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ATP-Binding cassette transporter A1 is involved in hepatic α-tocopherol secretion

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Abstract

Vitamin E (α-tocopherol) is an essential fat-soluble nutrient with antioxidant properties. α-Tocopherol transfer protein (α-TTP), the product of the gene responsible for familial isolated vitamin E deficiency, plays an important role in maintaining the plasma α-tocopherol level by mediating the secretion of α-tocopherol by the liver. However, the mechanisms underlying hepatic α-tocopherol secretion are not fully understood. This study was undertaken to elucidate the mechanism of α-tocopherol re-efflux from hepatocytes, the cells that have the most important role in regulating plasma-α-tocopherol concentrations. From in vitro experiments using [^{3}H]α-tocopheryl acetate and McARH7777 cells that stably express α-tocopherol transfer protein (α-TTP), the following results were obtained. First, addition of apolipoprotein A-I (apoA-I), a direct acceptor of the ATP-binding cassette transporter A1 (ABCA1)-secreted lipids, increased α-tocopherol secretion in a dose-dependent manner. Second, probucol, an antiatherogenic compound reported to be an inactivator of ABCA1 reduced hepatic α-tocopherol secretion. Third, ABCA1-RNAi suppressed hepatic α-tocopherol secretion. In a mouse in vivo experiment, addition of 1% probucol to the diet decreased plasma α-tocopherol concentrations. These results strongly suggest that ABCA1 is substantially involved in hepatic α-tocopherol secretion.

Keywords: α-Tocopherol; ATP-Binding cassette transporter A1 (ABCA1); α-Tocopherol transfer protein (α-TTP); Hepatocyte; Apolipoprotein A-I (apoA-I); Probucol

1. Introduction

Vitamin E is a major lipid-soluble, chain-breaking antioxidant in the body. Its main role is to protect the integrity of membranes by inhibiting lipid peroxidation. Vitamin E is the generic term for a group of four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ). Among the eight forms, α-tocopherol is biologically most active and is selectively retained in the body [1]. Despite the vital physiological requirement for vitamin E, our understanding of the mechanisms that regulate its levels in the body is limited. Current knowledge of intestinal vitamin E absorption is based on human and animal studies involving oral or intraduodenal administration of vitamin E followed by lymph and plasma analysis [2–7]. In most studies, around 20–50% of dietary vitamin E is believed to be absorbed [8]. Intestinal absorption of vitamin E requires the presence of bile acids. Vitamin E is absorbed by enterocytes and then is secreted in the form of chylomicrons into the lymphatic system. Anwar et al. [9] reported that vitamin E taken up by Caco-2 cells is secreted with chylomicrons and high-density lipoprotein (HDL). Chylomicrons containing vitamin E are secreted into the intestinal lymphatic system and are transported into the systemic circulation via the thoracic duct. Vitamin E reaching the plasma via chylomicrons is partly released during the hydrolysis of these particles by lipoprotein lipase [10]. Lipase-mediated hydrolysis of lipoprotein is needed for the uptake of vitamin E by peripheral cells. However, most of the vitamin E that reaches the liver is in the form of chylomicron remnants. Chylomicron remnants are taken up by endocytosis mainly in the liver, but probably also by several extrahepatic tissues. Once in the liver, the various isoforms of vitamin E undergo different metabolic fates. The cytosolic α-tocopherol transfer protein (α-TTP), a 31-kDa member of the Sec14 protein family, selectively binds α-tocopherol, whereas the other isoforms are metabolized by microsomal P450 [11,12] or excreted into the bile. A genetic defect in α-TTP is responsible for inducing a severe vitamin E deficiency characterized by a neurodegenerative disease, ataxia with

Abbreviations: α-TTP, α-tocopherol transfer protein; ABCA1, ATP-binding cassette transporter A1; apoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein.

