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Vitamin E forms inhibit IL-13/STAT6-induced eotaxin-3 secretion by upregulation of PAR4, an endogenous inhibitor of atypical PKC in human lung epithelial cells

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Abstract

Eotaxin-3 (CCL-26), a potent chemokine for eosinophil recruitment and contributing significantly to the pathogenesis of asthma, is secreted by lung epithelial cells in response to T helper 2 cytokines including interleukin 13 (IL-13). Here we showed that vitamin E forms, but not their metabolites, differentially inhibited IL-13-stimulated generation of eotaxin-3 in human lung epithelial A549 cells. The relative inhibitory potency was γ-tocotrienol (γ-TE) (IC50 ~15 μM) > γ-tocopherol, δ-tocopherol (IC50 ~25-50 μM) > α-tocopherol. Consistent with suppression of eotaxin, γ-TE treatment impaired IL-13-induced phosphorylation of STAT6, the key transcription factor for activation of eotaxin expression, and consequently blocked IL-13 stimulated DNA-binding activity of STAT6. In search of the upstream target of γTE by using inhibitor and siRNA approaches, we discovered that the atypical protein kinase C (aPKC) signaling, instead of classical PKC, p38 MAPK, JNK or ERK, played a critical role in IL-13-stimulated eotaxin generation and STAT6 activation. While showing no obvious effect on aPKC expression or phosphorylation, γ-TE treatment resulted in increased expression of PAR4, an endogenous negative regulator of aPKCs. Importantly, γ-TE treatment led to enhanced formation of aPKC/PAR4 complex that is known to reduce aPKC activity via protein-protein crosstalk. Our study demonstrated that γ-TE inhibited IL-13/STAT6-activated eotaxin secretion via up-regulation of PAR4 expression and enhancement of aPKC-PAR-4 complex formation. These results support the notion that specific vitamin E forms may be useful anti-asthmatic agents.

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Keywords

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Natural forms of vitamin E are eight structurally-related lipophilic antioxidants, which include α-, β-, γ- and δ-tocopherol (α-, β-, γ- and δ-T) and α-, β-, γ-and δ-tocotrienol (α-, β-, γ-and δ-TE). Research on vitamin E including potential protective effects on asthma has mostly focused on αT, the predominant form of vitamin E in tissues. Although severe αT deficiency appears to modulate airway allergic inflammation [1], inconsistent outcomes have been reported in clinical and animal studies regarding the role of α-T supplementation in airway diseases [2-6]. On the other hand, studies by us and others strongly suggest that other forms of vitamin E including γT, the major form of vitamin E in US diet, appear to have unique properties that are import to disease prevention or treatment [7, 8]. We have shown that γ-T, δ-T and γ-TE as well as their metabolites are more potent than α-T in inhibition of cyclooxygenase- and 5-lipoxygenase-catalyzed eicosanoids in cell-based studies and in an inflammation model in rats [9-13]. γ-T is also better than α-T in scavenging reactive nitrogen species and attenuating inflammation-related damage [11, 14, 15]. γT administered by nebulization is shown to improve pulmonary function in sheep with burn and smoke inhalation injury [16]. Recently, we have demonstrated that γ-T supplementation inhibited ovalbumin-induced airway inflammation in an asthma and allergic rhinitis model, respectively, in Brown Norway rats [17, 18]. In these studies, γ-T supplementation led to marked decrease of airway eosinophil infiltration and reduced proinflammatory cytokines [17, 18]. Despite these exciting findings, the mechanism(s) underlying γ-T-exerted inhibition of eosinophilia was not fully understood.

It is well recognized that interleukin-13 (IL-13), a key cytokine secreted by T helper 2 (Th2) lymphocytes, plays critical roles in the pathogenesis of allergic asthma [19]. IL-13 has been shown to regulate eosinophilic inflammation and mucus production, and promote epithelial damage and airway hyper-responsiveness [19-21]. As a central effector cytokine in the lung, IL-13 stimulates lung epithelial cells to release eotaxins-3 (CCL26) and other members of the eotaxin family including CCL11 and CCL24. Eotaxins are potent chemoattractants for eosinophils and cause airway eosinophilia, a hallmark of asthma. Emerging evidence suggests that CCL26 (or eotaxin-3), but not CCL11 or CCL24, likely accounts for eosinophil recruitment to asthmatic airways following allergen challenge in subjects with mild asthma [22]. Based on the observation that γT suppressed eosinophilia in the asthma model in rats [17, 18], we hypothesize that vitamin E forms including γT may modulate the secretion of eotaxin. Here we investigate the effect and mechanism of different forms of vitamin E and their metabolites on IL-13-stimulated eotaxin-3 secretion in human lung epithelial A549 cells.

MATERIALS AND METHODS

Materials

α-T (99%), γ-T (97-99%) and δ-T (97%) were purchased from Sigma (St Louis, MO). 2-(β-Carboxyethyl)-7, 8-dimethyl-6-hydroxychroman (γ-CEHC) was from Cayman Chemical (Ann Arbor, MI). γ-TE was a gift from BASF (Germany). Tissue culture reagents were from Invitrogen (Rockville, MD). Recombinant human IL-13 was purchased from R&D Systems Minneapolis, MN. Human interleukin-4 (IL-4) was from Atlanta Biologicals, Inc (Lawrenceville, GA). Inhibitors for PKCs (Gö-6983), MEK (U0126), p38 MAPK (SB202190), NFkB (Parthenolide), and JAK inhibitor I were from Calbiochem (La Jolla, CA). Highly specific inhibitors for classic PKCs (cPKC) and atypical PKCs (aPKC), i.e., myristoylated cPKC and aPKC pseudosubstrates (for cPKC: N-Myristoyl-Phe-Ala-Arg-Lys-

Gly-Ala-Leu-Arg-Gln-OH; for aPKC: N-Myristoyl-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu-OH), were purchased from Enzo Life Sci (Plymouth Meeting, PA). JNK inhibitor (SP600125) was from Biomol (Plymouth Meeting, PA). [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), inhibitor of JAK/STAT6 (Leflunomide), and all other chemicals were from Sigma (St Louis, MO).

