γ-Tocotrienol Inhibits TGF-β1-Induced Contractile Phenotype Expression of Human Airway Smooth Muscle Cells

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ABSTRACT

Background Tocotrienols, members of the vitamin E family, exist in four different isoforms (α, β, γ and δ tocotrienol) that have can be protective against brain damage, as well as having anticancer effects *in vivo* and *in vitro*. We have shown that γ-tocotrienol inhibits human airway smooth muscle cell proliferation and migration induced by platelet-derived growth factor (PDGF)-BB by suppressing RhoA activation. In this study, we tested whether γ-tocotrienol modulates transforming growth factor (TGF) -β-induced induction of human airway smooth muscle (ASM) into a contractile phenotype and concomitant synthesis of extracellular matrix proteins.

Methods ASM cells were stimulated with TGF-β1 (2 ng/mL) for 48 hours and the effect of γ-tocotrienol (50 μM) on α-smooth muscle actin, fibronectin and collagen I expression was assessed using Western blotting. The signaling pathways involved in TGF-β1 stimulation were also investigated.

Results TGF-β1 increased α-smooth muscle actin, fibronectin and collagen I abundance by 3- to 5-fold. This response was inhibited significantly by γ-tocotrienol. Furthermore, γ-tocotrienol suppressed RhoA activation, but did not affect Smad2 or Smad3 phosphorylation.

Conclusion These results indicate that γ-tocotrienol has potential for benefit in modulating on airway remodeling in asthma, likely via a mechanism involving the suppression of TGF-β activation of RhoA.

Key words airway smooth muscle; asthma; γ-Tocotrienol; remodeling; vitamin E

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Abbreviations: α-SMA,α-smooth muscle actin; ASM, airway smooth muscle; BSA, bovine serum albumin; DAPI, 4', 6-diamidino-2-phenylindole; EDTA, Ethylenediaminetetraacetic acid; FBS, fetal bovine serum; hTERT, human telomerase reverse transcriptase; Id1, inhibitor of differentiation 1; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; Rho, Ras homolog; TBS, cyto-Tris buffered saline; TGF, transforming growth factor

Airway wall remodeling is one of the characteristic features of asthma. The structural changes in airway remodeling include epithelial cell shedding, basement membrane thickening, submucosal fibrosis and myofibroblast accumulation, airway smooth muscle (ASM) cell hyperplasia and hypertrophy, and adventitial fibrosis.[1] Several inflammatory mediators play important roles in airway remodeling,[2, 3] and among these, transforming growth factor β1 (TGF-β) plays a predominant role through its capacity to induce extracellular matrix protein synthesis and profibrotic differentiation of ASM cells.[4–6] TGF-β1 binds to its dimeric serine/threonine kinase receptor which facilitates phosphorylation of Smad2 and Smad3 to initiate a canonical signaling cascade.[7] Sagara et al. have confirmed an association of TGF-β1/Smad2 signaling with subsequent airway remodeling in asthma.[8]

Current asthma therapies, such as inhaled corticosteroid, β2 agonists, muscarinic receptor antagonists and leukotriene receptor antagonists, have limited effects on airway remodeling.[9] Bronchial thermoplasty is a nonpharmacological procedure that targets ASM bundles.[10, 11] However, a recent study showed that the effects of bronchial thermoplasty on bronchial wall thickening may be transient.[12]

Vitamin E exhibits antioxidant activity and has been evaluated for its effect in asthma since oxidative stress is related to the pathogenesis of inflammatory airway disease.[13] Nonetheless, the therapeutic efficacy of vitamin E in asthma remains equivocal.[14, 15]

In nature, vitamin E exists in eight different isoforms; α, β, γ, and δ tocopherols and tocotrienols. The major component of vitamin E is α-tocopherol, whereas tocotrienols are less abundant. Beyond their antioxidant capabilities, tocotrienols also have pharmacological effects on various cell types. Tocotrienol has been shown to inhibit proliferation of breast, prostate, and lung cancer cell lines.[16] Yap et al. reported that γ-tocotrienol inhibits proliferation of a breast cancer cell line through downregulation of inhibitor of differentiation 1 (Id1),[17] which has been reported to be crucial for differentiation

of fibroblasts.[18, 19]

RhoA signaling is an important regulator of airway remodeling in asthma since activation of the RhoA/Rho kinase pathway is related to airway smooth muscle contraction, proliferation and cell migration.[20] We have reported that γ-tocotrienol inhibits proliferation and migration of ASM cells through inhibition of RhoA activation.[21] Schaafsma et al reported that the synthesis and secretion of extracellular matrix protein is mediated through RhoA activation in ASM cells.[22] Therefore, we considered that γ-tocotrienol could inhibit ASM cellular differentiation and synthesis of extracellular matrix protein.

