Published in final edited form as:

J Nutr Biochem. 2019 February ; 64: 101–109. doi:10.1016/j.jnutbio.2018.10.013.

Vitamin E δ -tocotrienol inhibits TNF- α -stimulated NF- κ B activation by up-regulation of anti-inflammatory A20 via modulation of sphingolipid including elevation of intracellular dihydroceramides

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Abstract

Nuclear factor- κ B (NF- κ B) regulates inflammation and cell survival, and is considered a potential target for anti-inflammatory and anti-cancer therapy. δ -Tocotrienol (δ TE), a vitamin E form, has been shown to inhibit NF- κ B, but the mechanism underlying this action is not clear. In the present study, we show that δ TE inhibited TNF- α -induced activation of NF- κ B and IL-6 in a dose- and time-dependent manner in Raw 264.7 macrophages. δ TE potently inhibited TNF- α -induced phosphorylation of transforming growth factor β -activated kinase 1 (TAK1), an upstream kinase essential for the activation of NF- κ B. Interestingly, δ TE significantly increased the expression of A20 and to a less extent, cylindromatosis (CYLD), both of which are inhibitors of NF- κ B. The importance of induction of A20 in δ TE's anti-NF- κ B effect is validated in A20 knockout cells where δ TE's inhibition of NF- κ B was largely diminished. In pursuit of the cause for A20 induction, we found that δ TE treatment caused rapid and persistent elevation of dihydroceramides, while decreased ceramides initially but increased ceramides during prolonged treatment. These changes of sphingolipids were accompanied by increased cellular stress markers. Importantly, δ TE's induction of A20 and inhibition of NF- κ B activation were partially counteracted by myriocin, a potent inhibitor of *de novo* synthesis of sphingolipids, indicating a critical role of sphingolipid modulation in δ TE-mediated effects. Since dihydroceramide has been shown to induce A20 and inhibit NF- κ B in RAW cells, we conclude that that δ TE inhibits NF- κ B activation by enhancing its negative regulator A20 as a result of modulating sphingolipids especially elevation of dihydroceramides.

Keywords

Tocotrienol; inflammation; vitamin E; NF- κ B; sphingolipids; cellular stress; A20

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INTRODUCTION

Nuclear factor (NF)- κ B plays a central role in immunity and inflammatory responses by regulating a remarkably diverse genes that regulate proliferation, immunity, anti-apoptosis, and negative feedback [1]. Under resting conditions, the two components of NF- κ B, p50 and p65, are sequestered in the cytosol by the inhibitory protein I κ B α . A variety of stimuli such as inflammatory cytokines, pathogen-associated molecules, and antigen receptors can activate NF- κ B [2]. For instance, TNF- α triggers activation of NF- κ B by binding to tumor necrosis factor receptor (TNFR), which recruits the following proteins to the receptor, i.e., TNFR type 1-associated death domain protein, receptor-interacting serine/threonine-protein kinase 1 (RIP1), TNFR-associated factor 2 (TRAF2) and baculoviral IAP repeat-containing protein 1 and 2 (cIAP1, 2). The conformational change of RIP1 leads to K63-linked poly-ubiquitination of RIP1, which results in recruitment of the IKK complex to the activated receptor and its upstream kinase including TGF- β -activated kinase 1 (TAK1). Activated TAK1 appears to be essential for IKK activation, which, in turn, catalyzes phosphorylation of I κ B α , and this event triggers degradation of I κ B α by proteasome. Thereafter, the p50/p65 dimer is dissociated with I κ B α and translocates to the nucleus where it activates the transcription of many genes.

While prompt activation of NF- κ B is essential for the host defense, timely turning down its activation is equally important for maintaining tissue homeostasis once the insult is eliminated. Aberrant NF- κ B activation has been associated with various chronic diseases including cancer. For instance, NF- κ B is found to be persistently activated in many types of lymphoid and myeloid malignancies [3, 4]. Constitutively active NF- κ B has been identified as a key link between chronic inflammation and cancer [5–7]. Consistently, inhibition of NF- κ B is shown to attenuate inflammation and cancer progression [8, 9]. For preventing excessive activation, NF- κ B is down regulated via a negative feedback loop mechanism, in which NF- κ B-target genes, such as A20 and CYLD (cylindromatosis), serve as negative regulators of NF- κ B signaling pathway via ubiquitin-editing activity or in a non-catalytic mechanism to block activation of IKK upstream signaling including TAK-1 [1, 10].