Eotaxin-3 generation by IL-13-stimulated A549 cells

Human lung A549 cells were obtained from American Type Culture Collection (Manassas, VA) and were routinely cultured in RPMI-1640 with 10% fetal bovine serum (FBS). Cells (3×10^5 per well) were seeded and allowed to attach overnight in a 24-well plate. Vitamin E stock solutions were initially made in DMSO and then diluted in 10 mg/mL of fatty acid-free bovine serum albumin. Confluent cells were pre-incubated with vitamin E forms for 14-16 h in DMEM containing 1% FBS and 0.05% DMSO (solvent), and then stimulated by 10 ng/ml of IL-13 for 24 h. Eotaxin-3 accumulation in the media was measured using a Quantikine Human Eotaxin-3/CCL26 Immunoassay kit (R&D Systems, Minneapolis, MN).

Evaluation of cellular dehydrogenase/reductase activity by MTT assays

The cellular metabolic status was evaluated by estimation of dehydrogenase/reductase activity that reduces MTT to form an insoluble purple product which was dissolved in DMSO and measured at 570 nm [23].

Western Blot

Cells were lysed in Tris-EDTA, 1% SDS, 1 mM DTT and 2 mM sodium vanadate with protease inhibitor cocktails (Sigma). The resulting solution was heated at 95°C for 5 min. Proteins (25-50 μg) were loaded on 10% pre-cast SDS-PAGE gels.

Resolved proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA) and probed by antibodies. Membranes were exposed to chemiluminescent reagent (Perkin Elmer, Waltham, WA) and visualized on a Kodak film.

Electrophoretic Mobility Shift Assay (EMSA)

Based on the functional STAT6-binding element in the promoter of human eotaxin-3 [24], biotin 3’ end-labeled DNA duplex of sequence (5’-GATCACAGAAttctctggaaTTGTTT-3’) was synthesized and labeled by Integrated DNA Technologies (IDT, Coralville, IA). EMSA was performed using the LightShift™ Chemiluminescent EMSA kit” (Pierce, Rockford, IL). Briefly, 8 μg of nuclear extracts isolated by NE-PER nuclear kit (Pierce, Rockford, IL) were incubated with 50 ng/μL Poly (dI•dC) and 40 fmol of biotin-labeled probes in 1X-binding buffer at room temperature for 20 min. Samples were separated on a 5% nondenaturing polyacrylamide gel (Bio-Rad, Hercules, CA), semi-transferred and cross-linked to Biodyne® Nylon transfer membranes (Pall Life Sciences, Ann Arbor, MI). The STAT6-probe complex was detected by a chemiluminescent nucleic acid detection module (Pierce, Rockford, IL) according to the manufacturer’s instructions. The STAT6-DNA complex was confirmed by competition experiments with 200-fold excess of unlabeled probes and super-shift with an anti-STAT6 antibody.

siRNA transfection

Human PKCλ/ι (s11110) and PKC ζ siRNA (s11128) were purchased from Ambion Applied Biosystems(Ambion, Carlsbad, CA). Transfection was performed with lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen Carlsbad, CA). Sixty-four hours after transfection, cells were stimulated with IL-13 (10 ng/ml) for 10min, collected and analyzed by Western blotting.

PKC activity

PKCζ kinase activity was measured using HTScan® PKCζ Kinase Assay Kit according to the manufacture's instructions (Cell Signaling, Danvers, MA). Briefly, tested compounds including γTE at 20 μM and 50 μM were incubated with 50 ng of recombinant PKCζ in 25 mM Tris-HCl (pH 7.5) with10 mM $MgCl_2$, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and 2 mM DTT at room temperature for 5 min. The reaction mixture was then added with the substrate peptide (1.5 μM) and ATP (200 μM) and incubated at room temperature 30 min. The reaction was stopped with 50 mM EDTA (pH 8). Phosphorylation of the substrate peptide was quantified by colorimetric ELISA with Phospho-PKA Substrate antibody.

Immunoprecipitation (IP)

Fresh cell pellets were lysed with 100 μL of lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, protease inhibitors and 2 mM sodium vanadate. After lysis, 100 μL of Tris-NaCl buffer was added to dilute NP-40 to 0.5%. Samples were incubated with PAR-4 antibody (Cell Signaling, Danvers, MA) on ice for 1.5 h with vortexing every 30 min and then rocking at 4 °C overnight. Samples were then incubated with 100 μL of Immobilized Protein G Slurry (Pierce, Rockford, IL) by gently rocking at 4°C for 2 h. After being thoroughly washed, the beads were mixed with loading buffer and incubated at 95 °C for 5 min. The supernatant was analyzed by Western blot.

Statistical analysis

One-way ANOVA and post hoc Tukey's multiple comparisons were used in statistical analyses.

RESULTS

Vitamin E forms, but not their metabolites, differentially inhibited IL-13-induced eotaxin-3 secretion in human lung epithelial A549 cells

In the early stage of the study, we found that IL-4 and IL-13, at 2 to 10 ng/ml, similarly stimulated eotaxin-3 secretion in a dose-dependent manner in human lung epithelial A549 cells (Data not shown). Compared with baseline controls in which less than 40 pg/10^6 cells of eotaxin-3 was detected, IL-13 (10 ng/ml) treatment led to a marked increase of eotaxin-3 secretion to 1300-5500 pg/10^6 cells. On the other hand, IL-1β (at 10 ng/ml) failed to cause any significant increase of eotaxin-3, despite being reported to induce eotaxin in a previous study [25]. In the subsequent studies, IL-13 was used to stimulate eotaxin-3.

We observed that vitamin E forms differentially inhibited IL-13-induced eotaxin-3 formation. At 25-50 μM, γ-T and δ-T decreased 35-50% secretion of eotaxin-3, while α-T showed 20-25% inhibition (Figure 1A). γ-TE was the strongest among all the vitamin E forms tested, with an IC50 of ~15 μM (Figure 1B). Besides lipophilic vitamin E forms, we also examined hydrophilic phenolic compounds including γ-CEHC, the terminal metabolite of γ-T, and resveratrol, a polyphenolic compound mainly found in grapes. γ-CEHC at 10 to 50 μM did not show significant effects on IL-13-induced eotaxin-3 (Figure 1C). Resveratrol inhibited 35% of eotaxin-3 production at 10 μM, but its inhibitory effect was not evident at 20 μM (Figure 1C).