In this study, we investigated if and how γ-tocotrienol inhibits TGF-β1 induction of a hypertrophic, contractile phenotype in human ASM cells, as well as assessing its impact on TGF-β1-induced biosynthesis of extracellular matrix proteins.

MATERIALS AND METHODS

Human ASM cells and culture conditions

ASM cells immortalized by stable expression of human telomerase reverse transcriptase (hTERT) were a kind gift from Dr. Andrew J. Halayko at the University of Manitoba. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37 ℃ in a humidified 5% CO_2 atmosphere. All experiments were performed in minimum of three different cell lines.

Stimulation of ASM cells with TGF-β1

ASM cells were seeded in a 60 mm dish and allowed to grow to confluence. The cells were then serum starved for 24 h and incubated with 10 to 50 μM γ-tocotrienol for 1 h, followed by stimulation with 2 ng/mL TGF-β1 for the intended duration.

Immunocytochemical analysis

ASM cells were seeded on coverslips and allowed to grow to confluence. The cells were then serum starved for 24 h and stimulated with TGF-β1 in the presence or absence of 50 μM γ-tocotrienol for 48 h. The cells were washed with CB buffer [10 mM 2-(4-Morpholino) ethanesulfonic acid, 150 mM NaCl, 5 mM glucose, and 5 mM $MgCl_2$] and fixed with 3% paraformaldehyde and 3% triton-X for 5 min. After washing with CB buffer, the coverslips were incubated with 5% bovine serum albumin (BSA) for 1 h, and then mouse anti-α-smooth muscle actin antibody (1:100; Sigma, St Louis, Mo) was added to the coverslips and incubated overnight at 4 ℃ in a humidified chamber. After washing with cyto-Tris buffered saline (TBS) with 0.1% Tween 20, the coverslips were incubated with Alexa Fluor 568 conjugated goat anti-mouse IgG antibody (1:50; Molecular Probes, Eugene, OR) for 1 h at room temperature. The nuclei were then stained with 300 nM 4', 6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 2 min. The coverslips were mounted onto slides with anti-fade (Molecular Probes) and evaluated using fluorescence microscopy (Olympus, Tokyo, Japan).

Preparation of cell lysates and Western blotting

Cells were washed with ice-cold phosphate buffered saline (PBS) and lysed in radioimmunoprecipitation assay buffer (RIPA) (50 mM Tris HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors). Equal amounts of protein were separated by electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare Lifescience, Buckinghamshire, UK). After blocking with 5% milk in TBS (20 mM Tris, 150 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, blots were incubated overnight at 4 ℃ with 0.1% TBS-T containing monoclonal anti-α-smooth muscle actin antibody (1:2000; Sigma), monoclonal anti-β-actin antibody (1:10000; Cell Signaling Technology, Danvers, MA), monoclonal anti-fibronectin antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-collagen1A2 antibody (1:1000; Cell Signaling Technology), monoclonal anti-Smad2 antibody (1:1000; Cell Signaling Technology), polyclonal anti-phospho-Smad2, anti-phospho Smad3, and anti-Smad3 antibody (1:1000; Cell Signaling Technology), monoclonal anti-Id1 antibody (1:200; Cell Signaling Technology) were used. After incubation with the primary antibodies, membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; GE Healthcare Lifescience) or anti-mouse IgG (1:3000; GE Healthcare Lifescience) for 1 h at room temperature. Immunoreactive species were visualized on an ImageQuant LAS 4000 mini (GE Healthcare Lifescience) with ECL reagents (GE Healthcare Lifescience) and bands were quantified by densitometry using TotalLab Quant software (Newcastle, UK).

RhoA activation assay

RhoA activity was measured using an Active Rho detection kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's protocol. Samples were separated by electrophoresis and transferred to PVDF membranes (GE Healthcare Lifescience). After blocking

with 5% milk in TBS containing 0.05% Tween 20 for 1 h at room temperature, blots were incubated with TBS-T containing the primary mouse monoclonal antibody for RhoA (1:1000; Santa Cruz Biotechnology) overnight at 4 ℃. The membranes were washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:10000) for 1 h at room temperature. Bands were visualized on an ImageQuant LAS 4000 mini with ECL reagents.

