Intracellular trafficking of vitamin E in hepatocytes: the role of tocopherol transfer protein

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Abstract **The term *vitamin E* denotes a family of tocopherols and tocotrienols, plant lipids that are essential for vertebrate fertility and health. The principal form of vitamin E found in humans, *RRR*-α-tocopherol (TOH), is thought to protect cells by virtue of its ability to quench free radicals, and functions as the main lipid-soluble antioxidant. Regulation of vitamin E homeostasis occurs in the liver, where TOH is selectively retained while other forms of vitamin E are degraded. Through the action of tocopherol transfer protein (TTP), TOH is then secreted from the liver into circulating lipoproteins that deliver the vitamin to target tissues. Presently, very little is known regarding the intracellular transport of vitamin E. We utilized biochemical, pharmacological, and microscopic approaches to study this process in cultured hepatocytes. We observe that tocopherol-HDL complexes are efficiently internalized through scavenger receptor class B type I. Once internalized, tocopherol arrives within ~30 min at intracellular vesicular organelles, where it colocalizes with TTP, and with a marker of the lysosomal compartment (LAMP1), before being transported to the plasma membrane in a TTP-dependent manner. We further show that intracellular processing of tocopherol involves a functional interaction between TTP and an ABC-type transporter.—** Qian, J., S. Morley, K. Wilson, P. Nava, J. Atkinson, and D. Manor. **Intracellular trafficking of vitamin E in hepatocytes: the role of tocopherol transfer protein. *J. Lipid Res.* 2005.** 46: **2072–2082.**

Supplementary key words antioxidants • lipid-transfer protein • lysosome

Vitamin E is a neutral plant lipid that is an essential nutrient in vertebrates. It is generally accepted that by virtue of its antioxidant activity, vitamin E is able to scavenge free radicals, alleviate oxidative stress, and thus promote normal cell function. Indeed, reduced vitamin E levels are associated with a plethora of pathologies, including infertility, hemolysis, and neuronal degeneration. Thus, adequate vitamin E intake is considered critical for health, and supplementation with the vitamin has become widespread in humans and companion animals (1, 2). There are eight chemically distinct forms of vitamin E, among which *RRR*-α-tocopherol (TOH) is considered the most biologically active and is selectively retained in the body (3). Despite the strict physiological requirement for vitamin E, our understanding of the mechanisms that regulate its levels in the body is limited. Dietary TOH is absorbed by enterocytes, and secreted to the circulation together with other lipids incorporated in chylomicra (4). Following endothelial catabolism of the chylomicra, vitamin E is taken up by parenchymal cells of the liver. It is in the liver that the key reactions that regulate vitamin E status take place. Specifically, the cytochrome P450 isoform CYP4F2 catabolizes tocols other than *RRR*-α-tocopherol into water-soluble products that are excreted in urine (5). The remaining vitamin form, *RRR*-α-tocopherol (TOH), is then secreted from the hepatocytes and delivered to peripheral tissues complexed to circulating lipoproteins. Hepatic tocopherol secretion is facilitated by the tocopherol transfer protein (TTP), a soluble polypeptide that binds TOH with high affinity and selectivity and catalyzes its transfer between lipid vesicles (6–8). In support of a critical role for TTP in regulating vitamin E status are the observations that humans who carry mutations in the *ttpA* gene display low plasma tocopherol levels and neurological disorders associated with elevated oxidative stress termed *ataxia with vitamin E deficiency* (AVED) (2, 3, 9, 10). Similarly, $TTP^{-/-}$ mice display low vitamin E levels, are infertile, and exhibit an AVED-like pathology (11, 12).

Details regarding the *intra*cellular transport of tocopherol are scarce. In many cell types, the vitamin accumulates primarily in lysosomes (13) and mitochondria (14), where high levels of oxidative stress are presumed to exist. Especially enigmatic are the paths for intracellular transport of vitamin E in hepatocytes, the cells that regulate whole-body distribution of tocopherol. In attempts to identify intracellular vitamin E transporters, multiple groups

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have reported that the only specific tocopherol binding activity in liver is that of TTP (6, 15–17). Arai and colleagues reported that when TTP is overexpressed in a cultured rat hepatocyte cell line, tocopherol secretion is markedly facilitated (18, 19). These authors concluded, on the basis of pharmacological sensitivity, that hepatocytes possess a novel and specific pathway for tocopherol secretion, independent of the route utilized for secretion of nascent very low density lipoproteins. Presently, nothing is known regarding the molecular mechanisms that underlie this activity.

We aim to delineate the pathway of vitamin E transport in liver cells, and the role that TTP plays in this process. Toward this goal, we report here our studies on the uptake, transport, and secretion of vitamin E in cultured hepatocyte cell lines that are capable of inducible expression of TTP.

EXPERIMENTAL PROCEDURES

Cell culture

McA-RH7777 cells and HepG2/C3A cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and additional 10% horse serum for McA-RH7777 cells. All transfections were done using Fugene6 (Roche Biochemicals Co.).

Inducible expression of TTP

Cell lines were generated using the TetOn system according to the manufacturer's instructions (Clontech). Briefly, HepG2 or McA-RH7777 cells were first transfected with the tetracycline regulatory element pTetOn and stable transfectants selected with neomycin (G418; 400 μg/ml). Then the cells were transfected with the pTRE2 vector containing the human TTP gene fused to a 5′ hemagglutin (HA) tag to aid in immunodetection. Double stable clones were selected with both neomycin and hygromycin (400 and 200 μg/ml, respectively), screened for inducible expression of HA-TTP by immunoblotting, and positive clones maintained in the presence of both antibiotics (200 μg/ml each). To induce TTP expression, doxycycline (Calbiochem, 1 μg/ml) was added to the growth media for 48 h.

Tocopherol complexes

Tocopherol-serum complexes were prepared according to the method of Asmis (20). Briefly, a glass scintillation vial was coated with a film containing the required amount of [^{14}C]α-tocopherol (0.18 μCi/ml) or NBD-tocopherol, and the solvent was evaporated under vacuum. After addition of FBS (tetracycline-free, Clontech), the vial was purged under nitrogen, sealed, and rotated at 4°C for 1 h at 10 rpm. DMEM was then added to the vial, to obtain a final serum concentration of 10% (v/v). The final concentration of [^{14}C]α-tocopherol and NBD-α-tocopherol was 3 μM and 50 μM, respectively.