Because A549 cells have been shown to metabolize vitamin E forms to long-chain carboxychromanols that are potent inhibitors of cyclooxygenases [12, 26], we examined whether these long-chain metabolites contribute to the inhibitory effect on eotaxin-3. Coincubation of vitamin E forms with sesamin (1 or 5 μM), which inhibits tocopherol hydroxylase-mediated vitamin E metabolism and prevents the accumulation of long-chain metabolites in the cultured media [12], did not significantly affect the inhibitory effect on

eotaxin-3 by γ-T or γ-TE (not shown). This strongly suggests that the un-metabolized vitamins, rather than their metabolites, are responsible for the observed inhibitory effects.

Since previous studies have reported that treatment of certain vitamin E forms impairs A549 cell viability when cells are cultured under sub-confluent conditions [27], it is important to examine whether the tested compounds affect cell viability in this study. Under the current experimental conditions where confluent A549 cells were used, none of these tested compounds had any significant effects on cell morphology based on microscopic examination. MTT assays showed slight decrease (10-15%) of cellular dehydrogenase/reductase activity in the cells treated with γ-T at 50 μM, and γ-TE or resveratrol at 20 μM for 36 hrs or longer.

STAT6, but not NFκB, was responsible for IL-13-stimulated eotaxin formation, and γ-TE inhibited IL-13-induced STAT6 phosphorylation and IL-13-stimulated DNA binding activity of STAT6

Both NFκB and STAT have previously been reported to regulate eotaxin-3 expression in various cell types [24, 25, 28]. Here we found that leflunomide, which has been shown to inhibit JAK-STAT6 signaling [28, 29], but not parthnolide, an inhibitor of NFκB [30], dose-dependently decreased IL-13 stimulated eotaxin secretion (Figure 2A). In addition, JAK inhibitor I that specifically inhibits JAK-STAT also markedly decreased eotaxin (data not shown). These results indicated that JAK-STAT but not NFkB is responsible for IL-13-induced eotaxin formation in A549 cells. Consistently, immunoblotting with anti-phospho-Tyr-641-STAT6 antibody revealed that IL-13 treatment caused a strong induction of STAT6 phosphorylation (Figure 2B), whereas no activation of STAT3 was observed (not shown). Interestingly, γ-TE treatment inhibited IL-13-induced STAT6 phosphorylation (Figure 2B). To test whether γTE affects STAT6 binding to the eotaxin-3 promoter, EMSA experiments were performed. Nuclear extracts were prepared from IL-13 treated cells in the presence or absence of γTE and incubated with biotin-labeled nucleotide probes containing STAT6-binding elements. As shown in Figure 2C, IL-13 induced formation of a STAT6-DNA complex, which was verified by the effective competition with unlabeled STAT6-recognition sequence and supershift with an antibody to STAT6. Importantly, consistent with inhibition of STAT6 phosphorylation and reduction of eotaxin-3, γTE suppressed IL-13-induced DNA binding activity of STAT6.

The atypical Protein kinase C (aPKC) signaling, but not p38 MAPK, MEK1/2, JNK or classic PKCs, was critical to IL-13-triggered STAT6 activation in A549 cells

The inhibition of STAT6 phosphorylation suggests that γTE likely affect upstream signaling(s) for STAT6 activation. Therefore, we searched for potential signaling that regulates both eotaxin formation and STAT6 phosphorylation. p38 MAPK (mitogen-activated protein kinases) and JNK (c-jun N-terminal kinase) signaling pathways have previously been reported to modulate STAT6-mediated gene expression [31, 32]. However, we found that their respective inhibitors, SB202190 (p38) and SP60012 (JNKs), had no effect on STAT6 phosphorylation (Figure 3A). On the other hand, PKC inhibitor Gö-6983, but not ERK inhibitor (U0126), potently inhibited IL-13-induced STAT6 phosphorylation and eotaxin-3 (Figure 3B and 3C), which are similar to γ-TE-caused effects.

Previous studies have demonstrated that the atypical PKCs, i.e., PKCζ and PKCλ/ι, are critically important to the activation of IL-4/STAT6 signaling in T cells [33-35]. Since IL-4 and IL-13 share similar receptors, we determined whether the classic PKCs or aPKCs are necessary for IL-13-triggered STAT6 activation. For this purpose, we used synthetic myristoylated peptides that are designed based on the pseudosubstrate sequence of individual protein kinase Cs. Since there is considerable sequence variability in the

pseudosubstrate domains between classic PKCs (PKCα or β) and aPKC (PKCλ/ι or ζ), these pseudosubstrate peptides serve as highly specific inhibitors for these PKC isoforms [36]. We found that the pseudosubstrate peptide for aPKC, but not its analog inhibitor for the classical PKCs, potently suppressed IL-13-stimulated STAT6 activation (Figure 4A). These data are consistent with the observation that Gö-6983, which inhibits cPKCs at nM and aPKCs at low μM, potently reduced IL-13-induced STAT6 activation only at ~10μM concentrations (Figure 3).

To further confirm the role of aPKCs in STAT6 signaling, we transfected cells with siRNAs for PKCλ/ι and PKC ζ, respectively. Figure 4B showed that down regulation of PKCλ/ι resulted in >80% reduction of aPKC levels detected with an antibody that presumably recognizes both aPKC isoforms, whereas downregulation of PKCζ only moderately reduced aPKC levels. These observations strongly suggest that compared with PKCζ, PKCλ/ι is predominantly expressed in A549 cells, which was consistent with the reports by Regala *et al.* [37]. This explains the relatively modest reduction of PKCζ in response to the siRNA treatment. Downregulation of either isoform led to decrease of STAT6 phosphorylation. Interestingly, PKCλ/ι downregulation caused marked reduction of STAT6 protein levels, which was similarly observed when cells were treated with Gö-6983 and pseudosubstrate peptide of aPKC (Fig 3 and Fig 4A). These siRNA experiments together with inhibitor studies demonstrate that aPKCs play critical roles in STAT6 phosphorylation and protein status in A549 cells.

γ-TE treatment enhanced PAR4 expression and PAR4/aPKC interaction

Given that the aPKCs appear to be critical to IL-13-induced STAT6 activation, we next examined whether γ-TE has any effect on aPKC-mediated signaling. In a cell-free assay, γ-TE did not show any effect on the aPKC activity (data not shown). Immunoblotting results indicated that aPKC expression or its phosphorylation was not affected by γ-TE or IL-13 treatment (Figure 5A).