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vitamin E deficiency [13,14]. α-TTP thus plays an essential role in maintaining plasma and tissue concentrations of α-tocopherol. The exact mechanism by which α-TTP regulates intracellular trafficking as well as secretion of α-tocopherol into the plasma and very low density lipoprotein (VLDL) particles is not fully understood. One hypothesis is that α-TTP facilitates VLDL-associated α-tocopherol secretion through a Golgi-mediated pathway. However, we reported that brefeldin A, which effectively inhibits VLDL secretion by disrupting the Golgi apparatus, had no effect on α-tocopherol secretion [15]. This result indicates that α-TTP-mediated α-tocopherol secretion is not coupled to VLDL secretion. Qian et al. [16] also confirmed that vitamin E is secreted by hepatoma cells by a non-Golgi α-TTP-mediated pathway. They showed that α-tocopherol arrives at intracellular vesicular organelles where it co-localizes with α-TTP and a marker of the lysosomal compartment (LAMP1) before being transported to the plasma membrane in an α-TTP-dependent manner and that α-tocopherol secretion from the hepatocytes is inhibited by glyburide, an inhibitor of several ABC transporters. Oram et al. [17] reported that efflux of α-tocopherol from cells to HDL apolipoproteins is mediated by ABCA1. However, none of the cell types they used (murine RAW264 macrophages, Tangier disease fibroblast and baby hamster kidney cells) were hepatocytes [17]. ABCA1 initiates the formation of mature HDL by facilitating apolipoprotein A-I (apoA-I) lipidation. Mutations in the gene encoding ABCA1 have been reported in Tangier disease, an autosomal recessive disorder characterized by almost complete absence of plasma HDL, deposition of cholesteryl esters in the reticuloendothelial system and aberrant cellular lipid trafficking [18–20]. Mice lacking a functional ABCA1 also have severe deficiency of fat-soluble vitamins including vitamin E [21]. However, in order to better understand the regulation of plasma α-tocopherol concentration, it is necessary to determine whether ABCA1 is involved in hepatic α-TTP-mediated α-tocopherol secretion. In this study, we obtained direct evidence that ABCA1 is involved in the secretion of α-tocopherol from hepatocytes.

2. Materials and methods

2.1. Materials

McARH7777 cells were purchased from American Type Culture Collection (Manassas, VA, USA). McA-TTP21 cells, namely, α-TTP-stable transformants of McARH7777 cells, were constructed previously [15]. [^{3}H]α-Tocopheryl acetate was purchased from Amersham. Fetal calf serum and horse serum were purchased from JRH Biosciences (Lenexa, KS, USA). Penicillin, streptomycin sulfate and L-glutamine were purchased from GIBCO (Gaithersburg, MD, USA). Human apoA-1 was purchased from Calbiochem (La Jolla, CA, USA). Phosphatidylcholine (egg) and dioleoyl-phosphatidylserine were purchased from Avanti (Alabaster, AL, USA). Dicethylphosphate, cholesterol and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Silica gel 60 TLC plate was purchased from Merck (Darmstadt, Germany). Probucol was purchased from Wako (Osaka, Japan). Adenoviral vectors, pAdEasy-1 and pShuttle-CMV were purchased from Qbiogene (Carlsbad, CA, USA). The siRNA expression vector, piGENE-tRNA-Pur, was purchased from iGENE (Ibaraki, Japan).

2.2. Animals and diets

Male 6-week-old C57BL/6Cr mice were purchased from Japan SLC (Hamamatsu, Japan). They were maintained under controlled conditions (temperature, 24±1°C; humidity, 55±5%) in plastic cages with sterilized paper for bedding. The animals were divided into the following two groups: (1) control feeding group (n=4) and (2) 1% probucol treatment group (n=4). The control diet was made by mixing 20 mg α-tocopherol into 1 kg of vitamin E-deficient diet. The 1% probucol diet was made by adding 1% v/w probucol to the control diet. The institutional animal care and use committee approved these studies.

2.3. Sampling

Both the control feeding group and the probucol treatment group received control diets for 2 weeks before the beginning of the experiments. They then received each diet for 4 weeks. Blood samples were collected after 6 h of fasting at the following three time points, namely, 0, 14 and 28 days after initiation of experimental diet feeding. Plasma total cholesterol of each sample was measured by using the Cholesterol E test (Wako).

2.4. Detection of α-tocopherol

Plasma α-tocopherol was extracted by chloroform/methanol (2/1, by volume). Lipids and vitamin E were extracted by centrifugation (15,000 rpm, at 4°C for 5 min) after mixing vigorously with a vortex mixer. Concentrations of α-tocopherol were detected using an HPLC system with an electrochemical detector (NANOSPACE SI-1, Shiseido, Tokyo, Japan). A Wakosil-2 5C18 RS column (Wako) was used with an eluent of methanol/$NaClO_4$ containing 50 mM $NaClO_4$ at a flow rate of 0.7 ml/min. The concentration of α-tocopherol was calculated by comparing the area under the curve with that of the standard.

2.5. Cell cultures

The rat hepatoma cell line McARH7777 cells were grown in Dulbecco's Modified Eagle's Medium with 2 mg/ml BSA containing 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 2 mM L-glutamine. McA-TTP21 cells were cultured in the same fashion as McARH7777 cells, as previously described [15].