Because of its critical role in inflammation and disease, NF- κ B is considered a target for prevention of chronic inflammation and possibly cancer. Different forms of vitamin E have been shown to have anti-inflammatory activities including inhibition of NF- κ B and have cancer prevention effects in animal models [11, 12]. For instance, we demonstrate that γ TE, a natural form of vitamin E rich in palm oil, inhibits NF- κ B activation in macrophages and cancer cells via upregulation of A20 by altering sphingolipid metabolism and induction of cellular stress [13]. Further, δ -tocotrienol (δ TE), a natural analog of γ TE rich in annatto seeds, has been shown to inhibit constitutive NF- κ B activation in pancreatic cancer cells [14] and TNF- α -triggered NF- κ B activation in breast cancer cells [15]. Consistently, δ TE supplementation inhibits pro-inflammatory markers in the serum of chicken or in *ex vivo* experiments with mouse peritoneal macrophages [16, 17]. Despite these interesting findings, the mechanisms underlying δ TE's anti-NF- κ B effect have not been elucidated. In the present study, we investigated the inhibitory effect and mechanisms of δ TE on NF- κ B activation in murine Raw 264.7 macrophages and mouse embryonic fibroblasts (MEFs). We found that δ TE at 5–20 μ M dose-dependently inhibited NF- κ B activation and was stronger

than its analog γ TE in this effect. We further showed that δ TE induced A20 and inhibited NF- κ B via modulation of sphingolipid metabolism including elevation of intracellular dihydroceramides (dhCers).

MATERIALS AND METHODS

Chemicals and reagents

δ TE (>97% pure) was a gift from American River Nutrition (Hadley, MA). Primary antibodies (Abs) against phospho-I κ B α , I κ B α , CYLD, p65, Actin and all the secondary Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Calyculin A, Abs for phospho-JNK, JNK, phospho-p38, p38, A20, phospho-p65, phospho-TAK1, TAK1, phospho-eIF2 α and eIF2 α were from Cell Signaling Technology (Beverly, MA). Sphingolipid standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Recombinant mouse TNF- α , Myriocin from *Mycelia Sterilia*, LPS and all other chemicals were from Sigma-Aldrich (St. Louis, MO). Cell culture media were obtained from American Type Culture Collection (Manassas, VA).

Cell culture

Murine RAW 264.7 macrophages from American Type Culture Collection were routinely maintained in DMEM with 10% FBS. Confluent cells were seeded and allowed to attach overnight at 4.5×10^6 per well in a 6-well plate or 0.8×10^6 per well in a 24-well plate. δ TE stock solutions were initially made in DMSO and then diluted in 10 mg/ml fatty acid-free BSA in PBS. Confluent cells were incubated in DMEM containing 1% FBS and 0.04% DMSO (control) or δ TE at various concentrations for indicated times and then stimulated by TNF- α at 10 ng/ml for 5 min.

A20^{-/-} and A20^{+/+} mouse embryonic fibroblasts (MEFs)

A20^{-/-} and A20^{+/+} MEFs were prepared from the knockout and wild-type mice, and were provided by Dr. Averil Ma from UCSF [18]. These cells were immortalized with SV40 large T antigen and cultured in DMEM with 10% FBS, 50 μ M beta mercaptoethanol, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine. Cell passages less than six were used in the study.

Western blot

Cells were lysed in a lysis buffer containing Tris-EDTA, 1% SDS, 1 mM DTT, 2 mM sodium vanadate, and protease inhibitor cocktails (Sigma-Aldrich). Cytosolic and nuclear proteins were extracted using the Pierce kit (Pierce, Rockford, IL). Proteins (20–50 μ g) were loaded on freshly prepared SDS-PAGE gels. Resolved proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) and probed by antibodies. Membranes were exposed to chemiluminescent reagent (PerkinElmer, Waltham, MA) and visualized on Kodak films. In all experiments, after being probed by antibodies for target proteins, PVDF membranes were stripped with antibody-stripping solution from EMD Millipore (Billerica, MA) and reimmunoblotted with antibodies for internal controls such as β -actin. Results of Western blots were quantified by AlphaEaseFC.

Lipid extraction

To measure sphingolipids, total lipids were extracted as previously described [19]. Briefly, cell pellets were re-suspended in methanol/chloroform /water (10:5:1 [v/v/v]), followed by addition of internal standards containing 0.5 nmoles of C12:0-ceramide (Cer), C17-sphingosine, C17sphinganine, C25:0-Cer, and C12:0-sphingomyelin from Avanti Polar Lipids (Alabaster, AL). After tip sonication, samples were incubated at 48°C overnight. The next day, 100 µl aliquots was taken out and dried under N₂ for measurement of total phospholipids (Wako Chemicals, Neuss, Germany). For the rest of the samples, 75 µl of 1 M KOH in methanol was added, sonicated for 30 min, and then incubated at 37°C for 2 h and dried in a nitrogen evaporator.