To compare the efficacy of HDL and LDL in delivering tocopherol to hepatocytes (Fig. 2A), the amount of endogenous tocopherol in each preparation was determined by gas chromatography/mass spectroscopy (5) and found to be 7.4 and 15.6 nmol/mg protein of HDL and LDL, respectively. These preparations were then loaded with [^{14}C]α-tocopherol (Amersham, 55 mCi/mmol) as described above, and an equal amount of total tocopherol (endogenous plus labeled) from each preparation was presented to the cultured cells, as described by Sattler and colleagues (21).

Tocopherol accumulation

HepG2-TetOn-TTP cells were seeded into each well in 24-well plates (4×10^5 cells/well), and TTP expression was induced with doxycycline. Serum or lipoprotein complexes of [^{14}C]*RRR*-α-tocopherol were then added to the culture media for the indicated time periods. At the end of each incubation, media was removed, and the cells were washed with DMEM and lysed in 20 mM HEPES (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% NP40, 20 mM sodium fluoride, 20 mM β-glycerophosphate, 1 mM sodium vanadate, and 200 μM PMSF. After measurement of the radioactivity in a scintillation counter, tocopherol accumulation was calculated as follows:

$$\text{tocopherol accumulation (\%)} = \frac{\text{CPM}_{\text{cells}}}{\text{CPM}_{\text{cells}} + \text{CPM}_{\text{medium}}} \times 100$$

To block uptake through the SR-BI receptor, cells were pretreated with the anti-SR-BI antibody (KKB-1, (22), generous gift of Karen Kozarsky, 1:200 dilution) for 3 h prior to loading of the cells with [^{14}C]tocopherol-HDL as described above.

Tocopherol secretion

HepG2-TetOn-TTP cells were grown in 24-well plates, and TTP expression was induced by doxycycline treatment. FBS complexed to [^{14}C]α-tocopherol was then added to the induction media (10% FBS, 0.18 μCi/ml; 3 μM tocopherol) for 36 h (loading period). The cells were then washed three times in complete medium and once in DMEM, and incubated with DMEM for the indicated duration (secretion period). The media was then collected, and the cells were washed twice in DMEM and lysed as described above. After measurement of the radioactivity in a scintillation counter, tocopherol secretion was calculated as follows:

$$\text{tocopherol secretion (\%)} = \frac{\text{CPM}_{\text{medium}}}{\text{CPM}_{\text{cells}} + \text{CPM}_{\text{medium}}} \times 100$$

At the end of a typical secretion experiment, secreted radioactivity was ~8,400 dpm per well.

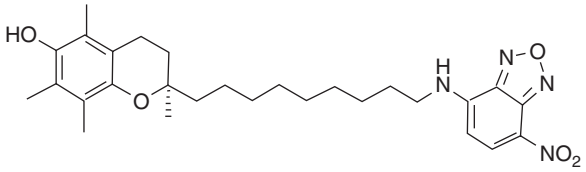
Fluorescence microscopy

Cells, cultured on dual-chamber microscope slides (Labtek), were rinsed three times with PBS, fixed in 4% formaldehyde, and permeabilized with 0.2% Triton X-100 before processing with the indicated stains or antibodies. Actin cytoskeleton and cell nuclei were visualized with direct fluorescence using Texas Red phalloidin and To-Pro3, respectively (Molecular Probes, Inc.). TTP protein was visualized using the AT-R1 mouse monoclonal antibody (generous gift of H. Arai, University of Tokyo, Japan) or anti-HA antibodies (monoclonal HA.11, Covance Inc.; or rabbit polyclonal, Santa Cruz, Inc.). Organelle markers were as follows: for endosomes, the early endosome antigen (anti-EEA1; Calbiochem); for lysosomes, lysosome-associated membrane protein (anti-LAMP1; Stressgen, Inc.); and for Golgi and endoplasmic reticulum, mannosidase (anti-mannosidase II; kindly supplied by Dr. K. Moremen, University of Georgia) and calnexin (anti-calnexin; Molecular Probes). Confocal images were collected with the appropriate excitation laser lines on a Leica TCS-SP2 microscope at the Cornell BioResource Center.

Fluorescent tocopherol

The preparation of C9-NBD-α-tocopherol followed the general synthetic approach reported in earlier work with tocopherol photoaffinity labels (23). In brief, Trolox methyl ester (methyl (2*S*)-6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydro-2-carboxylate) was protected on the C-6 phenol with *tert*-butyldimethylsilyl chloride, and the ester was reduced to the aldehyde with diisobutyl aluminum hydride in CH_2Cl_2. The aldehyde was then coupled with

8-hydroxyoctyltriphenylphosphonium bromide in a Wittig reaction using lithium hexamethyldisilylazide in dry tetrahydrofuran (THF). The resulting alkene was reduced with hydrogen gas and palladium on charcoal. The terminal primary alcohol was then transformed to an amine by mesylation (CH_3SO_2Cl, Et_3N, DMAP, CH_2Cl_2, 0°C to room temperature), followed by substitution with sodium azide in dimethylformamide, and catalytic reduction with hydrogen gas and 10% palladium on charcoal. The resulting *O*-6-silyl-protected nonylamine chromanol was then coupled to NBD-Cl (4-chloro-7-nitrobenz-2-oxa-1,3-diazole) by mixing the two in THF with two equivalents of Et_3N. The fluorophore is best stored as the silyl-protected phenol until needed and then deprotected using tetrabutylammonum fluoride in THF, followed by purification on silica gel. The structure of C9-NBD-α-tocopherol is shown below.



Analytical details are provided below for the silyl-protected material.

(2R)-((9-(4-nitrobenzo[c][1,2,3]oxadiazole-7-ylamino)nonyl)-3,4-dihydro-2,5,7,8-tetramethylchroman-6-yloxy)(tert-butyl)dimethylsilane. Dark orange oil, R_f = 0.36 (Et_2O-Hex, 1:1); ^1H NMR ($CDCl_3$) δ 8.46 (d, 1H, J = 9 Hz), 6.18 (br, 1H), 6.14 (d, 1H, J = 9 Hz), 3.45 (q, 2H, J = 6 Hz), 2.53 (t, 2H, J = 7 Hz), 2.07 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.77 (m, 4H), 1.39 (m, 6H), 1.28 (br, 8H), 1.20 (s, 3H), 1.02 (s, 9H), 0.09 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 145.8, 144.2, 144.0, 143.8, 143.8, 136.5, 125.8, 124.0, 123.5, 122.6, 117.4, 98.5, 74.4, 43.9, 39.5, 31.5, 30.0, 29.4, 29.3, 29.1, 28.5, 26.9, 26.0, 23.8, 23.5, 20.8, 18.5, 14.3, 13.4, 11.9, −3.3; MS (EI) *m/z* 624 (M^+). HRMS (EI): calculated for $C_{34}H_{52}N_4O_5Si$: 624.37069; found: 624.36959.