2.6. shRNA sequence against rat-ABCA1 mRNA

The shRNA sequence against rat-ABCA1 mRNA-specific sequences (GenBank accession number NM_178095) was designed. This oligonucleotide encodes an siRNA sense strand, loop (5′-CTTCCTGTC-3′) and an siRNA antisense strand. Custom oligonucleotides were synthesized as below: a template of stem loop: 5′-AGATGTCCTATCTCTGTTATGTTGATGATACTTCCTGTCATGTCATCAACATAACAGGGGTAGGGCATCTGGTACCATAGATATCTTTTTTT-3′; forward primer: 5′-CCAACTTTGAGCTCAGATGTCCTATCTCTGTTAT-3′; and the reverse primer: 5′-AAAAAAAGATATCTATGGTACC-3′.

2.7. Preparation of vectors and insertion of DNA for making the adenovirus expressing shRNA against rat-ABCA1

To knock down endogenous ABCA1 protein in McA-TTP21 cells, we constructed an adenovirus expressing shRNA against rat-ABCA1 (AdV-rABCA1-RNAi). We first inserted shRNA against rat-ABCA1 at multiple cloning sites downstream the $tRNA^{Val}$ promoter, a promoter to express the valine transfer RNA, of the piGENE-tRNA-Pur vector (iGENE, Ibaraki, Japan). With this vector, the shRNA fragment is transcribed by RNA polymerase III and connected to the tRNA.

To prepare the DNA insert (sense-loop-antisense) for shRNA, the insert was amplified by PCR using the templates and primers as described in Section 2.6. The resulting double-stranded DNA was inserted into the piGENE-tRNA-Pur.

To check the RNAi effect of this vector, we co-transfected HEK293cells with 1000 ng of this vector and 100 ng of pcDNA3 containing rat-ABCA1. After we confirmed that this vector can reduce the expression of ABCA1 to 15% compared to HEK293 cells transfected with pcDNA3 containing rat-ABCA1 only (data not shown), we inserted the sequence from the tRNA (Val) promoter to the T7 terminator of this vector into pShuttle-CMV. pShuttle-CMV is a transfer vector containing a multiple cloning site in which a cloned gene is under the control of the human CMV promoter for constructive recombinant expression in a wide variety of cell line. The pShuttle-CMV containing the sequence of shRNA against rat-ABCA1 and an adenovirus expression vector pAdEASY-1 were electroporated into *E. coli* BJ5183. Recombinant adenoviral constructs were transfected into E1-transformed HEK293 cells to produce viral particles. The recombinant adenoviruses were purified by using ViraKit AdenoMini-4.

2.8. Transduction of adenoviral vectors into cells

Cultured McARH7777 cells and McA-TTP21 cells at 70% confluence were infected with the respective adenoviruses in Dulbecco's Modified Eagle's Medium containing 2% FBS for 2 h. Cells were infected with the AdV-rABCA1-RNAi (rat-ABCA1-RNAi adenoviruses) at a rate of 10,000 particles per cell. As controls, cells were infected with the lacZ adenoviruses (AdV-lacZ) at the same rate. The cultures were supplemented with an equal volume of Dulbecco's Modified Eagle's Medium containing 20% FBS and incubated for an additional 4 days.

2.9. Preparation of cell membrane and Western blot analysis of ABCA1 protein

Cells were harvested in SET buffer containing 1% protease inhibitor cocktails (Sigma) and homogenized. The cell suspension was centrifuged at 1000×g for 10 min, and the supernatant was centrifuged at 100,000×g for 1 h to prepare the membrane fraction as a pellet. The proteins were separated by SDS-PAGE (7%) and analyzed by immunoblotting using the anti-ABCA1 monoclonal antibody KM3110 [22].

2.10. Preparation of liposomes

Multilamellar liposomes were prepared as described previously [15]. The liposomes were composed of PC/PS/DCP/cholesterol/[^{3}H]α-tocopheryl acetate (5 μCi/nmol)

