] NTE-122 and F-1394, nonselective ACAT inhibitors, prevent the progression of atherosclerosis in cholesterol-fed rabbits.[26] Moreover, the ACAT inhibitor avasimibe reduces the number of macrophages and matrix metalloproteinase expression in atherosclerotic lesions of hypercholesterolemic rabbits[27] and reduces atherosclerosis, in addition to its cholesterol-lowering effect in apoE*3-Leiden mice.[28] Sakashita et al.[29] demonstrated that ACAT-2 is also expressed in macrophage-derived foam cells in vitro and in vivo. Therefore, it is likely that tomatidine induced the remarkable inhibition of foam cell formation by inhibiting the activity of both ACAT-1 and ACAT-2 in the HMDM. However, because the presence of ACAT is essential for the cell's survival as cholesterol, an excessive inhibition of ACAT by synthetic ACAT inhibitors such as CP-113818 and Sandoz 58-035 and mice lacking ACAT has been shown to demonstrate unfavorable aspects.[30,31] Therefore, ACAT inhibitors that can be taken from the daily tomatidine supplementation is preferred to strong synthetic ACAT inhibitors.

In the present study, tomatidine also reduced the serum cholesterol levels (LDL cholesterol and total cholesterol) in apoE-deficient mice, and Friedman et al. reported that tomatine reduced the plasma LDL cholesterol in hamsters[12,13] and that tomatidine administration did not appear to induce any side effects in mice.[32] These results suggest that tomatidine may be the major bioactive compound responsible for reducing plasma cholesterol levels in tomatine-treated animals.

Because both of the ACAT isozymes are expressed in the liver and provide cholesteryl esters for VLDL, an ACAT1

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 a high-fat/high-cholesterol diet. Because we conducted animal experiments using apoE-deficient mice administered a normal diet, and tomatidine also inhibited ACAT activity in the HepG2 hepatic cell line (supplementary Figure 1 in the Supporting Information), it is reasonable that the administration of tomatidine inhibited the ACAT activity in the liver and macrophages rather than in the intestine and subsequently decreased the endogenous cholesterol production. Therefore, tomatidine may also decrease LDL cholesterol in apoE-deficient mice by inhibiting ACAT activity in the liver.

Our study provides the first evidence that tomatidine significantly ameliorates hyperlipidemia and atherosclerosis in apoE-deficient mice by inhibiting ACAT activity. There was a second paper about esculeogenin A indicating that steroidal alkaloids have inhibitory effects on foam cell formation by macrophages.[5] The results of that study also suggested that supplementation with tomatidine can prevent human atherosclerosis by inhibiting ACAT activity. Furthermore, the daily intake of tomatidine supplementation may also reduce the risk of cardiovascular diseases. The possibility that tomatidine may inhibit form cell formation and atherosclerosis by affecting targets other than ACAT activity will need to be examined in future projects.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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