Insig-dependent Ubiquitination and Degradation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Stimulated by $\delta$- and $\gamma$-Tocotrienols*

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Sterol-regulated ubiquitination marks 3-hydroxy-3-methylglutaryl coenzyme A reductase, a rate-determining enzyme in cholesterol synthesis, for endoplasmic reticulum (ER)-associated degradation by 26 S proteasomes. This degradation, which results from sterol-induced binding of reductase to ER membrane proteins called Insigs, contributes to the complex, multivalent feedback regulation of the enzyme. Degradation of HMG-CoA reductase is also stimulated by various forms of vitamin E, a generic term for $\alpha$, $\beta$, $\delta$, and $\gamma$-tocopherols and tocotrienols, which are primarily recognized for their potent antioxidant activity. Here, we show that $\delta$-tocotrienol stimulates ubiquitination and degradation of reductase and blocks processing of sterol regulatory element-binding proteins (SREBPs), another sterol-regulated action of Insigs. The $\gamma$-tocotrienol analog is more selective in enhancing reductase ubiquitination and degradation than blocking SREBP processing. Other forms of vitamin E neither accelerate reductase degradation nor block SREBP processing. In vitro assays indicate that $\gamma$- and $\delta$-tocotrienol trigger reductase ubiquitination directly and do not require further metabolism for activity. Taken together, these results provide a biochemical mechanism for the hypocholesterolemic effects of vitamin E that have been observed in animals and humans.

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)$^3$ reductase produces mevalonate, an important intermediate in the synthesis of cholesterol and essential nonsterol isoprenoids, which include ubiquinone, dolichol, heme, and the farnesyl and geranylgeranyl groups covalently attached to many cellular proteins (1). HMG-CoA reductase, a resident glycoprotein of the endoplasmic reticulum (ER), consists of two contiguous domains (2): a hydrophobic N-terminal domain consisting of eight membrane-spanning segments that anchors the protein to ER membranes (3); and a large C-terminal domain located in the cytosol contains the enzyme catalytic activity (4). The reductase is one of the most highly regulated enzymes in biology, as evidenced by its tight control through a multivalent regulatory system mediated by mevalonate-derived products (5). Part of this regulatory system involves sterol-regulated ubiquitination (6), which is mediated by the reductase membrane domain and leads to ER-associated degradation of the enzyme (7).

Degradation of reductase requires the action of at least one of two ER membrane proteins called Insig-1 and Insig-2 (7–11). The central event in this process is sterol-stimulated binding of the reductase membrane domain to Insigs, which in turn are bound to a ubiquitin ligase called gp78 (12). The membrane-anchored gp78 mediates ubiquitination of reductase, an obligate reaction for accelerated degradation of the enzyme (13). Nonsterol, mevalonate-derived products cannot stimulate ubiquitination or degradation of reductase in sterol-deprived cells but do augment degradation of the enzyme in cells replete with sterols (14–16). Metabolic evidence indicates that the nonsterol component of reductase degradation can be derived from the 20-carbon isoprenoid geranylgeraniol (GGOH), but not the 15-carbon isoprenoid farnesol (13). It is presently unclear whether the nonsterol potentiator of reductase degradation is GGOH itself or the pyrophosphate derivative, geranylgeranyl pyrophosphate, which may become attached to a specific protein that mediates the effect.

Insigs also play a prominent role in the sterol-dependent retention of SREBP cleavage-activating protein (SCAP) in the ER. SCAP, like reductase, contains a hydrophobic N-terminal domain with eight membrane-spanning regions and a large C-terminal domain located in the cytosol (17–19). SCAP is a sterol-responsive escort protein that associates with SREBPs and facilitates their translocation to the Golgi for sequential proteolytic processing. Proteolysis releases the N-terminal fragments of SREBPs into the cytosol, from which they enter the nucleus to enhance transcription of genes encoding cholesterol biosynthetic enzymes (including reductase) and the low density lipoprotein (LDL)-receptor, which mediates extracellular uptake of cholesterol-rich LDL particles (20). Sterols stimulate binding of Insigs to the sterol-sensing domain of SCAP, a stretch of ~170 amino acids containing five transmembrane domains that resemble the Insig binding site in reductase (18, 21). Insig binding does not trigger rapid degradation of SCAP,
but rather leads to retention of SCAP in the ER, thereby preventing delivery of bound SREBPs to the Golgi for proteolytic activation (22, 23). In the absence of proteolytic activation of SREBPs, rates of cholesterol synthesis and uptake are decreased.