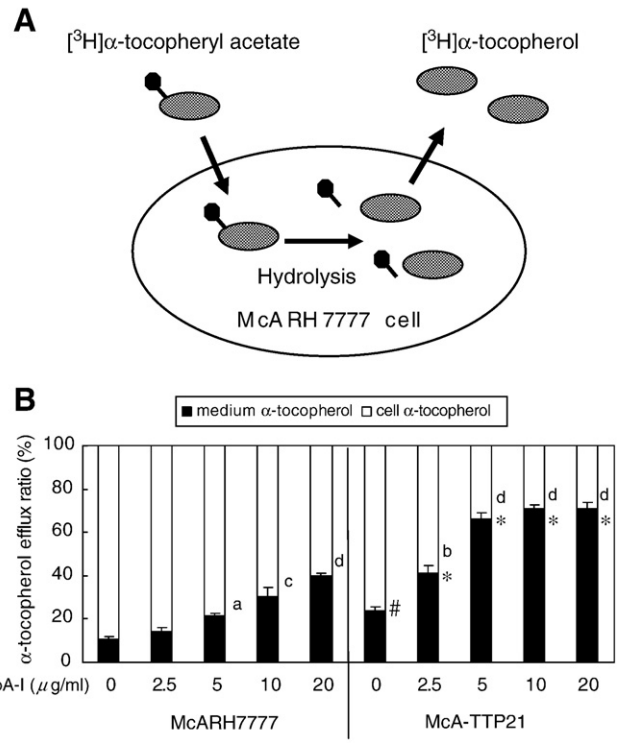

Fig. 1. (A) Assay of α-tocopherol secretion from McARH7777 cells. (B) Dose-dependent apoA-I-mediated secretion of α-tocopherol from McARH7777 cells and McA-TTP21 cells. (A) Alpha-tocopheryl acetate is hydrolyzed to generate α-tocopherol when entering cells. This α-tocopherol is secreted into the medium. To evaluate cellular α-tocopherol secretion, the amount of α-tocopherol in cells and medium was measured. (B) McARH7777 cells and McA-TTP21 cells were radiolabeled with [^{3}H]α-tocopheryl acetate, incubated for 24 h with medium containing 2 mg/ml BSA plus 0, 2.5, 5, 10 and 20 μg/ml apoA-I, respectively. We measured the amount of [^{3}H]α-tocopherol in cells (white) and medium (black). Data represent means±S.D. of triplicates expressed as percent of α-tocopherol secretion as described in the Materials and methods section. $^{a}P<.05$, $^{b}P<.01$, $^{c}P<.005$, $^{d}P<.001$ compared with apoA-I nontreated control (ANOVA, Tukey). $^{\#}P<.005$, $^{*}P<.001$ compared with McARH7777 cells treated with the same concentration of apoA-I (ANOVA, Tukey).

(molar ratio 50:50:10:75:2). The mixture of these lipids was dried under a stream of nitrogen, and dried lipids were resuspended and sonicated in 0.3 M glucose at room temperature.

2.11. Incubation of cells with radiolabeled lipids

Cells were plated at a density of 1×10^5 cells per well into 24-well collagen-coated plates. The medium was changed to DMEM containing 2 mg/ml BSA and 10 μg/ml apoA-I after overnight incubation. Following 6 h of incubation, the medium was removed, and cells were incubated for 24 h at 37°C in 0.5 ml medium with [^{3}H]α-tocopheryl acetate-containing liposomes (5 μCi per well). Alpha-tocopherol secretion (%) was calculated as follows: α-tocopherol secretion ratio (%)={[^{3}H]α-tocopherol in medium/([^{3}H]α-tocopherol in the cells+[^{3}H]α-tocopherol in medium)}×100 (%).

2.12. Lipid extraction and analysis by thin-layer chromatography

After incubation, extracellular and cellular lipids were extracted by hexane. Cells were washed twice with PBS and were lysed by adding 0.1 ml of 0.1% SDS. An aliquot was diluted with 0.4 ml of *aqua dest* and used for the following procedure. The media were centrifuged briefly (3500 rpm, 5 min, 4°C). The cell lysate or medium (0.5 ml) was added to 1 ml of 6% pyrogallol in ethanol. The mixture was diluted with 1.5 ml of 65% ethanol. Three milliliters of hexane was added and the mixture was mixed vigorously in a vortex mixer before being centrifuged at room temperature. The hexane layer was saved, and the hexane extracts were evaporated under nitrogen. The residue was dissolved in ethyl acetate and subjected to thin-layer chromatography on a silica gel plate. The plate was developed with $CHCl_3$. After development, plates were exposed and analyzed, using a bio-image analyzer (Fujifilm, Tokyo, Japan).

3. Results

3.1. ApoA-I increases hepatic α-tocopherol secretion in a dose-dependent manner

To examine the secretion of α-tocopherol from cultured hepatocytes, we used radiolabeled α-tocopheryl acetate (Fig. 1A), which is assumed to be hydrolyzed to generate α-tocopherol when entering cells. The α-tocopherol is secreted into the medium by the action of α-TTP. Thus, it should be possible to evaluate the secretion of α-tocopherol from cells by measuring the amount of α-tocopherol in the cells and in the medium [15,16].