Besides phosphorylation, aPKC activity is known to be regulated via protein-protein crosstalk [38]. For instance, it has been demonstrated that aPKC signaling is negatively regulated by PAR4 (prostate-apoptosis-response 4) in various cell types, where PAR4 selectively binds to aPKCs and inhibits their enzymatic activity [39]. Consistently, PAR-4 knockout resulted in enhanced PKCζ-mediated IL-4/STAT6 activation and exacerbated concavalalin A-induced liver damage [33]. Based on these studies, we examined potential effect of γ-TE on PAR-4 expression and PAR4-aPKC (PKCζ or PKCι/λ) interactions. Immunoblotting data showed that γ-TE treatment caused an increase of PAR4 expression (Figure 5B). To study the effect on PAR4-PKC interaction, cell lysates from vehicle- or γ-TE-treated cells were immunoprecipitated with Par4 antibody, and the immunoprecipitants were resolved by SDS-PAGE and analyzed by immunoblotting with an antibody for aPKCs (PKCζ and PKCλ/ι). An immunoreactive band corresponding to aPKCs was clearly stronger in the immunoprecipitants from cells treated by γ-TE than those from DMSO-treated controls (Figure 5C). This result indicates a stronger interaction between PKCζ/PKCλ/ι and PAR4 in γ-TE treated cells than vehicle-treated controls, which has been shown to cause impairment of aPKC signaling [39].

DISCUSSION

In the present study, we demonstrated that vitamin E forms but not their metabolites dose-dependently inhibited IL-13-induced eotaxin-3 secretion, and γ-TE was the most effective among the tested vitamin E forms. γ-TE treatment led to inhibition of IL-13-stimulated STAT6 phosphorylation and STAT6 DNA binding acitivity, which are critical to activation of eotaxin expression. Further mechanistic studies revealed that the aPKCs rather than

classic PKCs or other signaling is pivotal to IL-13-stimulated STAT6 activation in A549 cells. Interestingly, γ-TE treatment resulted in up-regulation of PAR4 expression, which has been shown to interact with atypical PKCs and consequently inhibits aPKC-mediated signaling [39]. Consistently, the immunoprecipitation results indicated that γ-TE treatment resulted in enhanced interaction between PAR4 and aPKC. Our study provides the first evidence that vitamin E forms suppressed IL-13/STAT6 activation by induction of PAR4 expression and enhancement of aPKC-PAR4 interaction that negatively regulates aPKCs (Figure 6) [39].

It is intriguing that previous studies have indicated that the atypical PKCs including PKCζ and PKCλ/ι play key roles in modulation of airway inflammation and may therefore be therapeutic targets for asthma [34, 35]. For instance, the expression of PKCζ increases during Th2 but not Th1 differentiation of CD4+ T cells. The loss of PKCζ blocks IL-4/STAT6 activation and decreases Th2 cytokine secretion. As a result, PKCζ-/- mice displayed profound inhibition of ovalbumin-induced allergic airway disease [34]. Recently, it is reported that PKCλ/ι knockout mice have impaired activation of NFκB in Th2 cells and dampened airway inflammation [35]. In the current study, we showed that both aPKC isoforms play important roles in IL-13-stimulated STAT6 activation and downregulation of PKCλ/ι appears to have profound effects on STAT6 protein status in human lung epithelial cells.

The atypical PKCs belong to a subgroup of the protein kinase C family. Unlike classic PKC isoforms, the aPKCs cannot be regulated by calcium, phorbol esters or diacylglycerol [40, 41]. Instead, the activity of aPKCs is shown to be controlled via their interaction with regulatory proteins. PAR4 appears to be an endogenous inhibitor for PKC activity by interaction with the zinc finger domain of PKCζ and PKCλ/ι [39]. Although PAR4 was initially discovered in prostate cells undergoing apoptosis [42], recent studies have revealed that PAR4 is generally expressed in many tissues including the lung [43]. Because of its regulation of aPKC-mediated signaling, PAR4 is believed to be able to modulate inflammatory responses [38, 44]. Thus, the loss of PAR4 is found to enhance PKCζ-mediated activation of IL-4/STAT6, and as a result, PAR4 knockout exacerbates hepatitis induced by Concanavalin A [33]. Our present study demonstrated that aPKCs are critical to IL-13-stimulated STAT6 phosphorylation in lung epithelial A549 cells, which mimics the regulatory role of PKCζ in IL-4/STAT6 activation in Th2 cells [34]. γ-TE treatment led to up-regulation of PAR4 and increased PAR4/aPKC crosstalk, which consistently correlates with impaired activation of IL-13/STAT6.

γ-TE has previously been shown to suppress NFκB activation via inhibition of IκBα phosphorylation in various cell types [45]. Because aPKC and PAR4 have been implicated in regulation of NFκB [38, 46], we investigated whether γ-TE modulates TNF-α-stimulated NFκB activation in A549 cells. However, γ-TE at 20 or 50 μM did not show any effects on TNFα-induced IκBα phosphorylation in confluent cells (Y Wang, unpublished observations). This is consistent with our previous observation that γ-TE does not have any effect on NFκB-upregulated cyclooxygenase-2 expression induced by IL-1β in A549 cells [12]. In addition, Regala *et al* [37] have demonstrated that knock down aPKC has no impact on TNF-α induced NFκB activation in A549 cells. Therefore, aPKC-regulated NFκB activation and γ-TE's inhibition of NFκB are likely cell-type dependent.

The present study was initially inspired by our recent observations that γ-T supplementation attenuated ovalbumin-stimulated eosinophilic infiltration in bronchoalveolar lavage fluid in Brown Norway rats [17, 18]. Because eotaxin is secreted by lung epithelium in response to IL-13 or IL-4 stimulation and its only known function is to recruit eosinophils to the airway, our current observation that vitamin E forms dose-dependently inhibited IL-13-activated

eotaxin secretion provides a molecular basis for the observed *in vivo* anti-asthmatic activity of γ-T [17, 18]. It is noteworthy that the IC50s of γ-T, δ-T and γ-TE for inhibition of IL-13-induced eotaxin secretion are pharmacologically achievable, as these concentrations have been reported in rodents and humans shortly after supplementation with these vitamin E forms [47-49]. Since compared with γT, δ-T and γ-TE are similarly or more effective in inhibition of eotaxin secretion (this study) and suppression of cyclooxygenase- and 5-lipoxygenase-mediated proinflammatory eicosanoids [12, 13], δ-T and γ-TE may have comparable or even stronger anti-asthmatic activity than γ-T, which warrants further investigation. In addition, future studies are necessary to elucidate the signaling that is responsible for PAR4 induction, and the role of vitamin E forms in modulating that signaling pathway(s).