Three classes of sterols are believed to control Insig-mediated reactions (ER retention of SCAP-SREBP and accelerated degradation of reductase): cholesterol, methylated sterols, and oysterols (11). Cholesterol, the bulk end-product of mevalonate metabolism, directly binds to the membrane domain of SCAP, inducing a conformational change in the protein that triggers Insig binding, thereby preventing escape of SCAP-SREBP complexes from the ER (24–26). Lanosterol, the first sterol intermediate in cholesterol synthesis, has no effect on the ER to Golgi transport of SCAP-SREBP, but this methylated sterol potently stimulates Insig-dependent ubiquitination and degradation of reductase (27). Oysterols, derived from the conversion of either endogenous or LDL-derived cholesterol, have dual actions in that they stimulate the acceleration of reductase degradation and promote ER retention of SCAP-SREBP. Thus, selective recognition of sterol ligands by reductase and SCAP may contribute to the ability of Insigs to mediate regulation of both proteins through distinct mechanisms.

Vitamin E is a generic term for eight naturally occurring forms of lipophilic compounds called tocopherols and tocotrienols. Tocopherols and tocotrienols share a polar chromanol ring that is linked to an isoprenoid-derived hydrocarbon side chain (Fig. 1A). Tocopherols carry a saturated phytol group that is derived from homogentisic acid and phytol pyrophosphate, whereas tocotrienols are thought to arise from the condensation of homogentisic acid and geranylgeranyl pyrophosphate (28). Tocopherols and tocotrienols can be subdivided into four isomers (α, β, γ, and δ) with regards to the numbers and position of methyl groups on their chromanol ring (Fig. 1A). Vitamin E is an essential component of the human diet, and various forms of the vitamin are synthesized exclusively by photosynthetic organisms. For example, tocopherols are generally present in common vegetable oils such as soybean, canola, wheat germ, and sunflower, whereas tocotrienols are concentrated (>70%) in cereal grains (i.e. oat, barley, rye, and rice brans), with the richest source found in palm fruits (29). The nutritive value of tocopherols and tocotrienols in food products emanates from their well-known antioxidant capacity, which helps to prevent oxidative damage to polyunsaturated fatty acids (30).

Tocotrienols, but not tocopherols, have been linked to additional beneficial therapeutic properties that include anti-thrombotic and neuroprotective activities and the ability to inhibit proliferation of breast cancer cells and lower serum cholesterol when administered in the diet of chickens, swine, rats, and hypercholesterolemic humans (31–37). Early studies revealed an association between the hypercholesterolemic activity of tocotrienol-rich extracts and decreased levels of hepatic HMG-CoA reductase activity (35). Subsequent structure-activity studies revealed that δ- and γ-tocotrienols were the most potent suppressors of reductase in primary rat hepatocytes and cultured HepG2 cells (39, 40). Importantly, these studies offered evidence for tocotrienols as direct, post-transcriptional suppressors that presumably mimic nonsterol isoprenoids in accelerating reductase degradation (41).

In the current study, we report the unexpected finding that δ- and γ-tocotrienols mimick sterols, rather than nonsterol isoprenoids, in promoting Insig-dependent ubiquitination/degradation of reductase. Furthermore, δ-tocotrienol, but not the γ-form, also effectively blocks cleavage of SREBPs. Other forms of vitamin E, which include all of the tocopherols and α-tocotrienol, have no measurable effect on reductase degradation or SREBP processing. In vitro ubiquitination assays indicate that γ- and δ-tocotrienols are directly recognized by the sterol-sensing system that mediates formation of the reductase-Insig complex and thereby initiates accelerated degradation of reductase. Considered together, these results provide a plausible mechanism for the hypocholesterolemic activity of tocotrienols that has been observed in animals and humans.