Reagents

γ-Tocotrienol was purchased from Cayman Chemical (Ann Arbor, MI). Y-27632 was purchased from Wako (Osaka, Japan). Simvastatin was purchased from Calbiochem (San Diego, CA).

Statistical Analyses

Data from individual assays are expressed as the mean ± SD. Statistically significant differences were assessed by ANOVA (Bonferroni multiple comparisons test) or Student's *t*-test with *P*-values < 0.05 considered significant.

RESULTS

γ-Tocotrienol inhibits TGF-β1-induced expression of α-smooth muscle actin

Western blotting and densitometry analysis showed that incubation of the ASM cells with TGF-β1 led to significant accumulation of α-SMA (Figs. 1A and 1B). Notably, co-treatment of cells with γ-tocotrienol was sufficient to inhibit TGF-β1-induced α-SMA expres-

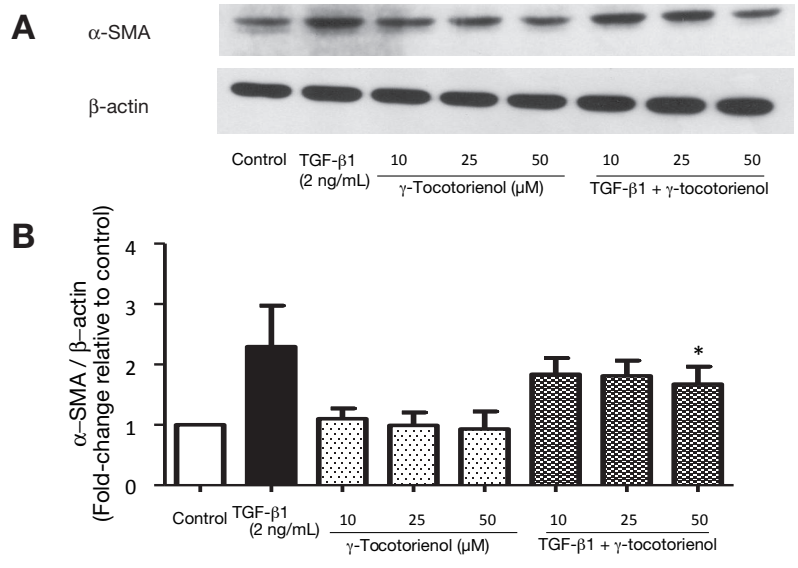
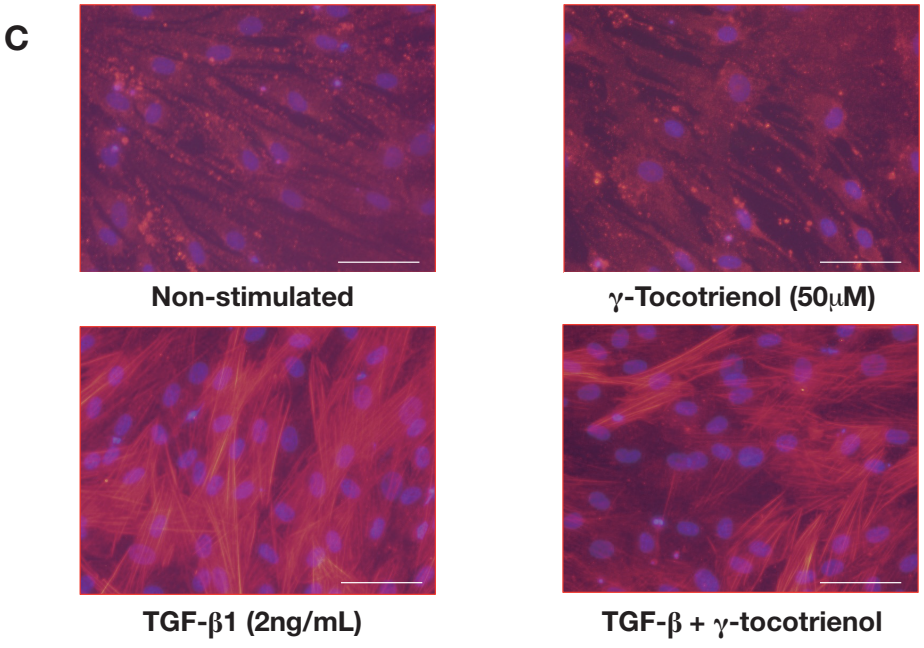