Acknowledgments

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ABBREVIATION

α-T, β-T, γ-T, or δ-T	α, β, γ, or δ-tocopherol
α-, β-, γ-and δ-TE	α-, β-, γ-and δ-tocotrienol
CEHC	7,8-dimethyl 2-(β-carboxyethyl)-6- hydroxychroman
aPKC	the atypical protein kinase C
cPKC	classic protein kinase C
IL-13	interleukin-13

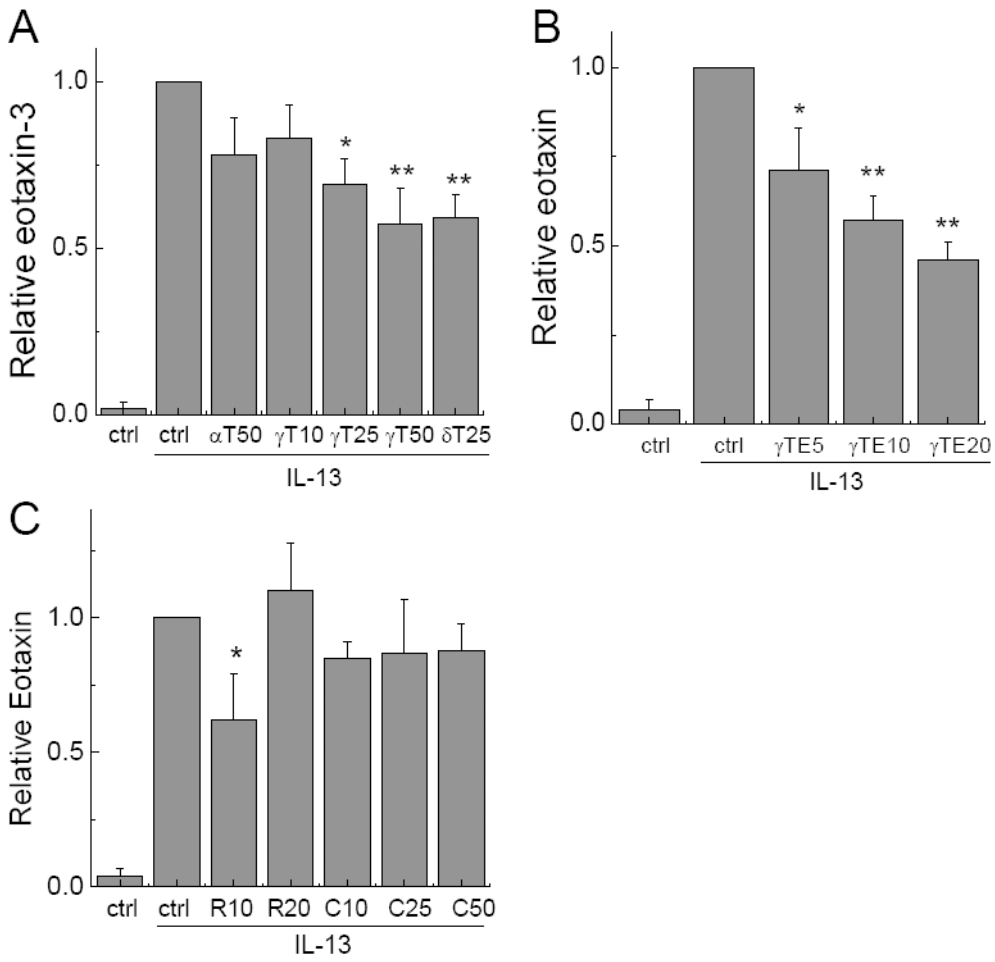

Figure 1. Effects of vitamin E forms, γ-CEHC and resveratrol on IL-13-induced eotaxin-3 secretion

After preincubated with tocopherols (**Panel A**) and γTE (**Panel B**) for 14-18 h, or γ-CEHC/resveratrol (**Panel C**) for 1 h, A549 cells were activated by IL-13 (10 ng/mL) for 24 h and media were collected to measure eotaxin-3 secretion. Results are the ratio of eotaxin secreted by cells treated with tested compounds to that of solvent controls. αT50, γT10, γT25, γT50, δT25, γTE5, γTE10 and γTE20 stand for the corresponding vitamin E forms at indicated concentrations (μM). R10 and R20 are resveratrol at 10 and 20 μM, respectively. C10, C25 and C50 are γ-CEHC at indicated concentrations (μM). Data are shown as mean values ± SD. *P<0.05 and **P<0.01 are significant difference between the treated and control cells. n >2 per bar.