**EXPERIMENTAL PROCEDURES**

**Materials**—We obtained MG-132, α-, β-, δ-, γ-tocopherols and tocotrienols from Calbiochem; horseradish peroxidase-conjugated anti-rabbit IgG from Jackson ImmunoResearch Laboratories; FLAG-ubiquitin from Sigma; and ubiquitin-aldehyde and ubiquitin-activating enzyme from Boston Biochem (Cambridge, MA). Other reagents were obtained from previously described sources (22). Lipoprotein-deficient serum (LPDS) (d > 1.215 g/ml) was prepared from newborn calf serum by ultracentrifugation (42).

**Expression Plasmids**—The following expression plasmids were described in the indicated reference: pCMV-Insig-1-Myc, which encodes amino acids 1–277 of human Insig-1 followed by six tandem copies of a c-Myc epitope tag under control of the cytomegalovirus promoter (CMV) (18); and wild-type, K89R/K248R, and YYF to AAAA versions of pCMV-HMG-Red-T7, which encodes the full-length hamster HMG CoA reductase (amino acids 1–887) followed by three tandem copies of the T7-epitope tag under control of a CMV promoter (13).

**Cell Culture**—SV589 cells, an immortalized line of human fibroblasts expressing the SV-40 large T-antigen (43), were maintained in monolayer at 37 °C in 5% CO₂. Stock cultures of SV-589 cells were grown in medium A (Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) fetal calf serum.

Stock cultures of Chinese hamster ovary-K1 (CHO-K1) cells were grown in monolayer at 37 °C in 8–9% CO₂, in medium B (1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 5% fetal calf serum.

**Ubiquitination of HMG-CoA Reductase in Intact Cells**—The conditions of incubations prior to harvesting of cells are described in the figure legends. At the end of the incubations, cells were harvested, lysed in detergent-containing buffer, and immunoprecipitations were carried out with polyclonal antibodies directed against the C-terminal domain of human HMG-CoA reductase as previously described (13, 44). Aliquots of the immunoprecipitates were subjected to 6% SDS-PAGE,
transferred to nylon membranes, and subjected to immunoblot analysis.

Transient Transfection, Cell Fractionation, and Immunoblot Analysis—Transient transfection of CHO-K1 cells with FuGENE-6 transfection reagent (Roche Diagnostics) was performed as described previously (7). Conditions of the incubations are described in the figure legends. At the end of the incubations, triplicate dishes of cells for each variable were harvested and pooled for analysis.

Pooled cell pellets were used to isolate nuclear extracts, 2 × 10⁵ g membrane fractions, or whole cell lysates; all fractions were subjected to 8% SDS-PAGE and immunoblot analysis was carried out as previously described (7). Primary antibodies used for immunoblotting were as follows: mouse monoclonal anti-T7 (IgGαβ, Novagen); mouse monoclonal anti-Myc (IgG fraction) from the culture medium of hybridoma clone 9E10 (American Type Culture Collection); IgG-A9, a mouse monoclonal antibody against the catalytic domain of hamster HMG-CoA reductase (45); IgG-P4D1, a mouse monoclonal antibody against bovine ubiquitin (Santa Cruz Biotechnology); IgG-1D2, a mouse monoclonal antibody against the N terminus of human HMG-CoA reductase inhibitor compactin (47), and a low level of mevalonate (50 μM). The sterol-depleted cells were then treated for an additional 5 h. Then, cells were harvested and subjected to cell fractionation as described under “Experimental Procedures.” Aliquots of the membrane (10 μg protein/lane) and nuclear extract fractions (35 μg protein/lane) were subjected to SDS-PAGE, transferred to nylon membranes, and immunoblot analysis was carried out with 5 μg/ml monoclonal IgG-A9 (against reductase) or 5 μg/ml monoclonal IgG-1D2 (against SREBP-2). Filters were exposed to film at room temperature for 1–60 s.