Measurement of sphingolipids using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for sphingolipid analysis was slightly modified based on a published protocol by Merrill et al. [19]. Briefly, all samples were analyzed using the Agilent 6460 triple quadrupole mass spectrometer coupled with the Agilent 1200 rapid resolution HPLC (Agilent Technologies, Santa Clara, CA) with identification of each sphingolipid by multiple reaction monitoring. The MS/MS conditions used for multiple reaction monitoring detection of sphingolipids were the same as previously described [19–21]. The LC column for sphingoid bases and ceramides was Agilent XDB-C18 (4.6 × 50 mm) with particle size of 1.8 µm, and the LC column for sphingomyelins was Agilent XDB-C8 (2.1 × 50 mm) with Zorbax 3.5 µm. Mobile phases A and B contain 5 mM ammonium formate and have, respectively, methanol-H₂O-formic acid (74:25:1 [v/v/v]) and methanolformic acid (99:1 [v/v]). Agilent MassHunter software was coupled with the LC-MS/MS for data acquisition and analysis.

Analyses of interleukin-6 (IL-6)

The concentration of IL-6 in the medium was quantified using immunoassay kits from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

Statistical analyses

One-way ANOVA was used for multiple group comparison. Student's *t* test was used to compare data between two groups. The p-p38 data (Figure 4B) did not follow normal distribution, and was therefore transformed via square root before statistical analyses. A *p* value <0.05 was considered significant.

RESULTS

δTE inhibited NF-κB activation, IL-6 and upstream TAK1 in macrophages

We compared the effect of four natural vitamin E forms, i.e., γ-tocopherol (γT), δ-tocopherol (δT), γTE and δTE, on TNF-α-stimulated NF-κB activation in RAW 264.7 cells. Among tested vitamin E forms, δTE showed the strongest inhibitory effect on TNF-α-triggered NF-κB activation, as indicated by decreased phosphorylation and degradation of IκBα in the cytosol and suppressed phosphorylation of p65 in the nucleus compared with

vehicle controls (Fig. 1A). The anti-NF- κ B effect was observed in cells pre-incubated with δ TE for 4 h, and also in 8- and 16-hr treated cells (Fig. 1B). In addition, δ TE inhibited NF- κ B activation in a dose-dependent manner (Fig. 1C). Consistent with inhibition of NF- κ B, δ TE also decreased LPS-stimulated IL-6 (Figure 1D), which is a NF- κ B target gene but requires additional transcriptional factor like CEBP for transcriptional activation [22]. In a recent pharmacokinetic study in human, the maximum achievable concentration of δ TE in the plasma was estimated to range from 5 to 17 μ M among subjects who took 600–1600 mg of δ TE [23]. Therefore, we tested the activities of δ TE at 10 or 20 μ M in the subsequent studies.

It is well established that TNF- α -stimulated phosphorylation of I κ B α is mediated by the IKKs, which are activated by the upstream TAK1 [24]. Our group previously showed that a specific inhibitor of TAK1, oxozeanol, markedly inhibited TNF- α -stimulated phosphorylation of I κ B α in RAW macrophages [13], indicating that activation of TAK1 is required for TNF- α -induced activation of NF- κ B. Therefore, we examined the effect of δ TE on TAK1 activation in the current study. We observed that, like γ TE [13], δ TE treatment blocked TNF- α -stimulated phosphorylation of TAK1 (Fig. 1E). This observation suggests that, similar to γ TE, δ TE likely targets the upstream signaling that is key to the activation of TAK1 and IKKs.

δ TE treatment up-regulated negative regulators of NF- κ B including A20 and CYLD

Previously we reported that γ TE inhibited NF- κ B signaling by up-regulating A20 [13]. Because like γ TE, δ TE also inhibited TNF α -induced TAK1 phosphorylation, a key upstream signaling that has been shown to be inhibited by overexpression of A20 [25], we reason that δ TE may affect the expression of A20 and other NF- κ B inhibitors. Indeed, we observed that δ TE treatment induced up-regulation of A20 after only 4 h incubation and this effect sustained with prolonged treatment (Fig. 2A). Besides A20, δ TE treatment also significantly enhanced the expression of CYLD especially after 4 hr incubation (Fig. 2B), in contrast to γ TE that did not show any impact on CYLD expression [13].