Uptake of NBD-tocopherol

McA-RH7777-TetOn-TTP cells in two-chamber slides were incubated with 50 μM NBD-tocopherol (complexed to FBS) for 2 h at 4°C, followed by chasing at 37°C with fresh DMEM-10% FBS for the indicated time periods prior to fixing and staining. NBD fluorescence was excited with the 453 nm line from an Ar^{2+} laser.

RESULTS

Generation of hepatocyte cell lines capable of inducible TTP expression

Detailed investigations of the biochemical functions of TTP in cells are hindered by the fact that the protein is not expressed in any known cell lines of hepatic origin. Furthermore, expression of TTP in freshly prepared primary hepatocytes declines precipitously following isolation (24). To facilitate studies of TTP in a physiologically relevant system, we generated human and rat cell lines that stably express TTP under the regulation of a tetracycline-inducible promoter. **Figure 1A** shows that the human hepatoblastoma HepG2-TetOn-TTP and the rat hepatoma McA-RH7777-TetOn-TTP clones that we isolated indeed exhibit strong expression of TTP upon treatment with doxycycline. Importantly, no "leaky" expression of the protein is observed in the absence of induction. To assess the functionality of TTP in these cell lines, we turned to the

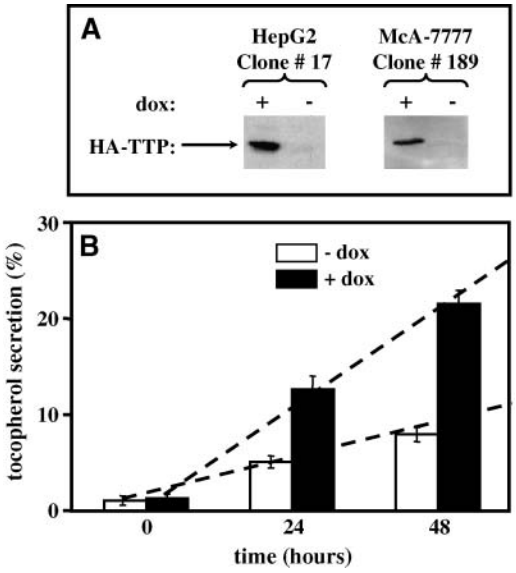


Fig. 1. Inducible expression of tocopherol transfer protein (TTP) in cultured hepatocytes. A: The indicated cell lines were generated as described in Experimental Procedures and cultured for 48 h in the presence or absence of doxycycline (dox) (1 μg/ml). TTP expression was assayed in lysates after 48 h of induction using anti-HA Western blotting. B: TTP-induced α-tocopherol secretion in HepG2-TetOn-TTP cells. Cells were loaded with [^{14}C]*RRR*-α-tocopherol for 36 h and washed, and the appearance of radioactivity in the media was assayed at the indicated times by scintillation counting. Shown are averages and standard deviation of quadruplicate wells. Data are representative of 10 independent experiments. When those data are fitted to linear functions (dashed lines), their slopes differ by 3.3-fold.

only known physiological activity of this protein, namely, the facilitation of tocopherol secretion (18). Indeed, upon induction of TTP expression in the HepG2-TetOn-TTP cell line, a pronounced (~3-fold) increase in secretion of tocopherol from the cells to the media is observed (Fig. 1B). Both the extent and the kinetic characteristics of the observed facilitation are similar to those reported by Arita et al. (18). Essentially identical results were observed in the McA-RH7777-TetOn-TTP cells (data not shown). We conclude that the TTP cell lines we have developed provide a proper experimental system for studying the biochemical functions of TTP under physiologically relevant conditions. Due to their clearer morphology, the McA-RH7777 cells are particularly suitable for microscopy studies. However, because McA-RH7777 cells are difficult to grow for the extended periods required for uptake and secretion assays (3–4 days past confluence), we utilize the HepG2-TetOn-TTP cells for these purposes.

Vitamin E accumulation by TTP-expressing cells

We first investigated the process of vitamin E accumulation, using radioactively labeled [^{14}C]α-*RRR*-tocopherol as a tracer. To mimic the physiological scenario, tocopherol was delivered to the cells complexed to serum lipoproteins (20) rather than from organic solvent stocks (25) or liposome preparations (18). We found that tocopherol accumulates in the cells in a time- and dose-dependent man-

ner, reaching saturation after ~36 h (data not shown). In serum, vitamin E is distributed between LDLs and HDLs (26–28). To compare the relative contributions of these fractions to vitamin E uptake by hepatocytes, we complexed purified lipoprotein preparations with radiolabeled vitamin E, and monitored its accumulation by the cells in the absence or presence of TTP expression. **Figure 2A** shows the time-dependent tocopherol import by HepG2 cells when the vitamin is presented as an LDL or as an HDL complex, and the effect of TTP expression on this process. Although tocopherol is efficiently taken up from both lipoprotein preparations (5–10% of available tocopherol internalized in 4 h), the rate of accumulation from HDL complexes exceeds that from LDL particles by ~2-fold. This finding is in agreement with the observations of Sattler and colleagues, who reported HDL to be the main delivery route for vitamin E in endothelial cells (29) and hepatocytes (21). Further support for this conclusion is the finding that treatment of cells with antibodies directed against HDL's cellular "port of entry," the scavenger receptor class B type I (SR-BI) (30), leads to pronounced inhibition of tocopherol accumulation (Fig. 2B). Importantly, induction of TTP expression does not increase tocopherol accumulation. Rather, expression of TTP leads to a small (10–20%) yet significant *reduction* in the accumulation of tocopherol from either LDL or HDL (Fig. 2A). This apparent attenuation of tocopherol accumulation is likely to result from TTP's activity in facilitating tocopherol efflux (Fig. 1B). The data indicate that a significant portion of vitamin E accumulation by hepatocytes involves interactions between HDL-bound tocopherol and the SR-BI receptor. We conclude further that TTP does not participate in tocopherol uptake from either HDL or LDL.