RESULTS

Fig. 1B shows the results of an experiment that compare the effects of various tocopherols and tocotrienols on the degradation of reductase and the processing of SREBP-2 in SV589 cells (43). The cells were first depleted of sterols by incubation for 16 h in medium containing lipoprotein-deficient serum, the reductase inhibitor compactin (47), and a low level of mevalonate (50 μM). The sterol-depleted cells were then treated for an additional 5 h with 2.5 μM 25-hydroxycholesterol or different concentrations of α-, γ-, or δ-tocopherol and tocotrienol plus 10 mM mevalonate, which allows for production of nonsterol isoprenoids that enhance reductase degradation (13). Following treatments, cells were harvested, subjected to fractionation, and aliquots of the resulting membrane and nuclear extracts were subjected to SDS-PAGE. Immunoblot analysis was subsequently carried out with antibodies against reductase (top panel) and SREBP-2 (bottom panel). As expected, 25-hydroxycholesterol accelerated degradation of reductase, as indicated by the disappearance of the reductase protein, and blocked SREBP-2 processing, as demonstrated by the disappearance of nuclear SREBP-2 (top and bottom panels, compare

FIGURE 1. Specificity of tocopherols and tocotrienols in stimulating HMG-CoA reductase degradation and blocking SREBP-2 processing. A, schematic representation of chemical structures for tocopherols and tocotrienols. B, SV-589 cells were set up on day 0 at 2 × 10⁵ cells per 100-mm dish in medium A containing 10% fetal calf serum. On day 2, the cells were switched to medium A containing 10% LPDS, 50 μM sodium compactin, and 50 μM sodium mevalonate. After 16 h at 37 °C, cells were refed identical medium containing the indicated concentration of 25-hydroxycholesterol or α-, γ-, or δ-tocopherol or tocotrienol plus 10 mM mevalonate and incubated for an additional 5 h. The cells were then harvested and subjected to cell fractionation, as described under “Experimental Procedures.” Aliquots of the membrane (10 μg protein/lane) and nuclear extract fractions (35 μg protein/lane) were subjected to SDS-PAGE, transferred to nylon membranes, and immunoblot analysis was carried out with 5 μg/ml monoclonal IgG-A9 (against reductase) or 5 μg/ml monoclonal IgG-1D2 (against SREBP-2). Filters were exposed to film at room temperature for 1–60 s.
The ubiquitination state of reductase was determined in sterol-deprived cells treated with 25-hydroxycholesterol or the various forms of tocopherols and tocotrienols in the presence of MG-132, which blocks proteasomal degradation of ubiquitinated proteins (Fig. 2B). Following treatments, detergent lysates were prepared and subjected to immunoprecipitation with polyclonal antibodies against reductase. The resulting immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with anti-ubiquitin (top panel) and anti-reductase (bottom panel). In the presence of 25-hydroxycholesterol, γ- or δ-tocotrienol, reductase became ubiquitinated as indicated by the high molecular weight smears of reactivity in the anti-ubiquitin immunoblot (top panel, lanes 2, 7, and 8). In contrast, ubiquitination of reductase was not stimulated by α-, γ-, or δ-tocopherols or α-tocotrienol to an appreciable extent (top panel, lane 3–6).

The Insig requirement for tocotrienol-stimulated degradation and ubiquitination of reductase is demonstrated in the RNA interference (RNAi) experiments of Fig. 3. SV-589 cells were transfected with duplexes of small interfering RNA (siRNA) targeting the control gene vesicular stomatitis virus glycoprotein or the combination of Insig-1 and Insig-2 and depleted of sterols for 16 h. For degradation experiments, cells were subjected to treatments with 25-hydroxycholesterol, α-, γ-, or δ-tocotrienol in the presence of 10 mM mevalonate (Fig. 3A). The combination of 25-hydroxycholesterol and 10 mM mevalonate led to complete degradation of reductase within 5 h in the control-transfected cells (top panel, lane 2). Similarly, γ- or δ-tocotrienol plus 10 mM mevalonate caused reductase to become fully degraded after 5 h (lanes 4 and 5). When siRNAs targeting Insig-1 and Insig-2 were introduced into the cells, the degradation of reductase mediated by 25-hydroxycholesterol or γ- or δ-tocotrienols was abolished (lanes 7, 9, and 10). Expression of a control protein, the transferrin receptor, did not change under any of the experimental conditions (bottom panel, lanes 1–10). Similar results were obtained for reductase ubiquitination (Fig. 3B). In these experiments, sterol-deprived siRNA-transfected cells were treated with MG-132 plus 25-hydroxycholesterol or tocotrienols for 1 h prior to lysis preparation, which was followed by reductase immunoprecipitation and immunoblot analysis. As expected, 25-hydroxycholesterol or γ- and δ-tocotrienols stimulated ubiquitination of reductase in the control cells (top panel, lanes 2, 4, and 5), and this ubiquitination was blocked by RNAi-mediated knockdown of Insigs (lanes 7, 9, and 10).