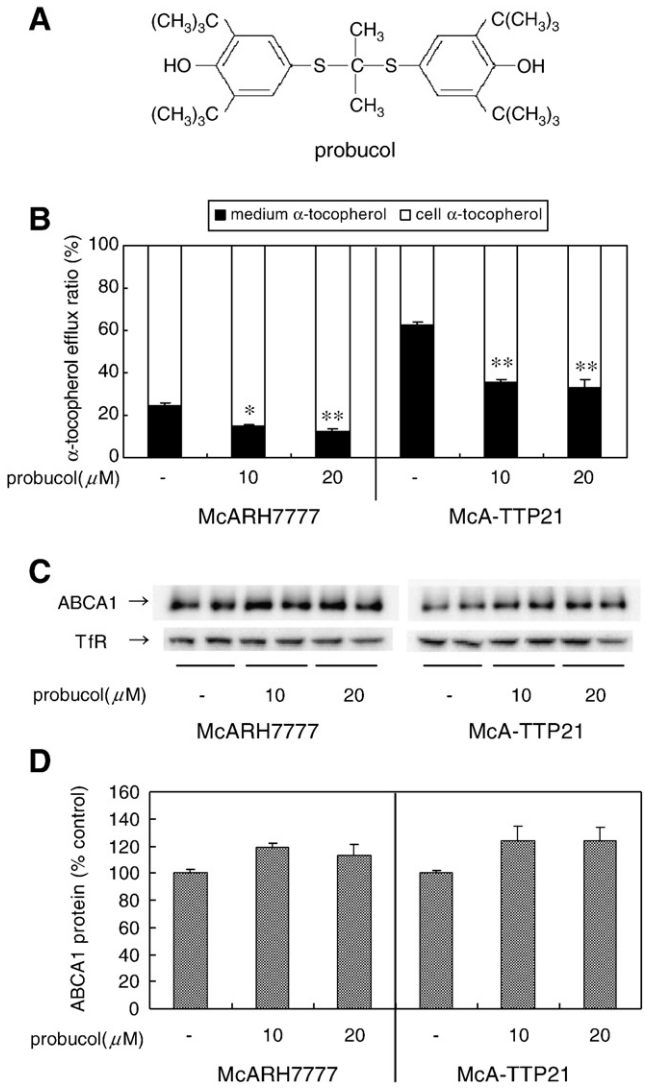

Fig. 2. Effect of probucol on α-tocopherol secretion and ABCA1 protein expression. (A) Chemical structure of probucol. (B) McARH7777 and McA-TTP21 cells were incubated with 0, 10 and 20 μM of probucol. After 1 h of incubation, [^{3}H]α-tocopheryl acetate-containing liposomes were added into the medium. After another 24 h of incubation, the radioactivity of [^{3}H]α-tocopherol in cells (white) and medium (black) was measured. Data represent means±S.D. of triplicates expressed as percent of α-tocopherol secretion. $^{*}P<.01$, $^{**}P<.005$, compared with probucol nontreated control (ANOVA, Tukey). (C) McARH7777 and McA-TTP21 cells were incubated under the same conditions as in (B). After 24 h of incubation, cells were harvested in SET buffer containing 1% protease inhibitor cocktails and homogenized. Seven micrograms of the membrane protein was analyzed by immunoblotting for ABCA1. Membrane fractions were prepared as described in Materials and methods. Immunoblot of transferrin receptor (TfR) served as a loading control. (D) Quantification of the ABCA1 protein was determined by densitometric analysis of the scanned images. Data represent means±S.D.