The inhibitory effect on NF- κ B activation by δ TE was diminished in A20-knockout mouse embryonic fibroblasts (MEFs)

To determine whether induction of A20 plays a role in δ TE's anti-NF- κ B effect, we examined how δ TE affect NF- κ B activation in the A20^{-/-} MEFs. Similar to the observations in macrophages, δ TE treatment significantly elevated A20 expression in the A20^{+/+} MEFs, whereas as expected, the level of A20 expression was very low in the A20^{-/-} cells (Fig. 3A). TNF- α induced more pronounced phosphorylation of I κ B α in the A20^{-/-} MEFs than that in the wild-type cells (Fig. 3B, compare *lanes 3* versus *4*), supporting the notion that A20 is a negative regulator of NF- κ B. Although δ TE inhibited TNF α -stimulated I κ B α phosphorylation in the A20^{+/+} cells (Fig. 3B, compare *lanes 3* versus *5*), it failed to significantly inhibit phosphorylation of I κ B α induced by TNF- α in the A20^{-/-} MEFs (Fig. 3B, compare *lanes 4* versus *6*). Further, we observed significantly diminished inhibition of NF- κ B by δ TE in the A20^{-/-} MEFs compared to A20^{+/+} cells (Fig. 3B, compare *lanes 5* versus *6*). These results, together with known functions of A20, support that A20 induction plays a key role in δ TE's inhibitory effect on NF- κ B activation.

δ TE treatment induced cellular stress, increased intracellular dihydroceramides (dhCers) and caused time-dependent changes of ceramides (Cers)

We have shown that γ TE upregulated A20 as a result of cellular stress-induced activation of basal NF- κ B [13]. In the current study, to understand why δ TE enhances A20 expression, we examined its potential impact on cell stress markers. We found that δ TE treatment induced phosphorylation of JNK, p38 and eIF2 α (Fig. 4A, 4B and 4C), indicating induction of cellular stress. It should be noted that we did not see obvious impact on cell morphology or viability by δ TE treatment in confluent macrophages based on microscopic examination and the MTT assays (data not shown). Interestingly, unlike γ TE which induced noticeable increase of I κ B α phosphorylation [13], we did not observe the similar effect by δ TE (data not shown).

Since vitamin E forms have been shown to induce cellular stress by modulation of sphingolipid metabolism including *de novo* synthesis of sphingolipids (Figure 5A) [13, 26–28], we next test whether δ TE has any impact on sphingolipids in macrophages by a sphingolipidomic approach using LC-MS/MS [26]. Compared with controls, δ TE significantly enhanced individual and total dhCer after 4 hr incubation and the elevation of dhCers sustained during prolonged treatment (Fig. 5B, Table 1). In contrast, δ TE significantly decreased Cers such as C22:0-, C24:0- Cer and total Cers after 4 hr incubation, but increased Cers including C16:0-Cer and total Cers after 16 hr treatment (Fig. 5C, Table 1). These data indicate that δ TE modulated sphingolipid metabolism in a similar pattern to γ TE, although γ TE did not have significant impact on Cers after 16 hr treatment in RAW cells [13].

Modulation of sphingolipids by δ TE played a significant role in its induction of A20 and inhibition of NF- κ B activation

To examine the role of sphingolipid modulation in δ TE-induced upregulation of A20 and anti-NF- κ B actions, we pharmacologically blocked *de novo* sphingolipid biosynthesis by myriocin, a specific inhibitor of serine palmitoyl-CoA transferase that catalyzes the rate-limiting reaction in *de novo* synthesis of sphingolipids. We have previously shown that co-incubation with myriocin prevented γ TE-induced increase of dhCer in the same cells [13]. Here, we found that myriocin significantly counteracted δ TE-induced upregulation of A20 (Fig. 6A). Furthermore, myriocin also significantly counteracted δ TE's inhibition of NF- κ B activation, as indicated by attenuated effect on δ TE's inhibition of I κ B α phosphorylation (Fig. 6B). These data suggest that modulation of sphingolipid metabolism by δ TE is critically involved in its induction of A20 and inhibition of NF- κ B.

DISCUSSION

A key finding of our current study is that δ TE inhibits NF- κ B activation by upregulation of anti-inflammatory enzymes A20 and to a less extent, CYLD (Figure 7). A20 and CYLD are well-recognized negative regulators of NF- κ B and are known to be upregulated via a negative feedback loop mechanism to prevent excessive activation of this transcription factor. The anti-NF- κ B activities of A20 and CYLD have been well characterized in mechanistic and animal studies. A20 has been shown to inhibit NF- κ B via its ubiquitin-