Fig. 4A shows an experiment in which the Insig requirement for degradation of overexpressed reductase was addressed. For this purpose, CHO-K1 cells were transfected with pCMV-HMG-Red-T7, an expression plasmid that encodes full-length reductase linked to three tandem copies of a T7 epitope tag. Following sterol depletion, cells were incubated in the absence or presence of 10 mM mevalonate plus either 25-hydroxycholesterol, α-, γ-, or δ-tocotrienol. As previously reported (7), overexpressed reductase was not subjected to 25-hydroxycholesterol-stimulated degradation (top panel, lane 2), and similar results were obtained with α-, γ-, and δ-tocotrienols (lanes 3–5). Consistent with the Insig requirement for regulated degradation of endogenous reductase, degradation of the overexp-

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**FIGURE 2.** δ- and γ-Tocotrienols mimic 25-hydroxycholesterol in stimulating degradation and ubiquitination of HMG-CoA reductase. SV-589 cells were transfected with duplexes of small interfering RNA (siRNA) targeting the control gene vesicular stomatitis virus glycoprotein or the combination of Insig-1 and Insig-2 and depleted of sterols for 16 h. For degradation experiments, cells were subjected to treatments with 25-hydroxycholesterol, α-, γ-, or δ-tocotrienol in the presence of 10 mM mevalonate (Fig. 3A). The combination of 25-hydroxycholesterol and 10 mM mevalonate led to complete degradation of reductase within 5 h in the control-transfected cells (top panel, lane 2). Similarly, γ- or δ-tocotrienol plus 10 mM mevalonate caused reductase to become fully degraded after 5 h (lanes 4 and 5). When siRNAs targeting Insig-1 and Insig-2 were introduced into the cells, the degradation of reductase mediated by 25-hydroxycholesterol or γ- or δ-tocotrienols was abolished (lanes 7, 9, and 10). Expression of a control protein, the transferrin receptor, did not change under any of the experimental conditions (bottom panel, lanes 1–10). Similar results were obtained for reductase ubiquitination (Fig. 3B). In these experiments, sterol-deprived siRNA-transfected cells were treated with MG-132 plus 25-hydroxycholesterol or tocotrienols for 1 h prior to lysis preparation, which was followed by reductase immunoprecipitation and immunoblot analysis. As expected, 25-hydroxycholesterol or γ- and δ-tocotrienols stimulated ubiquitination of reductase in the control cells (top panel, lanes 2, 4, and 5), and this ubiquitination was blocked by RNAi-mediated knockdown of Insigs (lanes 7, 9, and 10).

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pressed enzyme was restored upon co-expression of pCMV-Insig-1-Myc, which encodes human Insig-1 linked to six copies of a c-Myc epitope tag (lanes 7, 9, and 10).

Previously, we identified amino acid residues in the reductase membrane domain that when mutated, rendered the enzyme refractory to Insig-mediated, sterol-dependent ubiquitination and degradation (13). Mutation of a YIYF tetrapeptide sequence in the second transmembrane domain of reductase disrupted sterol-regulated binding to Insigs and blocked ubiquitination and degradation of the mutant enzyme. Lysines 89 and 248, located on the cytosolic face adjacent to membrane-spanning segments 3 and 7, respectively, were implicated as sites for sterol-regulated ubiquitination. Mutation of these lysine residues prevented reductase ubiquitination and blocked accelerated degradation. That data of Fig. 4B show that degradation-resistant forms of reductase harboring YIYF to AAAA (Insig-binding) or K89R/K248R (ubiquitation) mutations are resistant to Insig-mediated effects of 25-hydroxycholesterol, γ-, and δ-tocotrienols (upper panel, compare lanes 2–4 with 6–8 and 10–12).