To better characterize the cellular compartments that are involved in vitamin E uptake, we utilized a novel fluorescent analog of vitamin E, NBD-tocopherol (see Experimental Procedures). The 7-nitrobenz-2-oxa-1,3-diazol-4-yl-substituted tocopherol emits green fluorescence (λ_{max} emission = 530 nm) upon excitation with blue light (λ_{max} excitation = 466 nm). Furthermore, NBD-tocopherol resembles natural vitamin E, in that it binds reversibly and with high affinity to the TTP (K_d = 15 ± 3 nM as compared with 30 ± 4 nM for *RRR*-α-tocopherol; unpublished observations). We utilized NBD-tocopherol in conjunction with confocal fluorescence microscopy to monitor the temporal and spatial fate of tocopherol upon internalization by cultured hepatocytes. NBD-tocopherol was complexed to serum lipoproteins, and incubated with the McA-RH7777-TetOn-TTP cells on microscope slides at 4°C. To visualize the uptake process, cells were then transferred to 37°C, "chased" with plain media for a predetermined period, prepared for microscopy, and observed under a confocal fluorescence microscope. **Figure 3** shows a typical time-course, in which fluorescence from NBD-tocopherol and from Texas red-labeled phalloidin (staining cellular actin structures) were imaged after different incubation times. Following a 5 min incubation at 37°C, NBD-tocopherol was observed in bright fluorescent spots, ~20 nm in diameter, associated with the plasma membrane (Fig. 3). At longer incubation times, NBD fluorescence progressively translocated to the cell interior, reaching a perinuclear compartment after ~30 min (Fig. 3). The morphologic and kinetic characteristics of the internalization process suggest that the mechanism underlying vitamin E internalization involves the endocytic pathway (31).

Intracellular localization of TTP

To determine the intracellular localization of TTP, we employed confocal fluorescence microscopy utilizing monoclonal antibodies directed against either TTP (AT-R1 antibodies) or the amino-terminal hemaglutinin tag (anti-HA antibodies). As shown in **Fig. 4A**, **B**, both antibodies reveal that TTP is expressed in a punctate pattern that resembles vesicular structures surrounding the cell nucleus. To confirm that this intracellular distribution pattern is not an overexpression artifact, we also determined the localization of TTP in freshly isolated primary mouse hepatocytes. As can be seen in Fig. 4C, both the overall intensity and the spatial distribution pattern of endogenous TTP in primary hepatocytes are very similar to the pattern observed in transfected cell lines. To determine the intracellular organelle with which TTP is associated, we used antibody markers for the Golgi compartment (anti-mannosidase II) and the endoplasmic reticulum (anti-calnexin), as well as fluorescent probes for mitochondria (Mitotracker). We found that TTP is not associated with these compartments (data not shown). However, TTP exhibits partial colocal-

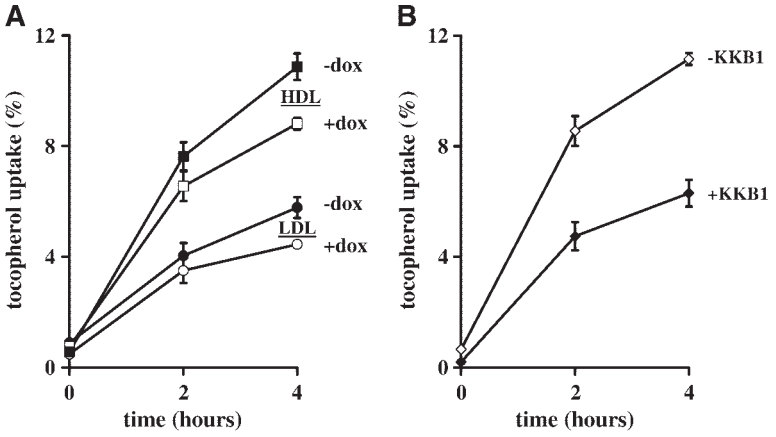


Fig. 2. Participation of HDL versus LDL in tocopherol accumulation by hepatocytes. A: The HepG2-TetOn-TTP cells were cultured in 24-well plates in the presence or absence of doxycycline (dox) (1 μg/ml) to induce TTP expression. After 48 h, [^{14}C]α-*RRR*-α-tocopherol (TOH) complexed to either HDL or LDL (see Experimental Procedures) was added for the indicated duration. At the end of each incubation period, the radioactivity present in the media and in the cells was counted and accumulation was calculated as described in the Experimental Procedures. Shown are averages and standard deviations of triplicate wells. Data are representative of two independent measurements. B: The experiment in A was repeated in the presence of anti-scavenger receptor class B type I (SR-BI) (KKB1) antibodies, to block the activity of the SR-BI HDL receptor.

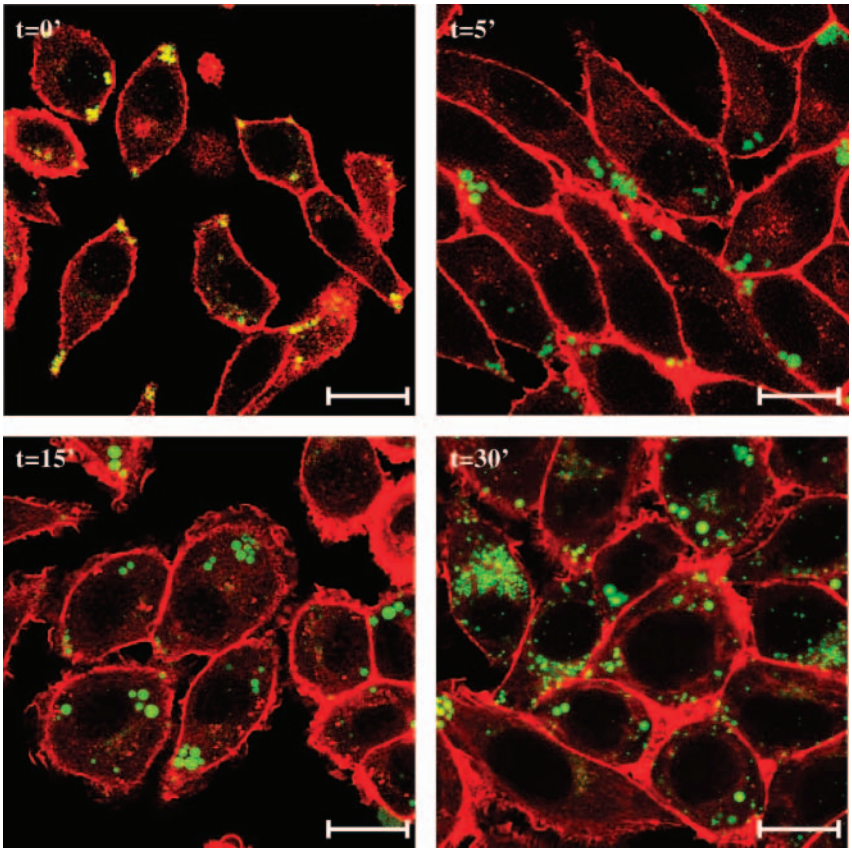


Fig. 3. Uptake of NBD-tocopherol cultured hepatocytes. McA-RH7777-TetOn-TTP cells cultured on microscope chamber slides were incubated with NBD-tocopherol (as serum complex) for 2 h at 4°C. The cells were then incubated at 37°C for the indicated times before processing for fluorescence microscopy. Red: Texas Red phalloidin (actin stain); green: NBD-tocopherol. Bar = 16 μm.