editing activity [29] including catalyzing de-ubiquitination of TRAF6 [30] and IKK γ [31], or via non-catalytic manners to block activation of TAK-1 and IKKs [25, 32]. Similarly, CYLD is reported to suppress NF- κ B via binding to IKK γ and de-ubiquitination of TRAF2 and, to a less extent, TRAF6 [33, 34]. Over-expression of A20 or CYLD leads to suppression of NF- κ B activity in various types of cells [25, 35], while A20-deficient mice exhibit unrestrained inflammation in multiple organs and are hypersensitive to both LPS and TNF- α [18]. In the present study, we show that δ TE blocked TNF α -stimulated TAK-1 phosphorylation and NF- κ B activation, and meanwhile elevated A20 in the cells. The pivotal role of A20 induction in δ TE's anti-NF- κ B action is validated in A20^{-/-} MEFs where δ TE failed to suppress TNF- α -triggered NF- κ B activation, whereas δ TE significantly inhibited NF- κ B activation in the A20^{+/+} MEFs. This observation also suggests that although δ TE treatment upregulates both A20 and CYLD, elevation of A20 likely plays a major role in δ TE's anti-NF- κ B effect.

Another key discoveries of our study is that modulation of sphingolipids, esp. elevation of dhCers, plays an important role in δ TE's exerted effects including induction of cellular stress, upregulation of A20 and anti-NF- κ B effect (Figure 7). We observe that δ TE treatment significantly increased intracellular dhCers after 4 hr incubation, and this effect was sustained during prolonged incubation for 8 or 16 hrs. dhCers have been shown to cause cellular stress [36], cell cycle arrest [37], or autophagy in various types of cancer cells [27, 38]. We have previously shown that C8-dhCer treatment resulted in upregulation of A20, increased cellular stress, and inhibition of TNF- α -induced I κ B α phosphorylation in macrophages [13]. These same actions are exerted by δ TE as observed in the current study and by γ TE as previously reported [13]. We therefore argue that elevated dhCers is likely responsible for δ TE-caused cellular stress, induction of A20 and inhibition of NF- κ B activation. Consistently, the pivotal role of modulation of sphingolipids in δ TE-mediated A20 upregulation and anti-NF- κ B is also supported by the observation that pharmacological blockage of *de novo* sphingolipids synthesis with myriocin, which prevents accumulation of dhCers, diminished δ TE's inhibitory effect on I κ B α phosphorylation and induction of A20. In contract to the effect on dhCers, δ TE's impact on Cers varied with the length of incubation. Specifically, δ TE treatment caused decrease of Cers after 4 h but increase of Cers after 16 h incubation. Since A20 upregulation was observed throughout 4–16 h treatment by δ TE, we argue that enhanced dhCers, but not the delayed increase of Cers, contribute to δ TE's induction of A20 and anti-NF- κ B activity.

Recently, we have shown that γ TE induced dhCer accumulation via inhibiting the activity of dehydroceramide desaturase (DEGS-1), which explains the observation that short-time treatment with γ TE caused elevation of dhCers and reduction of Cers in various cancer cells [21]. In the same study, we also found that γ TE had no effect on DEGS-1 protein expression [21]. Given the structural similarity of γ TE and δ TE, we propose that δ TE likely inhibits DEGS-1 activity. Although this possibility remains to be tested, we observed that in the present study, δ TE caused increase of dhCers and decrease of ceramides after 4 h incubation (Table 1), which is consistent with the hypothesis that δ TE may inhibit DEGS-1. During prolonged (16 h) treatment, δ TE increased ceramides in RAW cells, an observation similar to what we found in cancer epithelial cells where γ TE decreased Cers (due to its inhibition of DEGS-1 activity) during short-time incubation and enhanced Cers during prolonged

treatment [21]. While we are preparing this manuscript, Kim *et al* [39] reported that γ TE treatment decreased ceramides but had no effect on DEGS-1 gene expression in the resting macrophages, which is consistent with our observations [21]. Interestingly, γ TE inhibited LPS-induced elevation of sphingolipids and upregulation of DEGS-1 in activated macrophages [39], which is consistent with anti-inflammatory activities of this vitamin E form.

In the present study, δ TE showed stronger anti-NF- κ B effect than γ TE. This observation is in agreement a previous study where δ TE exerted the most potent inhibitory effect on NF- κ B activation among α -, β -, and γ -TE in pancreatic cancer cells [14], whereas γ TE is reported to be similar to or slightly stronger than δ TE in inhibiting NLRP3 inflammasome priming in macrophages [40]. It is noteworthy that although δ TE exerted similar effects to γ TE, such as increase of A20, induction of cellular stress and modulation of sphingolipid metabolism, δ TE significantly elevated CYLD level besides A20, unlike γ TE that did not have any impact on CYLD [13]. Although A20 appears to play a major role in δ TE's anti-NF- κ B actions (discussed above), it is possible that dual up-regulation of both A20 and CYLD by δ TE contributes to its stronger anti-NF- κ B activity than γ TE. Consistently, it has recently been shown that A20 and CYLD exhibit additive effects on suppression of NF- κ B in monocytic cells where knocking down both CYLD and A20 further increased the NF- κ B-dependent IL-8 mRNA expression compared with that of knocking down A20 alone [41].