Fig. 1. γ-Tocotrienol inhibits the TGF-β1-induced expression of α-smooth muscle actin. hTERT-ASM cells were pretreated with γ-tocotrienol for 1 h and then stimulated with TGF-β1 (2 ng/mL) for 48 h. Western blotting analysis showed that relative abundance of α-SMA was increased by TGF-β1 and γ-tocotrienol inhibited the TGF-β1-induced expression of α-SMA in a dose-dependent manner. Panel A shows a representative blot and panel B shows the results of densitometry analysis from seven cell lines. Data are expressed as mean value ± SD ($n = 7$). $*P < 0.05$ versus TGF-β1 (**A** and **B**). TGF-β1 enhanced α-SMA expression compared with control. γ-Tocotrienol inhibited the induction of α-SMA expression by TGF-β1. γ-Tocotrienol alone did not affect α-SMA expression (**C**). Bar = 50 μM. α-SMA, α-smooth muscle actin; TGF, transforming growth factor.



sion in a dose-dependent manner ($P < 0.05$) (Fig. 1B). In addition, immunocytochemical staining of the ASM showed that TGF-β1 markedly enhanced the density of α-smooth muscle actin (α-SMA) stress fibers, and though this accumulation was inhibited by γ-tocotrienol, γ-tocotrienol had no obvious impact on α-SMA fibers in the absence of TGF-β1 (Fig. 1C).

γ-Tocotrienol inhibits TGF-β1-induced expression of fibronectin and collagen I

TGF-β1 treatment greatly increased the abundance of fibronectin and collagen I protein (Figs. 2A and B). Induction of these extracellular matrix proteins was significantly inhibited by γ-tocotrienol (Figs. 2C and D), whereas γ-tocotrienol alone had no significant effect on the expression of fibronectin or collagen I.

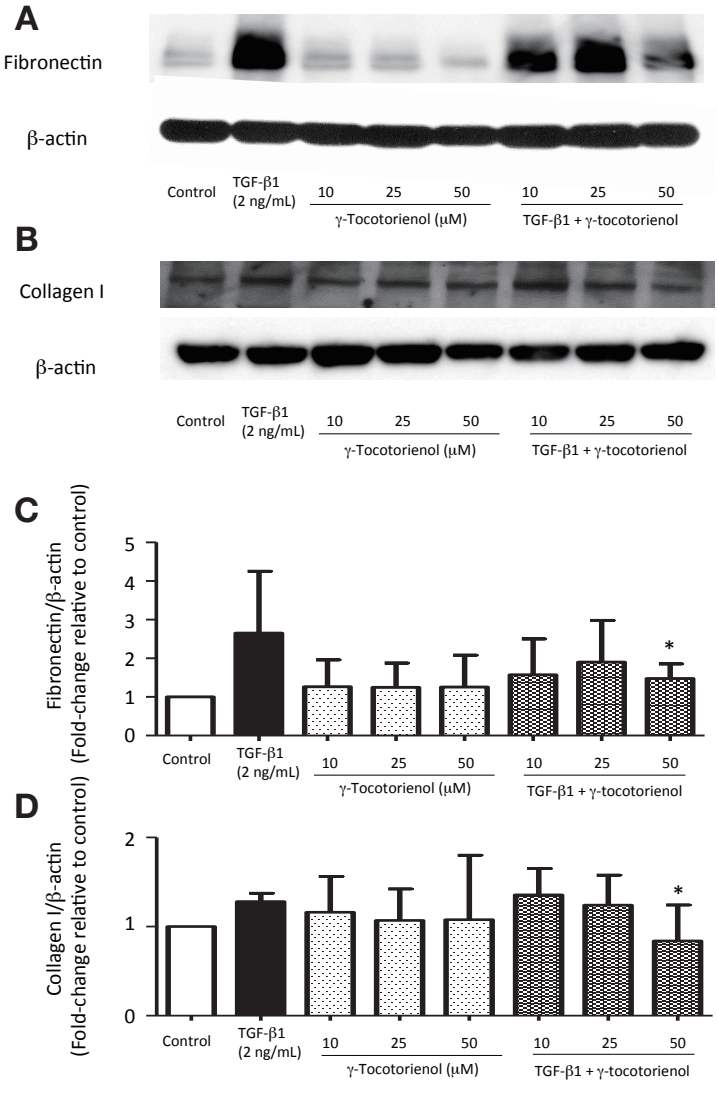


Fig. 2. γ-Tocotrienol inhibits the TGF-β1-induced expression of fibronectin and collagen I.
hTERT-ASM cells were pre-treated with γ-tocotrienol for 1 h and then stimulated with TGF-β1 for 48 h. TGF-β1 increased the abundance of fibronectin and collagen I (**A** and **B**). γ-Tocotrienol significantly attenuated the TGF-β1-induced expression of fibronectin and collagen I (**C** and **D**). γ-Tocotrienol alone did not affect the expression of fibronectin and collagen I (**C** and **D**). Data are expressed as mean ± SD ($n = 8$ for fibronectin, $n = 7$ for collagen I). $*P < 0.05$ versus TGF-β1. TGF, transforming growth factor.