The biochemistry of sterol-regulated ubiquitination of reductase can be examined in a permeabilized cell system (46). In this system, sterol-depleted cells are first permeabilized with digitonin to release >90% of cytosolic proteins into the supernatant upon centrifugation, leaving membrane proteins such as reductase associated with the pellet fraction. The pellet of permeabilized cells support ubiquitination of reductase that is stimulated by additions of 25-hydroxycholesterol and rat liver cytosol (46). In Fig. 5A, permeabilized SV589 cells were subjected to treatments with a mixture of FLAG-tagged ubiquitin, ubiquitin-aldehyde (to inhibit deubiquitinating enzymes) (48), an ATP-regenerating system, and 25-hydroxycholesterol or various concentrations of tocotrienols. The reactions were supplemented with rat liver cytosol to facilitate activation of FLAG-ubiquitin. Following incubation at 37°C, permeabilized cells were collected by centrifugation, lysed, and immunoprecipitated with a reductase antibody. Aliquots of the immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with a FLAG antibody (Fig. 5A, top panel) or a reductase antibody (bottom panel). Approximately equivalent levels of ubiquitinated reductase were generated by 25 μM 25-hydroxycholesterol (top panel, lane b) and 100 μM γ- or δ-tocotrienols.
Tocotrienol-stimulated Degradation of HMG-CoA Reductase

To trigger ubiquitination and subsequent degradation of the enzyme (7). Third, the tocotrienol-dependent ubiquitination was met by uptake of cholesterol-rich LDL particles from the plasma by the LDL receptor. Tocopherols, which contain a saturated phytyl side chain (Fig. 1A), neither accelerated reductase degradation nor lowered blood cholesterol. This finding led to the conclusion that, by virtue of their unsaturated isoprenoid side chains, tocotrienols accelerate reductase degradation by mimicking the action of nonsterol end-products of mevalonate metabolism (41).

The current data provide several lines of evidence that δ- and γ-tocotrienols mimic sterols, rather than nonsterol isoprenoids in stimulating ubiquitination/degradation of reductase. First, a mevalonate-derived nonsterol product (presumably GGOH) combined with either δ- or γ-tocotrienol to maximally stimulate degradation of reductase (Fig. 2A). Second, δ- and γ-tocotrienols effectively promoted ubiquitination of reductase in intact cells. This finding is important in view of our previous studies that indicated the action of nonsterol isoprenoids in reductase degradation did not involve enhanced ubiquitination of the enzyme (7). Third, the tocotrienol-dependent ubiquitination and degradation of endogenous reductase was abolished by RNAi-mediated knockdown of Insigs (Fig. 3). Moreover, tocotrienol-induced degradation of overexpressed reductase required the co-expression of Insig-1 (Fig. 4A). Fourth, mutant forms of reductase that were either defective in Insig binding or lacked the two lysine residues necessary for Insig-mediated ubiquitination of reductase were resistant to tocotrienol-stimulated degradation (Fig. 4B).