Given that A20 and CYLD are negative regulators of NF- κ B that controls immunity and cell survival, upregulation of these regulatory enzymes likely contributes to δ TE's ability to modulate inflammation and cancer. In particular, A20-deficiency in dendritic cells has been shown to develop arthritis and colitis [42], while A20 overexpression attenuates allergic airway inflammation in mice [43]. A20 or CYLD polymorphisms appear to be associated with inflammatory diseases such as Crohn's disease, arthritis, and autoimmune type I diabetes in humans [44, 45]. Furthermore, A20 and CYLD are recognized for their tumor suppressing functions because deficiency or mutation of A20 or CYLD is associated with lymphoma and familial cylindromatosis, respectively [45–47]. In light of our present study, beneficial effects of δ TE, esp. those via modulating NF- κ B or inflammation, may be rooted in its upregulation of A20 and CYLD. For instance, δ TE has been shown to block tumor development and suppress NF- κ B in pancreatic cancer cells and a xenograft model [14]. Furthermore, δ TE is shown to inhibit colitis-promoted colon cancer in mice [48]. In the future, studies should be conducted to investigate the effect of δ TE and γ TE on A20 and CYLD in relevant tissues under the whole body environment.

ACKNOWLEDGEMENTS

The authors would like to thank Amber S Jannasch for helps with LC-MS/MS analysis of sphingolipids.

FOOTNOTE: This work was in part supported by a NIH/NIEHS grant R01ES023349, an Incentive Grant from Purdue Institute of Inflammation, Immunology and Infectious Disease (PI4D) and a grant from Purdue Research Foundation.

ABBREVIATION

A20 tumor necrosis factor, alpha-induced protein 3 (TNFAIP3)

Abs	antibodies
Cer	ceramide
CYLD	cylindromatosis
δTE	delta-tocotrienol
dhCer	dihydroceramide
γTE	gamma-tocotrienol
eIF2α	translation initiation factor 2 α
ER stress	endoplasmic reticulum stress; gamma-tocotrienol
IKKs	I κ B kinases
JNK	c-jun N-terminal kinase
LPS	lipopolysaccharide
MEF	mouse embryonic fibroblasts
NF-κB	Nuclear factor- κ B
SM	sphingomyelin
TAK1	transforming growth factor β -activated kinase 1

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- * δ -tocotrienol (δ TE), a less known form of vitamin E, inhibited TNF- α -induced activation of NF- κ B and upstream kinase TAK-1 in macrophages.
- * δ TE significantly increased the expression of A20 and cylindromatosis (CYLD), both of which are inhibitors of NF- κ B.
- * Knockout of A20 largely diminished δ TE's anti-NF- κ B effect.
- * δ TE treatment significantly modulated sphingolipids including elevation of dihydroceramides, which was accompanied by increased cellular stress markers.
- * Modulation of sphingolipids plays critical roles in δ TE's mediated induction of A20 and inhibition of NF- κ B

Figure 1. δ TE inhibited NF- κ B activation, IL-6 and upstream TAK1 in RAW 264.7 macrophages. **Panel A:** RAW 264.7 cells were pretreated with γ T, δ T, γ TE, or δ TE at 20 μ M for 16 hr and then stimulated with 10 ng/ml TNF- α for 5 min. **Panel B:** RAW 264.7 cells were pretreated with δ TE at 20 μ M for 4, 8 or 16 hr and then stimulated with 10 ng/ml TNF- α for 5 min. **Panel C:** RAW cells were treated with δ TE at 5, 10, or 20 μ M for 16 hr before being stimulated by TNF- α as described in Panel A. **Panel D:** After pre-incubation with δ TE or γ TE at 5, 10, 15 and 20 μ M for 3 hr, RAW 264.7 cells were stimulated by LPS (0.1 μ g/ml) for 14 hr. Cell culture media were collected to measure IL-6. Relative IL-6 is the ratio of IL-6 secreted by cells treated with tested compounds to that of solvent control in the presence of LPS. Data are shown as mean $\pm$ SEM. The bars with different letters differ significantly (n = 5–6 per bar). **Panel E:** RAW cells were pretreated with δ TE for 16 h.

Calyculin A (CaA, a serine/threonine phosphatase inhibitor, 50 nM) was added immediately before cells were stimulated with 10 ng/ml TNF- α for 5 min. Cytosolic proteins were immunoblotted for evaluation of phospho-I κ B α , I κ B α , phospho-TAK1, and β -Actin and nuclear proteins for phospho-p65 and p65. These are representative data from n > 3 independent experiments.