ization with EEA1, a marker for the early endosome (data not shown), and significant colocalization with LAMP-1, a resident protein of the lysosome (Fig. 4D, F). The localization pattern of TTP did not change upon treatment of the cells with vitamin E, nor upon depletion of vitamin E by culturing in delipidated serum for 14 days (data not shown). Importantly, the intracellular localization of TTP strongly overlapped with that of NBD-tocopherol 30 min after internalization (**Fig. 5**). Taken together, these data suggest that vitamin E is internalized into vesicles that end up in the endocytic compartment (endosomes and lysosomes), where TTP is localized.

Functional studies of hepatic TTP-facilitated secretion

Because expression of TTP did not influence the kinetics or the extent of vitamin E accumulation by hepatocytes, the protein must function at a later step, during the delivery of tocopherol from the lysosome to the vitamin E secretory machinery. Indeed, when we determined the subcellular localization of NBD-tocopherol during the secretion period (2–3 h after loading), we observed a striking difference between cells that express TTP and those that do not. As can be see in **Fig. 6A**, in the absence of TTP expression, NBD-tocopherol remains in perinuclear particles for up to 3 h after loading (Fig. 6A, top panels). In TTP-expressing cells, on the other hand, within 3 h, most of the NBD-tocopherol is found associated with the plasma membrane, presumably near its site of secretion (Fig. 6A, bottom panels). Using image analysis, we quantitated the fraction of NBD-tocopherol fluorescence at the cell periphery (i.e., within 2 μm from cortical actin structures), and we present these data in Fig. 6B. We observed that in the absence of TTP, only a small fraction (20–30%) of NBD-tocopherol fluorescence is associated with the plasma membrane. In cells that express TTP, however, NBD-tocopherol is redistributed, and the majority of the vitamin (~90%) is found associated with the cell periphery 3 h after loading (Fig. 6B).

Taken together, these data suggest that TTP functions in the lysosome, possibly by facilitating the intermembrane transfer of vitamin E from endocytic organelles to transport vesicles. To substantiate this notion and to identify possible "players" that participate in the tocopherol secretion pathway, we utilized pharmacological agents that block known steps in intracellular lipid transport. In agreement with previous studies (19), TTP activity is abolished by treatment with chloroquine (**Fig. 7A**), a hydrophobic amine that accumulates in the lysosome and disrupts its function (32). Similarly, we found that TTP activity is abolished by pretreatment of the cells with the synthetic drug U18666A (Fig. 7A), known to disrupt lipid trafficking through the lysosome (33).