Effects of γ-tocotrienol on TGF-β1-induced cell signaling

As expected, TGF-β1 induced rapid and sustained phosphorylation of Smad2 and Smad3, which peaked 60-120 min after treatment. However, in contrast to effects on TGF-β1-induced α-SMA, fibronectin and collagen I, pretreatment with γ-tocotrienol did not prevent TGF-β1-induced phosphorylation of Smad2 and Smad3 (Fig. 3A and 3B).

γ-Tocotrienol inhibits TGF-β1-induced RhoA activation

We next examined the effects of pre-treatment of ASM cells with γ-tocotrienol on RhoA activation in response to acute exposure to TGF-β1. Notably, TGF-β1-induced activation of RhoA was inhibited significantly by γ-tocotrienol (Fig. 4).

γ-Tocotrienol does not change the TGF-β1-induced increase of Id1 expression

We also examined the effects of TGF-β1 and γ-tocotrienol on expression of the basic HLH transcription factor repressor Id1. After TGF-β1 stimulation Id1 abundance increased markedly, peaking from 3 to 6 h later, then returning to near base-line levels after 12 hours. In the presence of γ-tocotrienol, although TGF-β1 induced an increase in Id1 from 1-3 hours, this was lower than seen with TGF-β1-alone, and returned to baseline within 6 hours of TGF-β1 exposure However, these changes were not statistically significant (Fig. 5).

Effect of the ROCK inhibitor on Id1 expression induced by TGF-β1

Finally, we examined the relationship between RhoA activation and Id1 expression. ASM cells were pretreated with Y27632 (10 μM) or simvastatin (10 μM) for 1 h and then stimulated with TGF-β1 (2 ng/mL) for 3 h. While TGF-β1 significantly increased Id1 expression, this increase was not inhibited by Y27632 or simvastatin (Fig. 6).

DISCUSSION

In this study, we showed that γ-tocotrienol inhibited the TGF-β1-induced differentiation of human ASM cells into a contractile phenotype and the

concomitant synthesis of collagen I and fibronectin. Our results also revealed the signaling pathways that are affected by γ-tocotrienol in TGF-β1–exposed ASM cells, with a suppression of RhoA activation, but with no effects on Id1 expression or Smad phosphorylation. These findings are important because ASM cell differentiation and synthesis of extracellular matrix protein contribute to the airway remodeling observed in asthma. Our data indicate that γ-tocotrienol may prevent airway remodeling in asthma through inactivation of the RhoA/Rho kinase signaling pathway.

In nature, four different isoforms of tocotrienol exist (α, β, γ and δ). From these, we selected γ-tocotrienol since γ-tocotrienol has shown to inhibit breast cancer cells proliferation and ASM cells proliferation and migration.[21, 23] The effects of other isoforms on human ASM cells functions has not been investigated yet, other isoforms might have similar effects on human ASM cells and have beneficial effects on airway remodeling of asthma. Further study is needed to evaluate the effect of α, β and δ tocotrienol.

In this study, we used 10 to 50 μM of γ-tocotrienol as in our previous study.[21] We also confirmed that the maximum concentration of γ-tocotrienol (50 μM) had no cytotoxic effects on human ASM cells (data not shown). However, this concentration of γ-tocotrienol exhibits cytotoxic effect on other cell types.[24, 25] Moreover, clinical application of γ-tocotrienol by oral administration at this concentration might be difficult since serum level of tocotrienol was 0.54 ± 0.45 μM when hypercholesterolemia subjects received 250 mg/day of γ-tocotrienol supplementation by oral administration.[26] Inhalation of γ-tocotrienol might be one of the best delivery methods to reach effective concentration in the human lung.