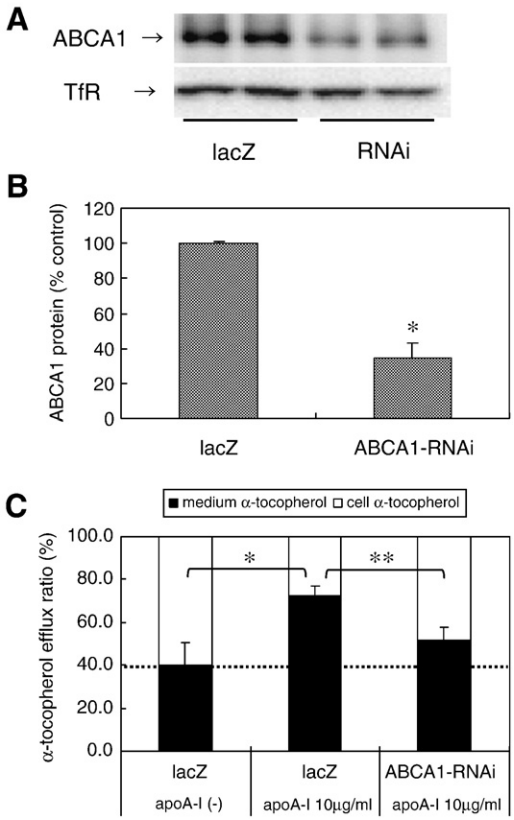

Fig. 3. Effect of RNAi of ABCA1 on the α-tocopherol secretion from McARH7777 and McA-TTP21 cells. (A) Expression of ABCA1 in McA-TTP21 cells which were infected with rat-ABCA1-RNAi adenoviruses (AdV-rABCA1-RNAi). Cells were infected with AdV-rABCA1-RNAi at a rate of 10,000 particles/cell. As controls, cells were infected with AdV-lacZ at the same rate. The cultures were incubated for 4 days as described in Materials and methods. After 4 days of incubation, cells were harvested and homogenized. Seven micrograms of the membrane protein was analyzed by immunoblotting for ABCA1 protein. Immunoblot of TfR served as a loading control. (B) Quantification of the rat ABCA1 protein in McA-TTP21 cells by densitometric analysis of the scanned images. Data represent means±S.D. $^{*}P<.01$, compared with AdV-lacZ-infected control (unpaired *t* test). (C) McARH7777 and McA-TTP21 cells were infected with 10,000 particles/cell of control AdV-lacZ or AdV-rABCA1-RNAi. After 3 days of incubation with each virus, AdV-lacZ-infected McA-TTP21 cells were radiolabeled with [^{3}H]α-tocopheryl acetate, incubated for 24 h with medium containing 2 mg/ml BSA with/without 10 μg/ml apoA-I. AdV-rABCA1-RNAi-infected McA-TTP21 cells were radiolabeled with [^{3}H]α-tocopheryl acetate, incubated for 24 h with medium containing 2 mg/ml BSA and 10 μg/ml apoA-I. After another 24 h of incubation, the radioactivity of [^{3}H]α-tocopherol in cells (white) and medium (black) was measured. Data represent means±S.D. $^{*}P<.005$, $^{**}P<.025$ (ANOVA, Tukey).

In this study, we used the rat hepatoma cell line McARH7777. Because the α-TTP expression level in McARH7777 cells is extremely low, we established an α-TTP stable transformant McARH7777 cell (the McA-TTP21 cell) [15]. We reported previously that the level of α-tocopherol secretion from McA-TTP21 cells is significantly higher than that from McARH7777 cells [15].

Previous studies showed that lipid-free apoA-I increases α-tocopherol secretion from cultured nonhepatic cells [17]. To confirm the participation of ABCA1 in hepatic α-tocopherol secretion, we tested the ability of apoA-I to remove radiolabeled α-tocopherol from McARH7777 and McA-TTP21 cells (Fig. 1B). In both McARH7777 and McA-TTP21 cells, α-tocopherol secretion was increased by addition of apoA-I in a dose-dependent manner. Moreover, α-tocopherol secretion from McA-TTP21 cells was significantly higher than that from McARH7777 cells at any apoA-I concentration. We observed small but appreciable amounts of α-tocopherol secretion from both McARH7777 and McA-TTP21 cells even in the absence of exogenous apoA-I. We found that the amounts of endogenous apoA-I secreted from McARH7777 and McA-TTP21 cells were negligible compared with the amount of added apoA-I (data not shown) under the present conditions, indicating that endogenous apoA-I secreted from cells does not contribute to the secretion of α-tocopherol. BSA (2 mg/ml) added to the culture (see Materials and methods) might serve as an acceptor of α-tocopherol secreted from the cells in the absence of exogenous apoA-I.

3.2. Probucol inhibits hepatic α-tocopherol secretion

Probucol (Fig. 2A) has been clinically used as an antiatherogenic compound, not only because of its lipid-lowering effect but also because of its ability to prevent atherogenic oxidation of low-density lipoprotein (LDL) as has been shown in in vitro [23,24] and in vivo models [25]. Wu et al. [26] as well as Favari et al. [27] reported that probucol inactivates ABCA1 by inhibiting the binding of apoA-I to the cells in a dose-dependent manner and by reducing the cellular lipid release. In this study, α-tocopherol secretion from McARH7777 and McA-TTP21 cells was inhibited by a treatment with 10 and 20 μM probucol (Fig. 2B). ABCA1 proteins in probucol-treated McARH7777 and McA-TTP21 cells showed an approximately 1.2-fold increase (Fig. 2C and D). Wu et al. [26] also reported that probucol does not affect ABCA1 mRNA levels but indeed leads to an increase in ABCA1 protein levels. They argued that probucol inhibits the proteolytic degradation of ABCA1 by calpain. Our results show that probucol does not decrease the ABCA1 protein level but reduces hepatic α-tocopherol secretion. Probucol did not decrease the level of α-TTP protein either (data not shown).