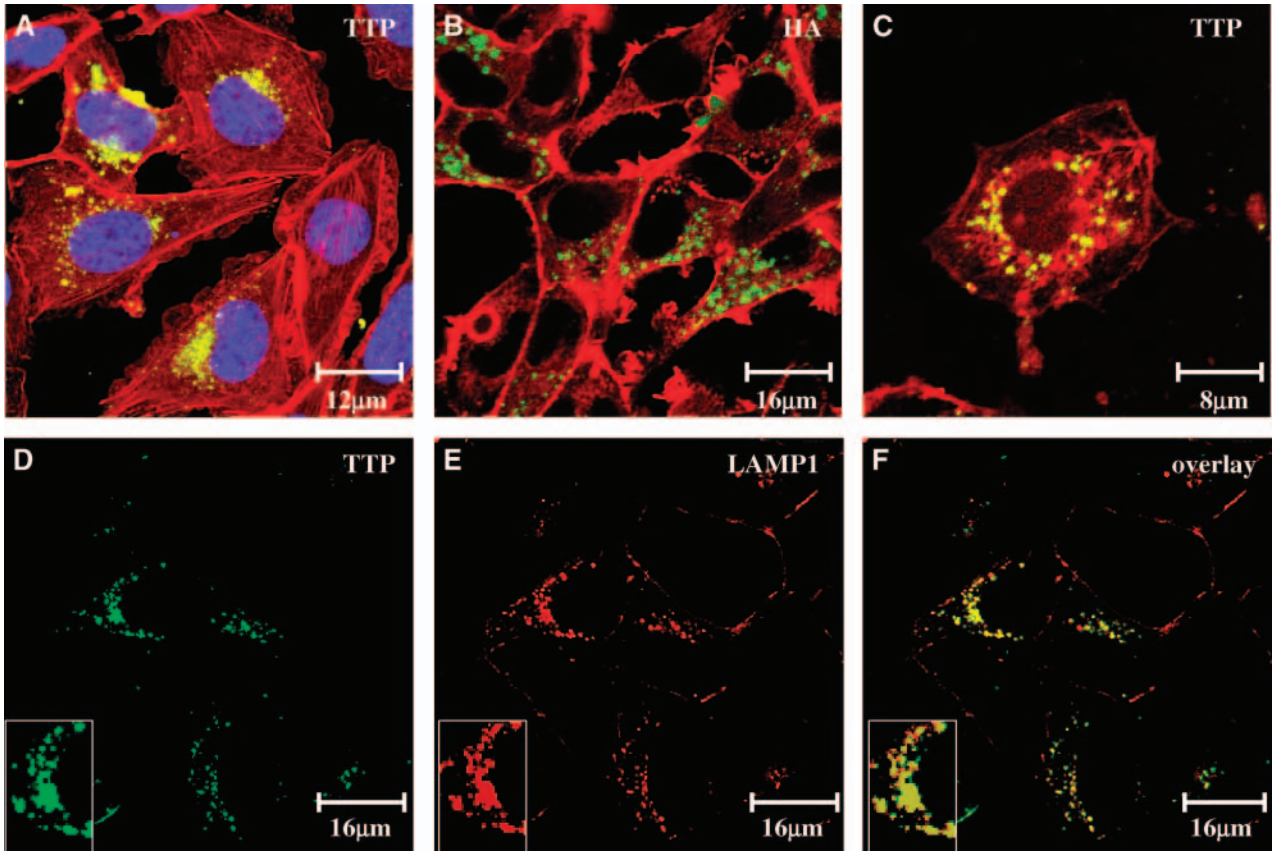


Fig. 4. Intracellular localization of TTP. A–C: McA-RH7777-TetOn-TTP cells treated with doxycycline for 48 h (A, B) or freshly isolated mouse hepatocytes (C) were cultured on microscope chamber slides and prepared for microscopy as detailed in Experimental Procedures. TTP was visualized with either anti-HA or anti-TTP antibodies (green), and the actin cytoskeleton was visualized with Texas Red phalloidin (red). D–F: Colocalization of TTP with the lysosomal marker LAMP1.

Because the transporter ABCA1 was shown to increase vitamin E efflux in some cells (34), it could make an important contribution to TTP activity. We found that treatment of the cells with the ABC inhibitor glyburide completely abolished TTP-mediated tocopherol secretion (Fig. 7A). Similar experiments implicated ABCA1 in the secretion of cholesterol, phospholipids, and apolipoproteins (35). Our data therefore complement and extend studies reported by Oram, Vaughan, and Stocker (34), and implicate ABCA1 in the TTP-dependent secretion of vitamin E in hepatocytes.

Additional evidence in support of a functional interaction

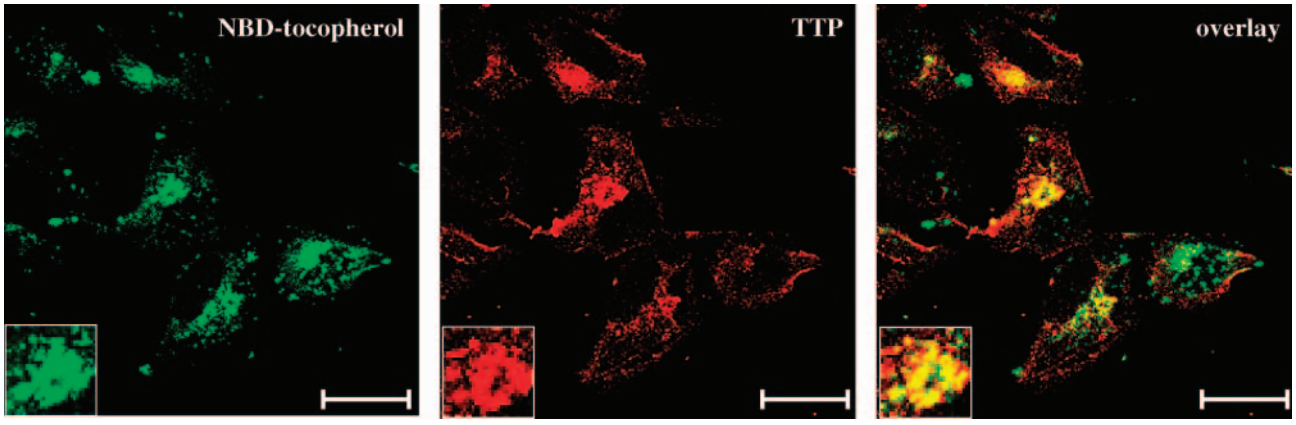


Fig. 5. Colocalization of TTP and NBD-tocopherol. McA-RH7777-TetOn-TTP cells were cultured on microscope slides, and TTP expression was induced for 48 h. NBD-tocopherol was then added to the media as described in Fig. 3. After 30 min at 37°C, cells were prepared for microscopy as described in Experimental Procedures. Green: NBD-tocopherol; red: anti-TTP antibodies (AT-RI). Bar = 16 μm.