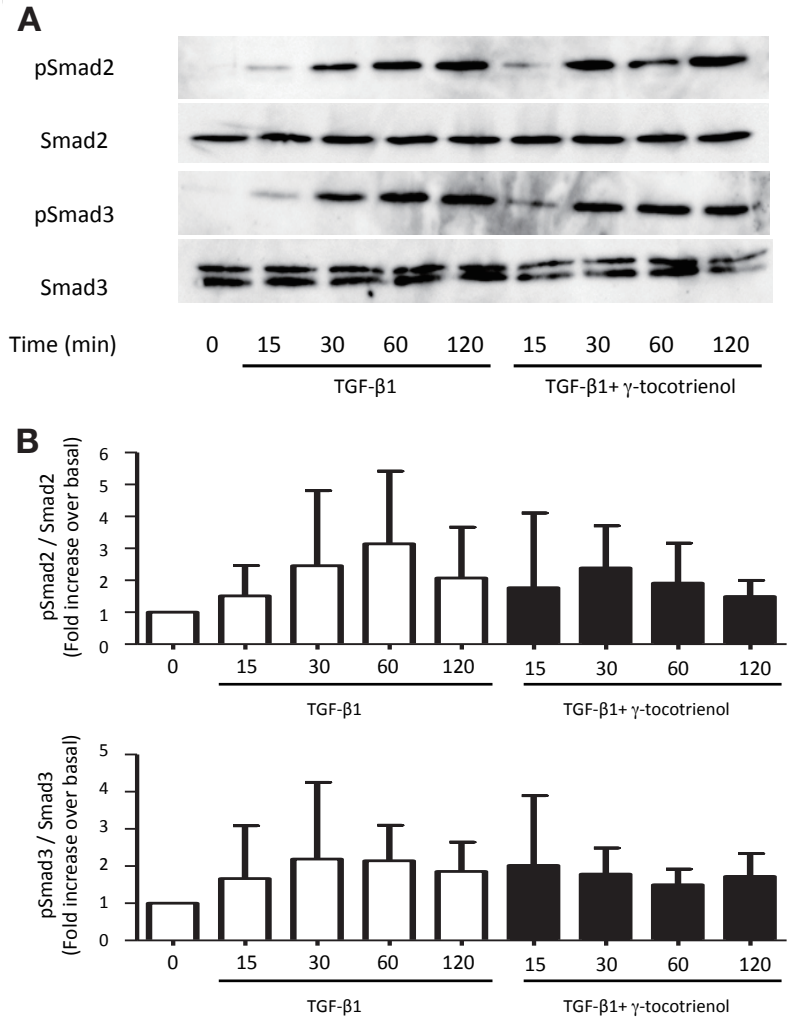


Fig. 3. γ-Tocotrienol does not inhibit TGF-β1-induced phosphorylation of Smad2 and Smad3.
A: Phosphorylation of Smad2 and Smad3 peaked 60 to 120 min after stimulation with TGF-β1 (2 ng/mL). γ-Tocotrienol (50 μM) did not inhibit the TGF-β1-induced phosphorylation of Smad2 and Smad3. **B**: The phosphorylated Smad2 and Smad3 bands were quantified by densitometry and expressed as the fold change, relative to control. Neither phosphorylation of Smad2 nor Smad3 were inhibited by γ-tocotrienol. Data are expressed as mean ± SD (n = 5). TGF, transforming growth factor.

Id1 is a member of the helix-loop-helix proteins that act as negative regulators of DNA-binding of basic helix-loop-helix transcriptional factors.[27] Id1 binds caveolin-1 and induces epithelial-mesenchymal cell transition and migration of prostatic cancer cell lines.[28] Furthermore, caveolin-1 is required for expression of the contractile phenotype.[29] Id1 is a critical mediator of TGF-β1-induced transdifferentiation in a rat hepatic stellate cell line.[30] Furthermore, Je et al. reported that Id1 inhibits TGF-β1-induced collagen expression in a dermal fibroblast cell line.[31] Since Id1 is decreased by γ-tocotrienol in breast cancer cell lines,[17] we considered that downregulation of Id1 was involved in the mechanism underlying the γ-tocotrienol inhibition of the fibrotic phenotype change of ASM cells. However, γ-tocotrienol did not change the expression of Id1 in this study. Therefore, Id1 is not involved in this inhibitory effect of γ-tocotrienol on the profibrotic changes induced by TGF-β1 in ASM cells.