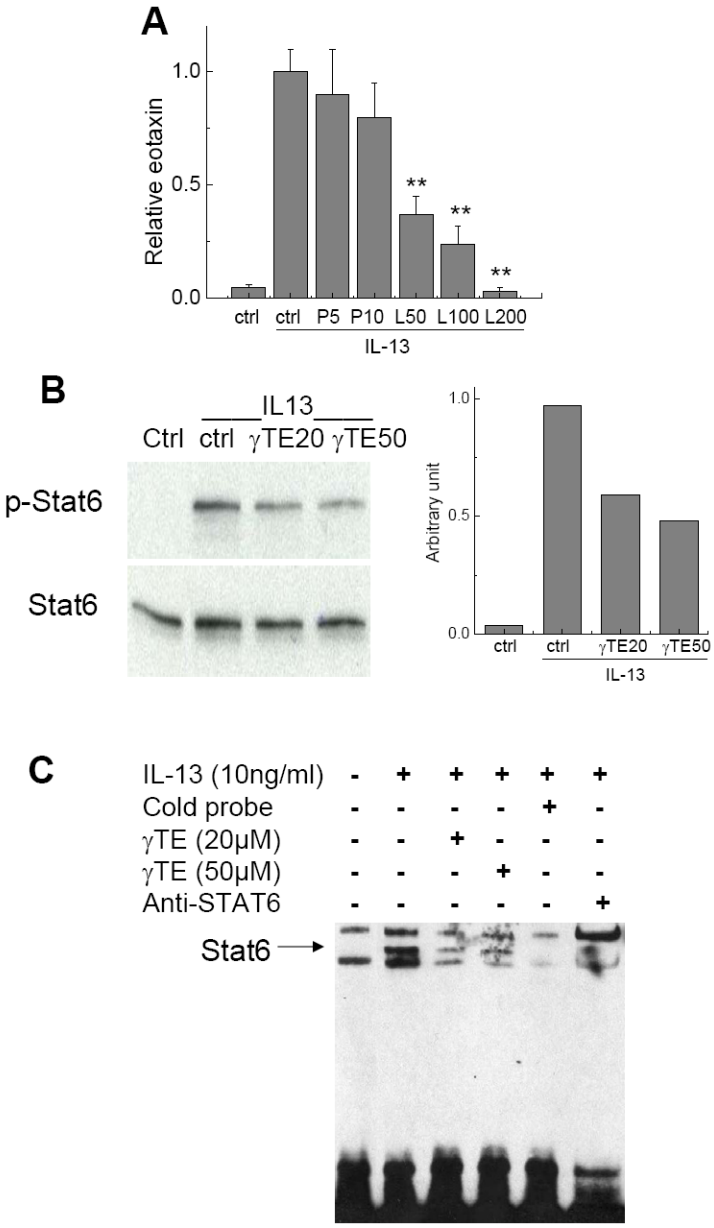

Figure 2. Panel A - The inhibitor for JAK-Stat6 (Leflunomide, LF) but not NFkB (Parthnolide, P) inhibits IL-13-induced eotaxin secretion

A549 cells were preincubated with LF and P at indicated concentrations (μM) for 1 h and stimulated by IL-13 for 24 h. Eotaxin formation was measured by ELISA. Data are shown as mean values ± SD. *P<0.05 and **P<0.01 are difference between treated and control cells. n >2 per bar. **Panel B -γ-TE treatment partially impaired IL-13-stimulated STAT6 phosphorylation**. Cells were preincubated with γ-TE at indicated concentrations (μM) or DMSO (0.05%) for 14-16 h, and stimulated with IL-13 (10ng/ml) for 20 min. Immunoblotting was performed with anti-phosphor-Tyr-641-STAT6 or anti-STAT6 and the densitometry was shown (right). **Panel C** – γ-TE inhibited IL-13 stimulated STAT6 DNA binding activity. Cells were pretreatment with γTE for 18h and stimulation with IL-13 for 30 min. Nuclear extracts were isolated and EMSA was conducted as described in Methods and Materials.

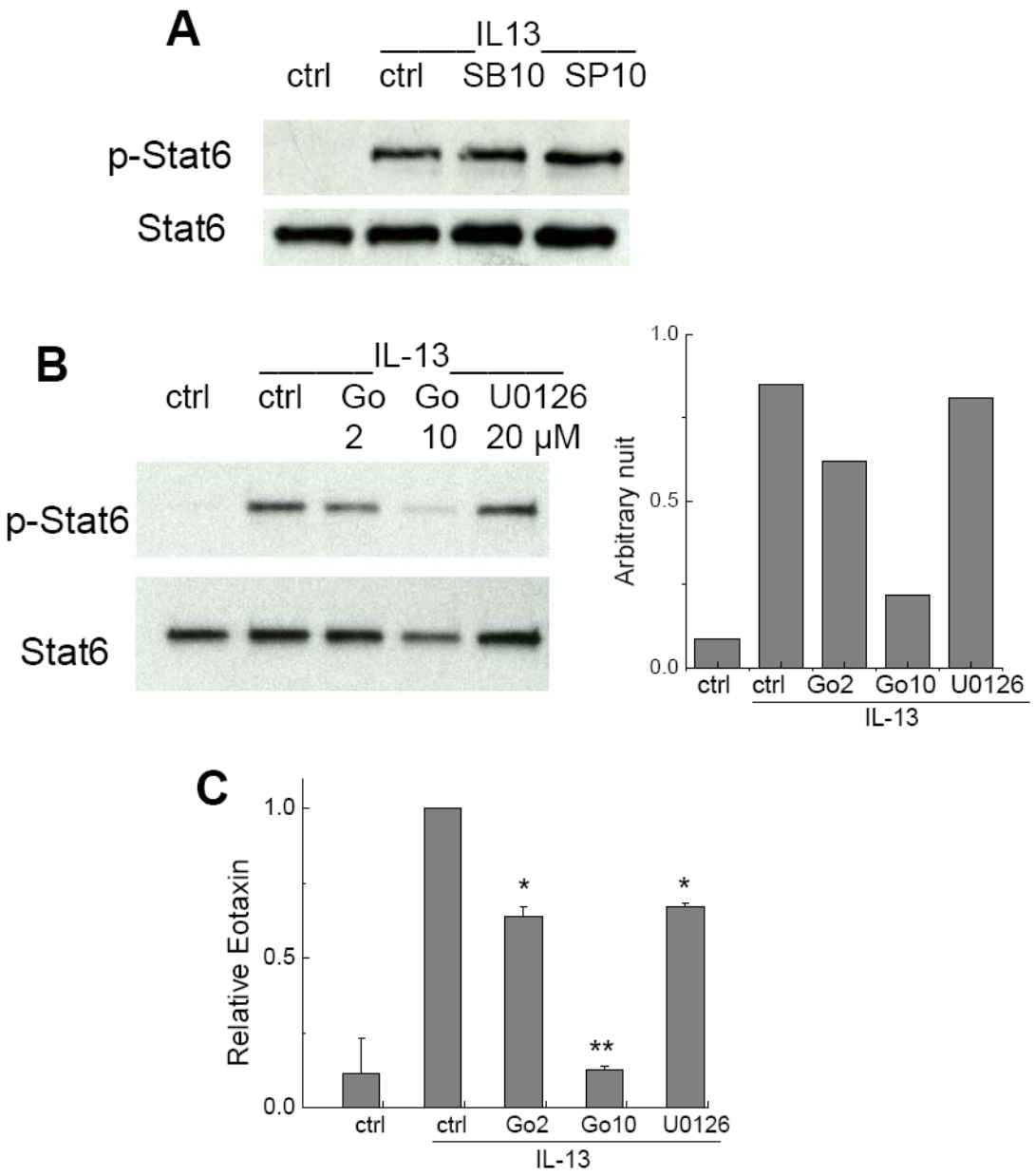

Figure 3. Panel A: Inhibitors for p38 (SB) or JNK (SP) had no effect on IL-13-stimulated STAT6 activation