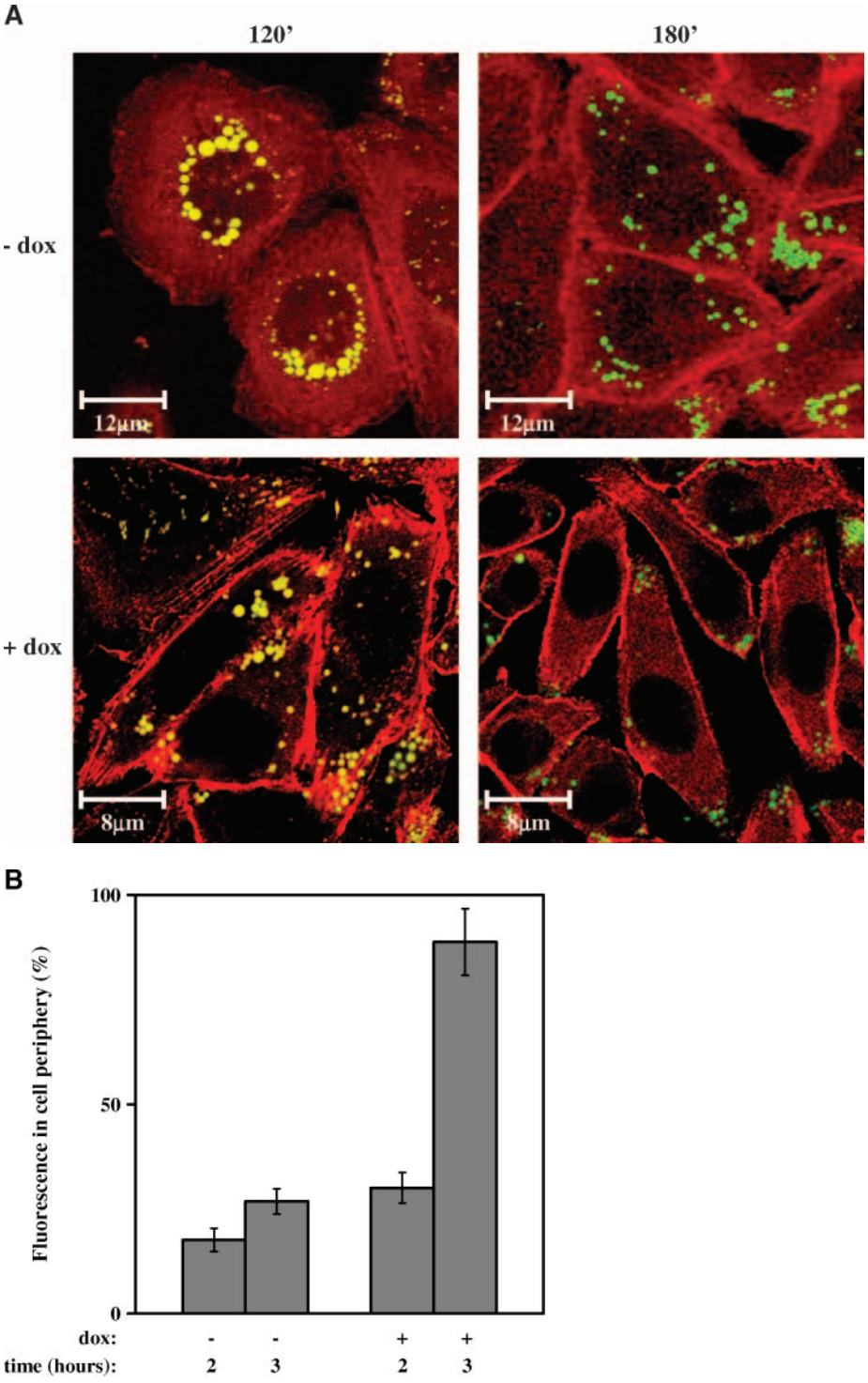


Fig. 6. TTP-dependent translocation of NBD-tocopherol from lysosomes to the plasma membrane. McA-RH7777-TetOn-TTP cells were cultured on microscope slides, TTP expression was induced with doxycycline (dox) where indicated, and NBD-tocopherol was delivered to the cells as described in Fig. 3. After incubation for 45 min at 37°C ("loading"), the cells were washed and incubated with DMEM containing 10% FBS for the indicated times before processing for direct fluorescence. A: Confocal images of NBD-tocopherol fluorescence (green) and actin (red) during secretion phase of the experiment. Bar = 10 μm. B: Quantitation of tocopherol translocation to the plasma membrane. NBD fluorescence within 2 μm cortical actin was quantitated and is shown here as a fraction of total NBD fluorescence in the cell. Shown are averages and standard deviations measured from 40 individual cells for each condition.

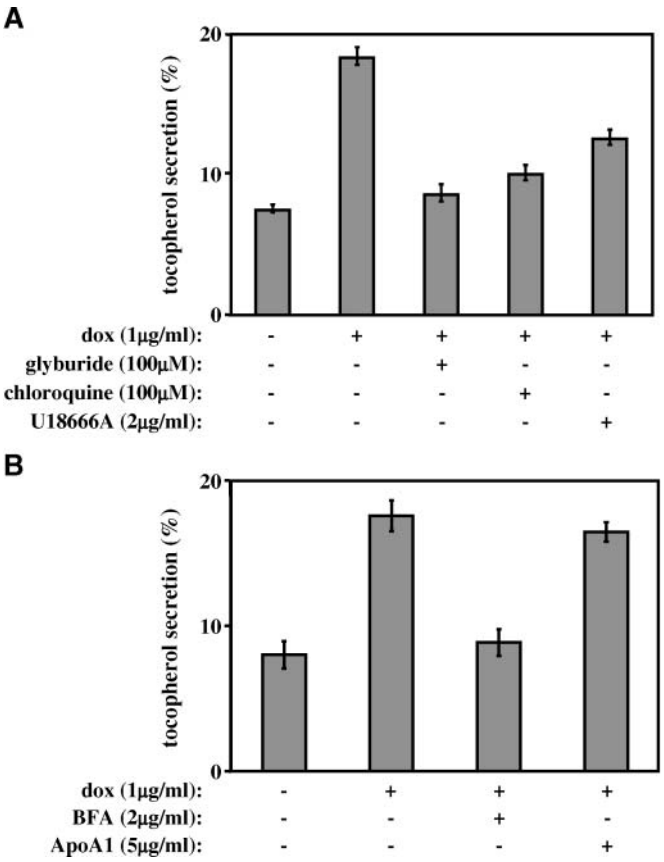


Fig. 7. Modulators of hepatic vitamin E secretion. HepG2-TetOn-TTP cells were cultured in triplicate 24-well plates, TTP expression was induced with doxycycline (dox) where indicated, and the cells were loaded with radiolabeled tocopherol for 36 h. The secretion of labeled tocopherol after 24 h was measured as described in Fig. 1B, in the presence of the indicated treatments.

between TTP and ABCA1 is the observation that apolipoprotein A-I (apoA-I), a direct acceptor for ABCA1-secreted lipids (35), is important for TTP-mediated tocopherol secretion. We observed that when the secretion experiment is done in the absence of serum (Fig. 7B), TTP activity is sensitive to the action of the Golgi inhibitor brefeldin A (BFA), known to disrupt the biosynthesis and export of apoA-I (36). However, TTP activity is not affected by BFA when apoA-I is added to the culture media (Fig. 7B). We interpret these results as supporting evidence for the TTP-dependent transfer of vitamin E to ABCA1, and, in turn, to apoA-I in the media.

DISCUSSION

The importance of vitamin E and TTP to health is evident from the clinical symptoms that accompany mutations in the *ttpA* gene. Humans carrying such mutations display very low circulating tocopherol levels, and varying degrees of neuropathy that lead to ataxia (2, 37). Similarly, mice in which TTP expression is disrupted exhibit diminished plasma and tissue vitamin E levels, and display similar neurological symptoms, in addition to infertility (11, 12, 38). Although these observations clearly implicate TTP as a major mediator of vitamin E action, the exact functions of TTP are still poorly understood. Arai and colleagues demonstrated that stable expression of TTP in McA-RH7777 cells is accompanied by facilitation of tocopherol secretion to the media (18). These authors concluded that TTP somehow regulates the activity of a novel secretory pathway in the liver. However, the molecular mechanisms by which TTP carries out this function are presently unknown.

To better understand TTP's mechanism of action, we sought to characterize each step in the intracellular trafficking of vitamin E in hepatocytes (uptake, intracellular transport, and secretion) and to examine the role that TTP may play in each of these processes. Toward that end, we developed hepatic cell lines from human (HepG2) and rat (McA-RH7777) origins that are stably transfected with the TTP gene under the regulatory action of an inducible promoter. These cell lines displayed an ~3-fold increase in the rate of tocopherol secretion to the media upon induction of TTP expression (Fig. 1).