We demonstrated that the TGF-β1-induced activation of RhoA was prevented by γ-tocotrienol. RhoA and its downstream Rho kinase play a key role in ASM cells contraction and airway hyperresponsive-

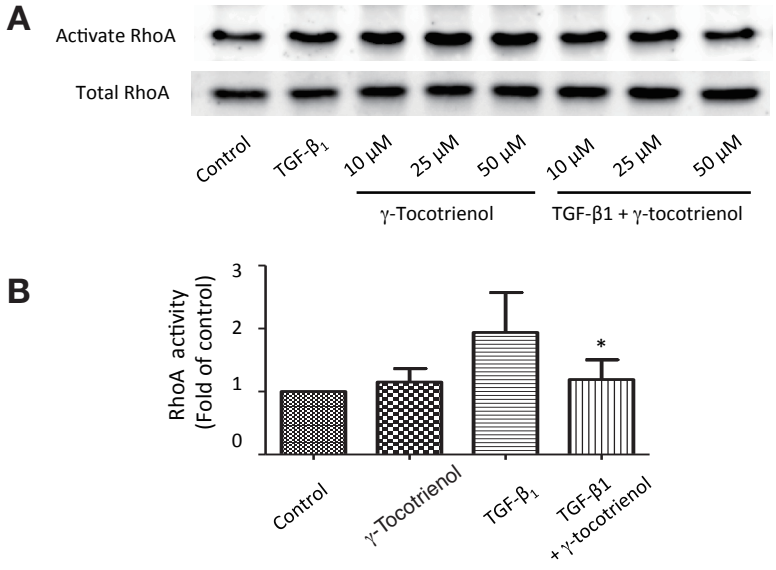


Fig. 4. γ-Tocotrienol inhibits TGF-β-induced activation of RhoA.
A: Effect of γ-tocotrienol on the activation of RhoA induced by TGF-β1 was studied. Rho activation peaked 5 min after TGF-β1 stimulation; this effect was inhibited by γ-tocotrienol (10 to 50 μM). **B**: The activated RhoA bands were quantified by densitometry and expressed as a fold change relative to control unstimulated. The TGF-β1-induced activation of RhoA was inhibited significantly by γ-tocotrienol. Data are expressed as mean ± SD (n = 5). $*P < 0.05$ versus TGF-β1. Rho, Ras homolog; TGF, transforming growth factor.

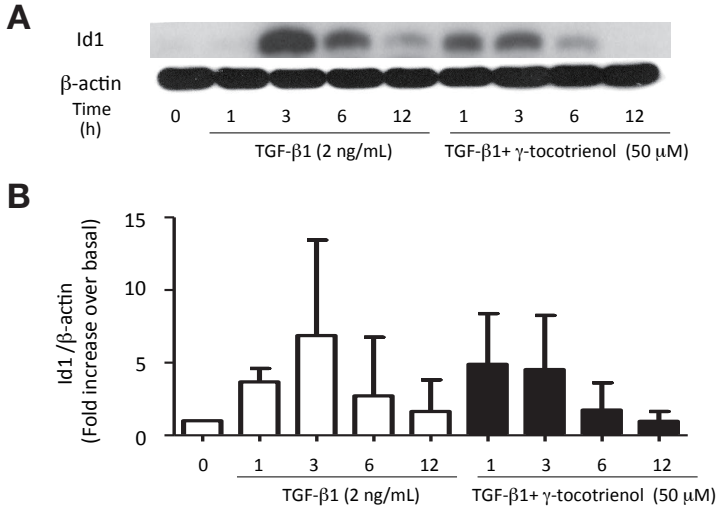


Fig. 5. Effect of γ-tocotrienol on Id1 expression induced by TGF-β1.
hTERT-ASM cells were pretreated with γ-tocotrienol for 1 h and then stimulated with TGF-β1 (2 ng/mL). TGF-β1 increased the expression of Id1 from 3 to 6 h. γ-Tocotrienol did not affect Id1 expression at any of the time points. Panel **A** shows a representative blot and panel **B** shows the results of densitometry analysis from six cell lines. Id1, inhibitor of differentiation; TGF, transforming growth factor.