A549 cells were pre-incubated with SB or SP (10 μM) for 1 h, and activated by IL-13 (10 ng/mL) for 10 min. Western blotting was conducted to measure STAT6 phosphorylation. **Panel B: Inhibition of PKC but not MEK resulted in blocking IL-13-stimulated STAT6 phosphorylation**. A549 cells were preincubated with Gö-6983 (Go, a PKC inhibitor) at 2 or 10 μM, or U0126 (a MEK inhibitor) at 10 μM or vehicle (ctrl) for 1 h, and stimulated by IL-13 for 10 min. Western blotting was conducted to assess STAT6 phosphorylation and its densitometry data were shown (right). **Panel C: Inhibitory effects of inhibitors for PKC (Go) and MEK (U0126) on IL-13-induced eotaxin secretion**. Gö-6983 at 2 or 10 μM and U0126 at 10 μM were preincubated with A549 cells for 1 h. Cells were then treated by IL-13 (10 ng/mL) for 24 h and eotaxin secretion into the media was measured. Data are

shown as mean values ± SD, *P<0.05, **P<0.01: difference between treated and control cells. n=2 or more per bar.

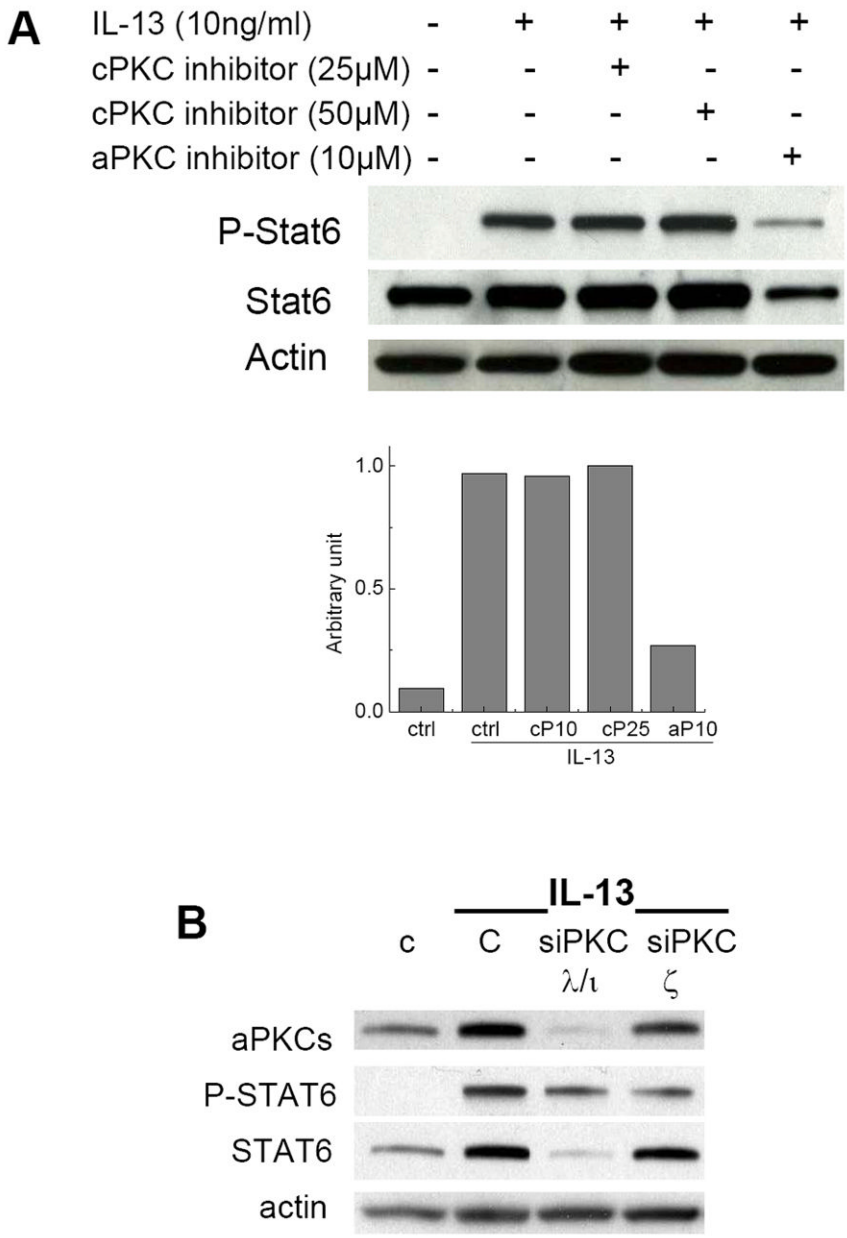

Figure 4. Panel A - The pseudosubstrate peptide for the atypical PKCs (aPKC) but not the classic PKCs (cPKC) suppressed STAT6 activation

A549 cells were incubated with pseudosubstrate peptide specific for aPKCs or cPKCs at indicated concentrations for 30 min, and then stimulated by IL-13 for 10 min. Western blotting was conducted to probe the effects on STAT6 phosphorylation. **Panel B – Downregulation of aPKCs affected STAT6 activation and expression**. Cells were transfected with control siRNA (C), siRNAs of PKCλ/ι or PKCζ. Sixty-four hours later, cells were activated by IL13 (10 ng/ml) for 10 min.