In human plasma, vitamin E is distributed between LDLs and HDLs (26–28). Hence, tocopherol uptake can occur through either the LDL receptor pathway or the SR-BI scavenger HDL receptor. Here, we show that tocopherol uptake from HDL is more efficient than that from LDL (Fig. 2) in HepG2 cells. Furthermore, we observe that a specific anti-SR-BI antibody markedly inhibits tocopherol accumulation, in a manner similar to its action on cholesterol internalization (22). These results confirm and extend previous reports suggesting that SR-BI-mediated uptake of vitamin E from HDL is physiologically important (21, 29). This notion is also supported by the observations that in vivo, disruption of SR-BI expression causes a reduction in tocopherol levels (39), whereas defects in the LDL receptor do not impact tissue uptake of vitamin E (40). Furthermore, our data demonstrate that TTP is not essential for tocopherol uptake from LDL or HDL in hepatocytes. We therefore conclude that TTP's site of action is at a later step in the intracellular processing of tocopherol.

To gain better insight into the dynamics of vitamin E internalization, we synthesized NBD-tocopherol, a novel vitamin E analog that can be visualized by direct fluorescence microscopy. Using the NBD-labeled tocopherol in conjunction with confocal microscopy, we observe that after a rapid (approximately <5 min) internalization, tocopherol-containing particles translocate into the cell interior, eventually arriving at the late endocytic compartment, where they colocalize with TTP (Figs. 3, 5). The subcellular distribution of the TTP protein, on the other hand, is static, and is not affected by the presence of vitamin E. TTP displayed a distinct, punctate pattern that overlaps with markers of the late endocytic pathway, specifically lysosomes (Fig. 4). Interestingly, lysosomes are the intracellular organelle in which the highest tocopherol concentrations are found (13, 41, 42). In contrast to our observations, it was previously reported that the localization pattern of ec-

topically expressed TTP is diffuse throughout the cells, and shifts to an endosomal punctate pattern only upon treatment with chloroquine or bafilomycin (19). We observe that TTP is *constitutively* associated with the late endocytic compartment, regardless of vitamin E status and in the absence of ionophore treatment (Fig. 4). On the basis of these data, we conclude that TTP has one primary site of action: in vesicular component(s) of the late endocytic pathway, to which newly acquired vitamin E is destined.

Using a novel, fluorescently labeled tocopherol, we were able to visualize NBD-tocopherol arrival in the lysosome following its internalization from serum complexes (Fig. 3). We were then able to observe how NBD-tocopherol translocates from the lysosomes to the cell surface in a TTP-dependent manner (Fig. 5).

We obtained additional insight into the molecular mechanism of action of TTP through the use of pharmacological inhibitors. We find that treatment with lysosomotropic agents interferes with TTP function (Fig. 7A), in agreement with our observations on TTP localization. Glyburide, an inhibitor of ABC transporters, also abrogated TTP-dependent secretion of vitamin E (Fig. 7A), suggesting that TTP functionally cooperates with ABCA1. Finally, we find that in the absence of serum, TTP action is sensitive to BFA treatment, and this sensitivity is lost when apoA-I is added to the media (Fig. 7B). Thus, TTP action requires a soluble lipoprotein that functions as an extracellular acceptor, and this requirement is fulfilled by apoA-I, known to interact with (and be lipidated by) the ABCA1 transporter (43). The involvement of ABCA1 in tocopherol secretion has been previously suggested by the observation that tocopherol efflux is defective in Tangier fibroblasts lacking a functional ABCA1 transporter, and that ectopic expression of the transporter restores this activity (34). Also, mouse models in which expression of the *abca1* gene is disrupted have undetectable levels of tocopherol in plasma (44). Our data extend these observations to tocopherol secretion from hepatocytes, and thus establish ABCA1 as an important component of the specific pathway regulated by TTP.

It has been reported that SR-BI plays an important role in the efflux of sterols to lipid acceptors (22, 45, 46). We examined the role that this activity plays in tocopherol secretion. Indeed, we found that vitamin E efflux is enhanced by incubation of the cells with HDL. Furthermore, HDL-induced efflux was inhibited by anti-SR-BI antibodies. However, TTP-dependent secretion of vitamin E was not affected by these treatments (data not shown). We conclude that although transport of vitamin E to HDL via SR-BI occurs in hepatocytes, this process is not a component of the regulated, TTP-mediated secretion of tocopherol.

The exact molecular function of TTP in tocopherol secretion remains unclear. We hypothesize that newly arrived vitamin E is endocytosed and reaches the lysosome, where the lipoprotein particle is degraded (47). In the lysosome, TTP binds the vitamin, and the geometry of its binding pocket (48, 49) dictates preferential association with the *RRR*-α-tocopherol form. The binding of lysosomal tocopherol to TTP is likely to involve additional factor(s), because TTP is only weakly associated with the lysosome and does not possess a classic lysosomal targeting sequence. TTP then targets tocopherol to transport from the lysosome to the cell surface.

Our observations that TTP is constitutively associated with the lysosome lead us to discount a model in which TTP carries its ligand through the cytosol to the port of exit at the cell surface. Because the major specific *RRR*-α-tocopherol binding activity in liver cytosol is that of TTP (6, 15–17, 50, 51), it is unlikely that another soluble protein "shuttles" *RRR*-α-tocopherol in the cell. Rather, we propose that TTP functions to incorporate vitamin E into transport vesicles that form in the late endocytic compartment and then travel to the plasma membrane. In support of this model is the observation that hepatic secretion of vitamin E was abolished by treatment with colchicine, known to disrupt microtubule function (52).

The sensitivity of tocopherol secretion to glyburide (Fig. 7) strongly suggests the involvement of an ABC-type transporter in the process. Similar pharmacological sensitivity has often been used to specifically implicate ABCA1 (e.g., 53). ABCA1 could contribute to tocopherol secretion in a number of possible ways. The transporter could facilitate tocopherol efflux by directly transporting it across the plasma membrane, as suggested for phospholipids and cholesterol (54). Alternatively, ABCA1 could interact with vitamin E in the cytosol, during its documented recycling between the plasma membrane and endocytic vesicles (55). Finally, ABCA1 could facilitate tocopherol transport indirectly. Recent reports demonstrate that the expression of ABCA1 markedly enhances overall vesicular traffic (44, 56). Future studies will be aimed at deciphering the steps of tocopherol transport after its release from the lysosome, and the exact role of TTP in these events.

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