Figure 2. δ TE up-regulated negative regulators of NF- κ B, A20 and CYLD in RAW 264.7 macrophages.

Panel A and B: RAW 264.7 cells were treated with δ TE at 20 μ M for 4, 8, or 16 hr. Proteins of whole cell lysis were immunoblotted with antibodies for CYLD, A20 or β -Actin. Data are representative results from > 4 independent experiments. The intensity of A20 and CYLD was quantified by AlphaEaseFC and normalized by β -Actin. * p < 0.05 between solvent control and δ TE group (mean $\pm$ SEM, n = 4–5 independent experiments).

Figure 3. δTE's anti-NF-κB effect in A20^{+/+} and A20^{-/-} MEFs.

Panel A: A20^{+/+} and A20^{-/-} MEFs were treated with δTE (10 μM) for 4 hr, and A20 expression (in whole cell lysis) was detected by Western blotting. Panel B: After preincubation with δTE (10 μM) for 4 hr, cells were stimulated by TNF-α (10 ng/mg) for 5 min and then collected for probing phospho-IκBα (cytosol) by Western blotting. *p<0.05 indicates difference between δTE and solvent controls (mean ± SEM, n = 5–6 independent experiments).

Figure 4. δTE treatment induced cellular stress.

RAW 264.7 cells were incubated with δTE at 20 μM for 4, 8 or 16 hr. Total proteins for p-JNK or JNK (Panel A) p-p38 (Panel B) and p-eIF2α (Panel C) were analyzed by immunoblots. * $p < 0.05$ indicates statistical differences between δTE and solvent controls (mean $\pm$ SEM, $n = 4$ independent experiments).

Figure 5. δ TE treatment increased intracellular dhCer and Cer.

Panel A: *De novo* synthesis of sphingolipids. R = Acyl group with C16 -C26. Panel B and C: RAW 264.7 cells were treated with δ TE (20 μ M) or solvent (Ctrl) for 4, 8 or 16 hr.

Sphingolipids were analyzed by LC-MS/MS. Total dhCers (B) and Cers (C) are shown as means $\pm$ SEM (n = 4 independent experiments). *p < 0.05, **p < 0.01 between control and δ TE-treated cells (A paired sample t-Test was used for statistical analyses).

Figure 6. Myriocin counteracted δTE-caused induction of A20 and inhibition of NF-κB.

Panel A: RAW 264.7 cells were incubated with δTE (20 μM) for 16 hr in the presence or absence of 6 μM myriocin (myr). Whole proteins were extracted for A20 expression analysis by Western blotting (mean ± SEM, n=5 independent experiments). Panel B: RAW cells were incubated with δTE (20 μM) for 16 hr with or without 6 μM myriocin (myr), and then were stimulated with TNF-α for 5 min. Cytosolic proteins were used for detecting IκBα phosphorylation. **p < 0.01 and *p < 0.05 indicate between δTE and ctrl or δTE and δTE + myr (mean ± SEM, n=5 independent experiments).

Figure 7. Proposed mechanism underlying δTE's anti-NF-κB activation.

δTE modulates sphingolipid metabolism including elevation of intracellular dihydroceramides, which leads to cellular stress as indicated by increased p-JNK, p-38 and p-eIF2α. These events further result in the upregulation of A20 and to a less extent CYLD, which are responsible for inhibiting TNFα-stimulated activation of TAK1 and NF-κB.

Table 1.
The effect of δ TE on sphingolipids in RAW264.7 cells

RAW cells were treated with δ TE at 20 μ M or vehicle (Ctrl) for 4, 8 or 16 hr. Lipids were extracted and sphingolipids were measured by LC-MS/MS (Materials and Methods). All data are mean $\pm$ SEM. The paired sample t Tests were used to analyze results between solvent control and treatment groups.