3.3. Effect of RNAi of ABCA1 on hepatic α-tocopherol secretion

We attempted to knock down endogenous ABCA1 protein in McA-TTP21 cells (Fig. 3A and B). We constructed an adenovirus expressing shRNA against rat-ABCA1 (AdV-rABCA1-RNAi) and infected it into McA-TTP21 cells (see Section 2.7). Based on immunoblotting, ABCA1 expression levels in AdV-rABCA1-RNAi-infected McA-TTP21 cells were only 35% of the levels in control AdV-lacZ-infected McA-TTP21 cells (Fig. 3B). We measured the amount of radiolabeled α-tocopherol in the cells and medium.

We assumed that the apoA-I-dependent increase in α-tocopherol secretion equals the value obtained by subtracting the value of α-tocopherol secretion in the absence of apoA-I (~40%, left bar in Fig. 3C) from that of α-tocopherol secretion in AdV-lacZ-infected McA-TTP21 cells treated with apoA-I (~72%, central bar in Fig. 3C). The apoA-I-dependent increase in α-tocopherol secretion was significantly lowered by ABCA1-RNAi (~50%, right bar), indicating that ABCA1 participates in the transport of α-tocopherol. While the apoA-I-independent α-tocopherol secretion from McA-TTP21 cells was 10% (Fig. 1B), the apoA-I-independent α-tocopherol secretion from AdV-lacZ-infected McA-TTP21 cells increased up to 40% (Fig. 3C). It is likely that incubation with the adenovirus over 4 days increased α-tocopherol leakage from these cells. At present, we have no explanation for these results, but similar results were obtained in every experiment. Because the apoA-I-independent α-tocopherol secretion from AdV-lacZ-infected McA-TTP21 cells was not decreased by ABCA1 depletion (data not shown), other machineries such as membrane particles shed off from the cells may serve as an acceptor for α-tocopherol efflux in these cells.

3.4. Probucol decreases murine plasma α-tocopherol concentration

Furthermore, we investigated whether probucol can decrease murine plasma α-tocopherol concentration. Male mice were fed with a 1% (v/w) probucol or control diet for 4 weeks. Compared with

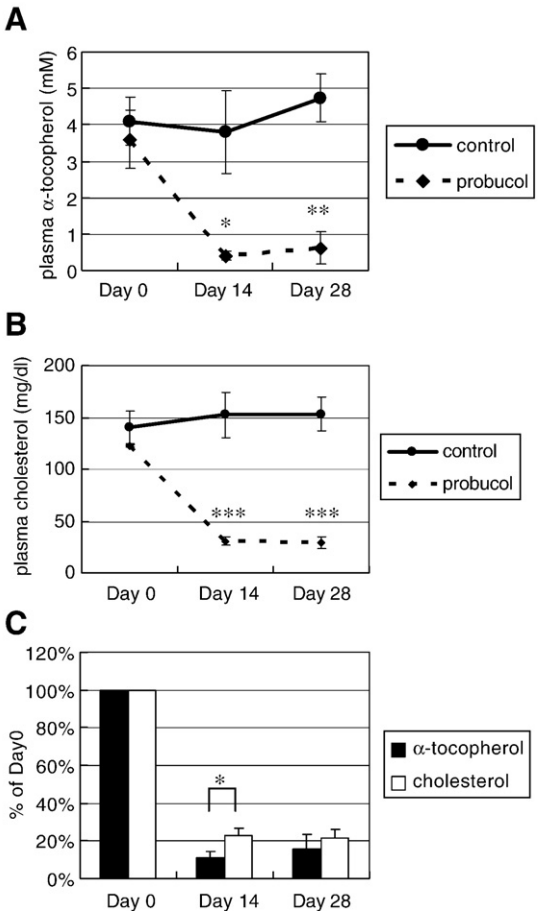

Fig. 4. Probucol reduces the α-tocopherol and cholesterol concentration in murine plasma. (A) Male 6-week-old C57BL/6Cr mice were divided into the following two groups: (1) control feeding group (n=4) and (2) 1% v/w probucol treatment group (n=4). Blood samples were collected after 6 h of fasting at the following three time points: 0, 14 and 28 days after they were fed each diet. Plasma α-tocopherol was extracted by chloroform/methanol. Concentrations of α-tocopherol were detected using an HPLC system. Data represent means±S.D. $^{*}P$<.025, $^{**}P$<.01, compared with Day 0 (paired t test). (B) Plasma concentrations of total cholesterol on Days 0, 14 and 28 were measured. Data represent means±S.D. $^{***}P$<.0025, compared with Day 0 (paired t test). (C) Comparison of the concentration of α-tocopherol and total cholesterol in probucol-treated murine plasma. The results are expressed as a percentage of each concentration on Day 0, respectively. Data represent means±S.D. $^{*}P$<.05 (ANOVA, Tukey).