Both δ- and γ-tocotrienols stimulated in vitro ubiquitination of reductase in isolated membrane fractions supplemented with ubiquitin-activating enzyme (Fig. 5B). Tocotrienols are known to be metabolized through successive β-oxidation reactions that follow an initial hydroxylation of the ω-methyl group, which is catalyzed by the cytochrome P450 enzymes CYP3A4 and CYP4F2 (54–56). Because the in vitro reductase ubiquitination reactions were not supplemented with NADPH, an essential cofactor for CYP3A4 and CYP4F2 activity, it is unlikely that an intermediate of tocotrienol metabolism triggered reductase ubiquitination. Rather, the results of Fig. 5B indicate that tocotrienols themselves trigger Insig binding and lead to the ubiquitination and subsequent degradation of reductase.
A comparison of the structures of tocotrienols and tocopherols reveal important insights into their regulation of reductase. Tocopherols (α-, β-, δ-, and γ) neither stimulated reductase degradation nor inhibited processing of SREBP-2 (Fig. 1B), results that are consistent with previous reports (41). These findings indicate that the isoprenoid-like phytanyl side chain of tocotrienols is a crucial determinant of activity, and suggest that either 1) the isoprenoid side chain presents the chroman ring for recognition in the reaction that stimulates binding of reductase to Insigs or 2) the isoprenoid side chain is recognized in combination with the chroman ring. Notably, the unsaturated side chain of tocotrienols appears to allow for efficient penetration and better distribution in cell membranes (57), which may contribute to the differences observed in the potency of tocotrienols and tocopherols in stimulating reductase ubiquitination in vitro. Substitution of the chroman ring appears to add an additional level of specificity to the action of tocotrienols (see Fig. 1A). For example, the substitution of a methyl group in the R2 position (α- and β-tocotrienols) abolishes regulatory activity with respect to reductase degradation and SREBP processing. γ-Tocotrienol, which contains a methyl group in R1 position and a hydrogen in R2 position, affects reductase degradation, but does not efficiently block SREBP-2 processing (Fig. 1B). On the other hand, δ-tocotrienol, in which the R1 and R2 positions are substituted with hydrogen, regulates both reductase and SCAP-SREBP (Fig. 1B).

Perhaps the tocotrienol binding pocket in reductase (or an associated protein) is larger than the corresponding region in SCAP (or a SCAP-associated protein) and accommodates the more bulky methyl group of γ-tocotrienol.

The mechanism underlying recognition of δ- and γ-tocotrienols by the same machinery that mediates sterol-stimulated degradation of reductase and ER retention of SCAP-SREBP is currently unknown. Tocotrienols may bring about these reactions through binding to an accessory protein, similar to the recently proposed mechanism for the action of 25-hydroxycholesterol on SCAP (26). Alternatively, tocotrienols may directly bind the sterol-sensing domains of reductase and SCAP, triggering a conformational change in the proteins that allows Insig binding. A similar mode of action has been recently demonstrated for the effect of cholesterol on the activity of SCAP (25). The complete resolution of these issues awaits demonstration of either direct binding of tocotrienols to the sterol-sensing domains of reductase and SCAP, or the identification of an intermediary protein(s) that, upon binding to tocotrienols, associate with SCAP and/or reductase, thereby recruiting Insigs to their respective sterol-sensing domains.

An association between elevated plasma LDL-cholesterol and the development of coronary heart disease in humans is well established (58). Competitive reductase inhibitors called statins have proven useful in lowering blood cholesterol, thereby reducing the incidence of coronary events and prolonging the lives of those with pre-existing coronary heart disease (59–61). The cholesterol-lowering action of statins results from depletion of liver cholesterol, which relieves Insig-mediated inhibition of SCAP-SREBP. As a result, SREBPs are processed to their mature nuclear forms, which in turn, activate transcription of the LDL receptor gene, leading to an increase in LDL receptors that remove LDL from the circulation. At the same time, statins also reduce the mevalonate-derived regulatory products in the liver that normally govern reductase activity through the multivalent feedback system (14). The absence of such regulatory products prompts a buildup of active reductase protein that becomes progressively more difficult to inhibit (62, 63). In theory, a reagent that stimulates degradation of the accumulated reductase without effecting SCAP-SREBP would potentiate the therapeutic effectiveness of statins or, in some cases, provide an alternative therapy.

The current results indicate that γ-tocotrienol possesses these characteristics and provide a mechanistic explanation for the hypcholesterolemic activity of tocotrienols observed in animals and humans. The efficacy of γ-tocotrienol may be limited by a rapid rate of metabolism, which appears to be mediated by the induction of CYP3A4 through the action of the pregnane X receptor (PXR), a nuclear receptor that plays a substantial role in xenobiotic detoxification (64, 65). Notably, the 1,1-bisphosphonate ester SR-12813, which accelerates reductase degradation and lowers blood cholesterol in animals (8, 66, 67), also binds to and activates PXR (38, 55). Thus, understanding the specificity and mechanistic action of γ-tocotrienol on Insig-mediated degradation of reductase and dissociating these effects from those on the activation of PXR may provide insights into the development of new cholesterol-lowering drugs that treat and/or prevent coronary heart disease.

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