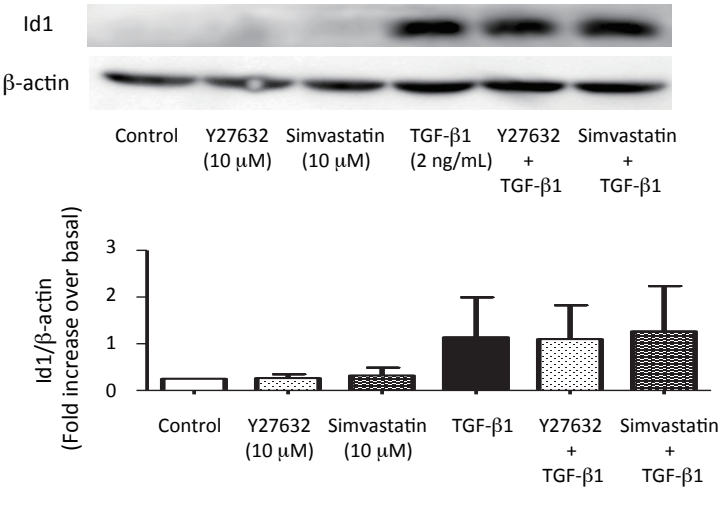


Fig. 6. Simvastatin and the Rho-kinase inhibitor Y-27632 do not inhibit the TGF-β1-induced expression of Id1.
hTERT-ASM cells were pretreated with Y27632 (10 μM) or simvastatin (10 μM) for 1 h and then stimulated with TGF-β1 (2 ng/mL) for 3 h. TGF-β1 increased the expression of Id1, but this was not affected by either Y27632 or simvastatin. Data are expressed as mean ± SD (n = 4). Id, inhibitor of differentiation; TGF, transforming growth factor.

ness.[32] In ASM cells, simvastatin suppresses the TGF-β1-induced expression of collagen I and fibronectin.[22] Schaafsma et al. reported that PDGF induced RhoA activation and ASM cell contraction.[33] We also reported that γ-tocotrienol inhibits the PDGF-BB-induced ASM cells proliferation and migration via inactivation of RhoA.[21] These data suggest RhoA play a key role in ASM cells functions and γ-tocotrienol might inhibit RhoA activation and as a result, inhibit airway remodeling.

Cheung et al. reported that Id1 induced epithelial cell invasion, which was regulated by Rho GTPases.[34] In this study, we demonstrated that inhibition of the RhoA/ROCK pathway by simvastatin or Y27632 did not affect the induction of Id1 by TGF-β1. Our results indicate that γ-tocotrienol directly inhibits RhoA activation without upregulating or downregulating Id1. However, the precise mechanism by which RhoA is inactivated by γ-tocotrienol in the ASM remains unclear. Qureshi et al. reported that tocotrienols lower serum cholesterol,[35, 36] which is mediated by inhibition of HMG-CoA reductase by stimulating the ubiquitination and degradation of reductase and by blocking sterol regulatory element-binding proteins.[37] Future investigations will determine whether these pathways are involved in ASM cells.

We haven't studied the role of reactive oxygen species (ROS) in this study. It has been reported that vitamin E has antioxidant activity and γ-tocotrienol decreased PDGF-BB-induced ROS levels in human airway smooth muscle cells.[21, 38] Since TGF-β1 generates ROS and ROS is related to airway remodeling in asthma and fibrosis in several organs,[13, 39] reduction of ROS might be one of the inhibitory mechanisms of γ-tocotrienol in this study.

The effect of current asthma therapies on airway remodeling in asthma has been investigated in several studies (reviewed in 40). Since the feature of ASM remodeling correlates with asthma severity,[41] therapies targeting ASM are important. Bergeron et al. reported that inhaled corticosteroid (ICS) decrease α-smooth muscle area in peripheral airways. However, ICS did not decrease collagen III or α-smooth muscle area in the central airways nor did it affect the number of TGF-β positive cells in the submucosa.[42] Chakir et al. also reported that oral corticosteroid did not change TGF-β, collagen I and III levels in moderate to severe asthma.[43] Therefore, new drugs targeting the TGF-β1-induced profibrotic changes in airway mesenchymal cells are an unmet need. γ-Tocotrienol exhibits anti-proliferative and inhibitory effects on migration of ASM cells. Furthermore, we have demonstrated the anti-fibrotic effect of γ-tocotrienol in TGF-β1 stimulated ASM cells. Thus, γ-Tocotrienol might be possible therapeutic option targeting airway remodeling in asthma.

In conclusion, we demonstrated the effect of γ-tocotrienol on the TGF-β1-induced differentiation of human ASM cells and extracellular matrix deposition. We also defined the key elements of the downstream signaling cascade inhibited by γ-tocotrienol in the ASM cells activated by TGF-β1. These new findings suggest that γ-tocotrienol could become a therapeutic agent to regulate airway remodeling in asthma.

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The authors declare no conflict of interest.

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