plasma α-tocopherol concentrations prior to probucol treatment, plasma α-tocopherol concentrations were significantly reduced after 2 and 4 weeks of probucol treatment (Fig. 4A). Notably, the probucol-induced reduction of plasma α-tocopherol concentrations was significantly larger than the reduction of plasma total cholesterol concentrations after 2 weeks of treatment with probucol (Fig. 4B and C). These results suggested that probucol inhibited ABCA1 and directly reduced plasma α-tocopherol concentrations in an in vivo model. The reduction of plasma α-tocopherol can therefore be regarded as a direct effect of ABCA1 inhibition and not merely as a secondary effect due to the reduction of plasma lipoproteins, a major reservoir of plasma α-tocopherol.

4. Discussion

The present study using both in vitro and in vivo studies showed that ABCA1 is substantially involved in hepatic α-tocopherol secretion. Three lines of evidence support this conclusion. First, apoA-I, a direct acceptor for ABCA1-secreted lipids, increased α-tocopherol secretion from cultured hepatocytes in a dose-dependent manner. Second, probucol, an inactivator of ABCA1 via inhibition of cellular apoA-I binding, inhibited α-tocopherol secretion from hepatocytes and decreased murine plasma α-tocopherol concentrations. Third, ABCA1-RNAi suppressed hepatic α-tocopherol secretion.

Orso et al. [21] reported that ABCA1$^{(-/-)}$ mice are deficient in fat-soluble vitamins such as vitamins A, E and K1, but they did not determine whether this was due to a secondary effect of the decreased plasma lipoprotein levels or the reduction of ABCA1-mediated secretion of these vitamins. On the other hand, Elinder et al. [28] found that treatment of hypercholesterolemic patients (N=303) with probucol for 3 years reduced serum vitamin E by 14%. Bird et al. [29] reported that 0.5% probucol decreased plasma concentrations of vitamin E in LDL receptor-deficient mice. Choy et al. [30] reported that dietary supplementation with 1% probucol decreased the concentration of α-tocopherol in the aortic arch and descending aorta of ApoE$^{-/-}$ mice. They did not show plasma data, but suggested that low plasma α-tocopherol concentrations influence α-tocopherol concentrations in aortic tissue. The present results show that both plasma cholesterol and α-tocopherol concentrations decreased after probucol treatment and that the reduction in α-tocopherol levels was larger than the reduction in plasma cholesterol levels. These findings confirm that probucol does not reduce plasma α-tocopherol levels by merely reducing plasma lipoproteins as the main reservoir of α-tocopherol, but selectively inhibits ABCA1, leading to a direct α-tocopherol reduction. This provides evidence that not only cholesterol, but also α-tocopherol is a direct substrate for ABCA1. Furthermore, in vitro experiments in hepatocyte cultures show that ABCA1 plays an important role in the secretion of α-tocopherol.

As we previously reported [15], α-tocopherol is also secreted to a certain extent from cultured hepatocytes not expressing α-TTP (see Fig. 1B, where apoA-I amounts to zero). It is not clear whether α-TTP-independent secretion is limited to cultured hepatocytes or occurs also in the liver under in vivo conditions. However, we hypothesize that this α-TTP-independent secretion of α-tocopherol from hepatocytes is also mediated by ABCA1, since this secretion was stimulated by apoA-I (Fig. 1B) and inhibited by probucol (Fig. 2B).

Qian et al. [16] observed that fluorescent-labeled tocopherol arrives in the lysosome following its internalization from serum complexes. Tocopherol translocates from lysosomes to the cell surface in an α-TTP-dependent manner. It seems unlikely that α-TTP delivers α-tocopherol to ABCA1 directly, since we could not show a direct protein–protein interaction between α-TTP and ABCA1 (unpublished observations). We hypothesize that α-TTP transports α-tocopherol to the plasma membrane, where it is picked up by ABCA1 and excreted from the hepatocyte.

Based on our results, we recommend that physicians, when treating hyperlipidemic patients with probucol, check the patients' plasma α-tocopherol concentrations and consider giving them vitamin E supplements. Alternatively, it is possible that that probucol will replace α-tocopherol in its function as an antioxidant due to its strong antioxidative capacity. Further studies are needed to determine whether probucol can replace vitamin E in its function as a fat-soluble antioxidant or whether it has physiological roles.

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