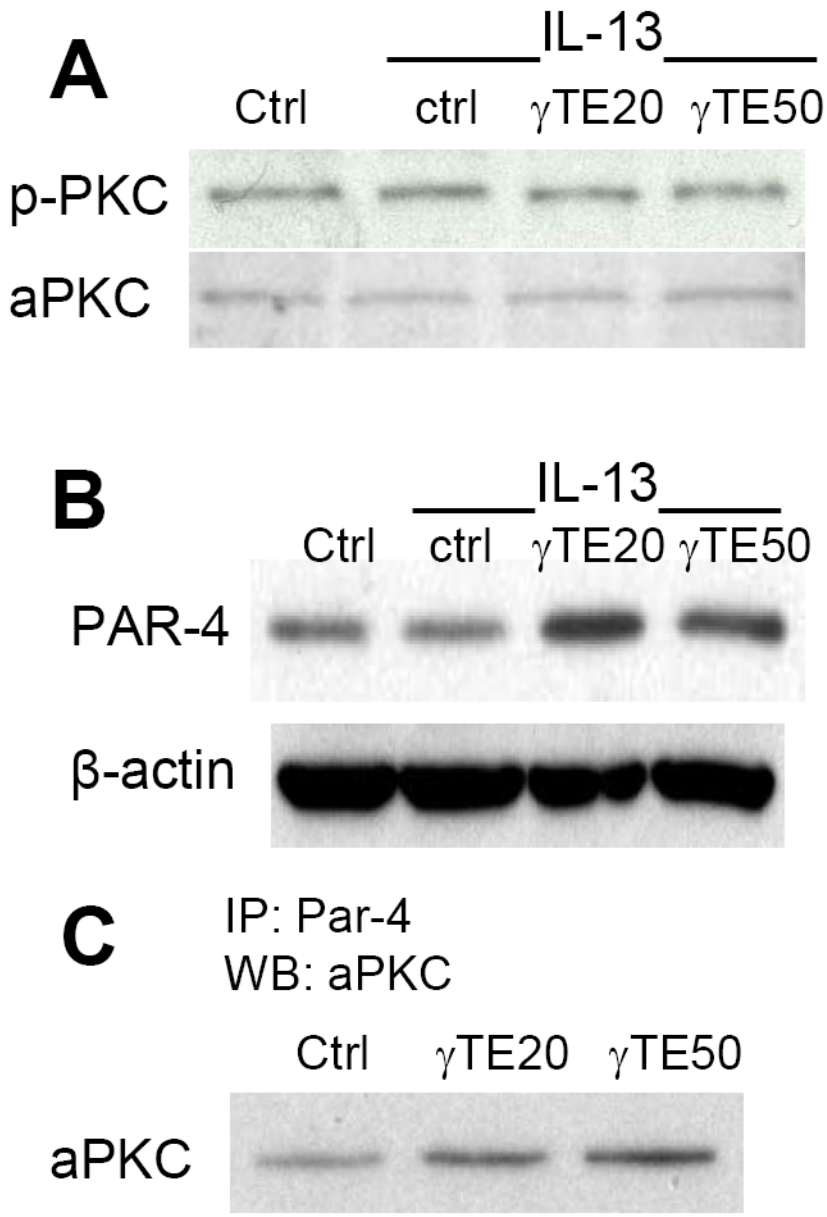

Figure 5. The effects of γ-TE on atypical PKC (aPKC) expression and its phosphorylatd state (p-aPKC) (Panel A), PAR4 expression levels (Panel B) and potential crosstalk between aPKCs and PAR4 by immunoprecipitation (IP) (Panel C)

A549 cells were incubated with γ-TE at 20 or 50 μM for 14-16 h, stimulated by IL-13 (10 ng/ml) for 10 min, and collected for Western blotting. In some studies, immunoprecipitation was performed for cells treated with γTE or solvent controls using antibody for PAR4. The precipitated proteins were analyzed by Western blot using antibody for aPKCs.

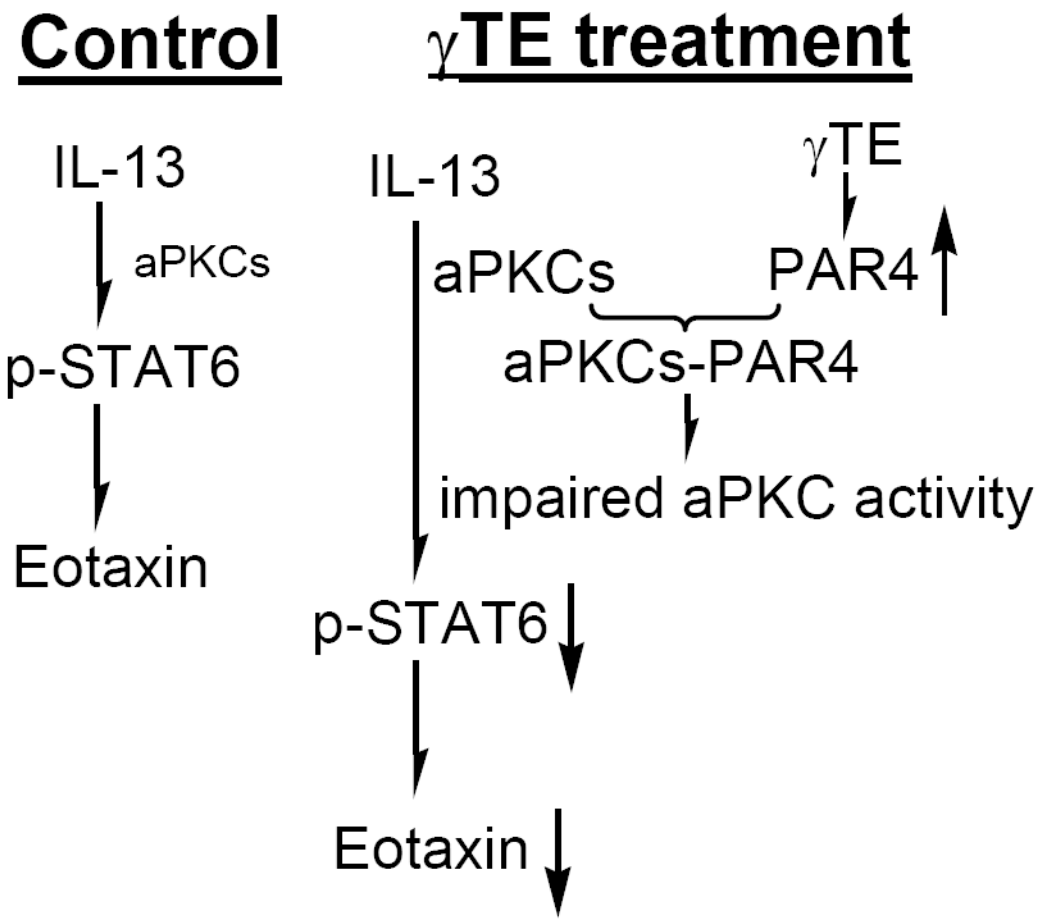

Figure 6. The mechanism underlying γ-TE-caused inhibition of eotaxin

Our data indicate that aPKCs are critically important to STAT6 phosphorylation and eotaxin generation. γ-TE treatment enhanced PAR4 expression and aPKC-PAR4 complex formation, which negatively regulates aPKCs activity and therefore resulted in decreased eotaxin secretion.