Ceramides (pmol/ μ g PC)	Ctrl 4 h	δ TE 4 h	Ctrl 8 h	δ TE 8 h	Ctrl 16 h	δ TE 16 h
C16:0-Cer	1.38 $\pm$ 0.22	1.18 $\pm$ 0.27	1.53 $\pm$ 0.19	1.77 $\pm$ 0.26	1.09 $\pm$ 0.08	1.74 $\pm$ 0.13 **
C18:0-Cer	0.20 $\pm$ 0.05	0.15 $\pm$ 0.04 *	0.18 $\pm$ 0.03	0.16 $\pm$ 0.03	0.12 $\pm$ 0.01	0.14 $\pm$ 0.02 *
C20:0-Cer	0.11 $\pm$ 0.02	0.09 $\pm$ 0.02 **	0.11 $\pm$ 0.025	0.083 $\pm$ 0.014	0.064 $\pm$ 0.009	0.076 $\pm$ 0.010 *
C22:0-Cer	0.57 $\pm$ 0.17	0.48 $\pm$ 0.16 *	0.60 $\pm$ 0.16	0.49 $\pm$ 0.15	0.27 $\pm$ 0.04	0.38 $\pm$ 0.08 *
C24:1-Cer	1.10 $\pm$ 0.45	0.89 $\pm$ 0.40 *	1.05 $\pm$ 0.45	0.71 $\pm$ 0.30	0.88 $\pm$ 0.27	0.71 $\pm$ 0.27
C24:0-Cer	1.10 $\pm$ 0.33	0.99 $\pm$ 0.35	1.50 $\pm$ 0.54	1.27 $\pm$ 0.50 *	0.76 $\pm$ 0.20	1.35 $\pm$ 0.38 **
C26:1-Cer	0.015 $\pm$ 0.004	0.015 $\pm$ 0.005	0.017 $\pm$ 0.005	0.014 $\pm$ 0.005	0.0086 $\pm$ 0.003	0.0083 $\pm$ 0.002
Total Cer	4.48 $\pm$ 1.15	3.80 $\pm$ 1.21 *	4.98 $\pm$ 1.33	4.50 $\pm$ 1.17	3.18 $\pm$ 0.41	4.40 $\pm$ 0.68 **

dhCers (pmol/ μ g PC)	Ctrl 4 h	δ TE 4 h	Ctrl 8 h	δ TE 8 h	Ctrl 16 h	δ TE 16 h
C16:0-dhCer	0.40 $\pm$ 0.10	0.65 $\pm$ 0.18 *	0.29 $\pm$ 0.06	0.70 $\pm$ 0.13 *	0.043 $\pm$ 0.009	0.24 $\pm$ 0.06 **
C18:0-dhCer	0.031 $\pm$ 0.009	0.039 $\pm$ 0.010	0.017 $\pm$ 0.004	0.033 $\pm$ 0.007 **	0.0026 $\pm$ 0.0006	0.011 $\pm$ 0.003 *
C20:0-dhCer	0.014 $\pm$ 0.004	0.020 $\pm$ 0.006 *	0.0086 $\pm$ 0.003	0.016 $\pm$ 0.004 **	0.00037 $\pm$ 0.0001	0.0033 $\pm$ 0.001 *
C22:0-dhCer	0.068 $\pm$ 0.014	0.109 $\pm$ 0.025	0.049 $\pm$ 0.009	0.10 $\pm$ 0.01 **	0.0040 $\pm$ 0.0003	0.022 $\pm$ 0.003 **
C24:1-dhCer	0.035 $\pm$ 0.010	0.060 $\pm$ 0.021	0.025 $\pm$ 0.007	0.047 $\pm$ 0.013 *	0.0045 $\pm$ 0.0011	0.020 $\pm$ 0.006 *
C24:0-dhCer	0.062 $\pm$ 0.012	0.10 $\pm$ 0.02	0.057 $\pm$ 0.012	0.12 $\pm$ 0.03 **	0.0052 $\pm$ 0.0012	0.049 $\pm$ 0.010 **
Total dhCer	0.61 $\pm$ 0.12	0.98 $\pm$ 0.24 *	0.45 $\pm$ 0.06	1.02 $\pm$ 0.16 **	0.060 $\pm$ 0.008	0.35 $\pm$ 0.06 **

Sphingoid bases (pmol/ μ g PC)	Ctrl 4 h	δ TE 4 h	Ctrl 8 h	δ TE 8 h	Ctrl 16 h	δ TE 16 h
Sph	0.53 $\pm$ 0.09	0.50 $\pm$ 0.11	0.54 $\pm$ 0.05	0.39 $\pm$ 0.07 *	0.32 $\pm$ 0.05	0.25 $\pm$ 0.03
dhSph	0.15 $\pm$ 0.02	0.16 $\pm$ 0.04	0.15 $\pm$ 0.02	0.16 $\pm$ 0.02	0.066 $\pm$ 0.006	0.090 $\pm$ 0.005
Sph-P	0.018 $\pm$ 0.003	0.019 $\pm$ 0.005	0.038 $\pm$ 0.007	0.028 $\pm$ 0.008	0.013 $\pm$ 0.005	0.011 $\pm$ 0.002

* P < 0.05

** P < 0.01

indicate significant differences between control and δ TE-treated cells. Cer: Ceramide; dhCer: dihydroceramide; Sph: sphingosine; dhSph: dihydrosphingosine; Sph-P: sphingosine 1 phosphate; dhSph-P: dihydrosphingosine phosphate; PC: Total phospholipids