Modulation of NF-κB and Nrf2 control of inflammatory responses in FHs 74 Int cell line is tocopherol isoform-specific

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Elisa I, Kitts DD. Modulation of NF-κB and Nrf2 control of inflammatory responses in FHs 74 Int cell line is tocopherol isoform-specific. Am J Physiol Gastrointest Liver Physiol 305: G940–G949, 2013. First published October 17, 2013; doi:10.1152/ajpgi.00269.2013.—The present study investigates the relative ability of α-, γ-, and δ-tocopherol (Toc) to modulate cell signaling events that are associated with inflammatory responses in fetal-derived intestinal (FHs 74 Int) cells. Secretion of the proinflammatory cytokine IL-8 in FHs 74 Int cells was stimulated in the following order: α-Toc < γ-Toc < δ-Toc. A similar proinflammatory response was observed when inflammation was induced in FHs 74 Int cells. Modulation of IL-8 expression by Toc corresponded to an isoform-specific modulation of NF-κB and nuclear factor-erythroid 2-related factor 2 (Nrf2) cell signaling pathways involved in expression of proinflammatory cytokines and antioxidant enzymes, respectively. δ-Toc and, to a lesser extent, γ-Toc activated NF-κB and Nrf2 signaling, as indicated by the greater nuclear translocation of transcription factors. Activation of NF-κB signaling by γ- and δ-Toc was accompanied by upregulation of NF-κB target genes, such as IL-8 and prostaglandin-endoperoxide synthase 2, with and without a prior IFNγ-PMA challenge. Nevertheless, γ- and δ-Toc, particularly δ-Toc, concurrently downregulated glutamate-cysteine ligase, a Nrf2 target gene that encodes for glutathione biosynthesis. This observation was substantiated by confirmation that γ- and δ-Toc were effective at decreasing glutamate-cysteine ligase protein expression and cellular glutathione content. Downregulation of glutathione content in fetal intestinal cells corresponded to induction of apoptosis-mediated cytotoxicity. In conclusion, γ- and δ-Toc are biologically active isoforms of vitamin E and show superior bioactivity to α-Toc in modulating cell signaling events that contribute to a proinflammatory response in fetal-derived intestinal cells.

IL-8; Nrf2; NF-κB; tocopherol; inflammation

Since the discovery of vitamin E in 1922, eight structurally related compounds, including four tocopherol (α-, β-, γ-, and δ-Toc) and four tocotrienol isomers, have been identified (8, 21). Of these, α-Toc has garnered considerable public interest, largely because this form of vitamin E has the greatest relative biological activity and, also, because it is the dominant form of vitamin E in the systemic circulation (6, 7). Similarly, human milk contains mostly α-Toc, minor levels of γ-Toc, and a barely quantifiable level of δ-Toc (18). In contrast, infant formula consists of substantial amounts of γ- and δ-Toc, which can be attributed to the use of specific sources of vegetable oils to constitute the lipid component of the infant food, along with mixed Toc isoforms (1, 11, 19, 47). It should also be noted that the major form of vitamin E consumed in the United States is γ-Toc, although the major circulating Toc is α-Toc (53). Given the high concentrations of γ- and δ-Toc in infant formula, it can be implied that more vitamin E is consumed by formula than breast-fed infants and also that the mixture of vitamin E forms consumed by formula-fed infants is markedly different from that consumed by breast-fed infants. The potential health effects of differences in Toc isoform proportions and related concentrations in the infant formula are not known, since little information exists on the biological activity of non-α-Toc isoforms in infant cells. Since dietary Toc isoforms are only metabolized in the liver after intestinal absorption, thus leading to excretion of non-α-Toc, the intestine represents a unique organ where all intact forms of vitamin E exist in concentrations that can manifest a physiological effect (31, 49).

The current study aims to determine the effect of γ- and δ-Toc, relative to α-Toc, in modulating baseline and IFNγ-PMA-induced inflammatory responses (10) in infant intestinal cells. A primary fetal-derived intestinal cell line (FHs 74 Int) was employed, with IL-8 secretion used as the end-point measure for inflammation, and the effect was evaluated in recognition of the relative cellular uptake of each Toc isoform (19). Two stress-activated cell signaling pathways, known to contribute to expression of the proinflammatory cytokine IL-8, namely, NF-κB and nuclear factor-erythroid 2-related factor 2 (Nrf2) pathways (2, 30, 46), were also examined. Activation of the NF-κB pathway leads to expression of proinflammatory cytokines, including IL-8 (30). On the other hand, the Nrf2 signaling pathway regulates expression of the antioxidant enzymes that boost cellular defense systems and mitigate an inflammatory response (2, 39). These two signaling pathways have also been reported to work together to modulate the overall inflammatory response (4, 35). The affinity of Toc isoforms to modulate Nrf2 and NF-κB signaling pathways was therefore investigated as a possible mechanism regulating IL-8 expression. Lastly, the extent to which different Toc isoforms are taken up by the cells and the effect of Toc isoforms on cell viability were evaluated in response to the relative potential to modulate inflammation.

Materials and Methods. RRR-α-Toc was purchased from Fisher (Fairlawn, NJ) and (+)-γ-Toc, (+)-δ-Toc, protease inhibitor cocktail (P8340), and EGF from Sigma-Aldrich (St. Louis, MO). FHs 74 Int cells (CCL-241) and Hybri-Care medium were purchased from Cedarlane (Hornby, ON, Canada). NF-κB and Nrf2 TransAM ELISA kits (catalog nos. 40096 and 50296, respectively) were obtained from Active Motif (Carlsbad, CA), NE-PER nuclear protein extraction kit (product no. 78835) from Pierce (Rockford, IL), and DC protein assay from Bio-Rad Laboratories (Hercules, CA). Detection limits for NF-κB and Nrf2 TransAM ELISA are 0.15 and 0.5 μg of nuclear extract per well, respectively. Custom PCR array (CAPH10994) and IL-8 single-analyte ELISA kits were purchased from Qiagen (Valencia, CA) and APO-BrDU terminal deoxynucleotidyl transferase dUTP-mediated nick end labeling (TUNEL; catalog no. AU1001) and glutathione enzyme-based (catalog no. 703002) assay kits from Phoe-
nix Flow Systems (San Diego, CA) and Cayman Chemical (Ann Arbor, MI), respectively. Rabbit polyclonal anti-glutamate-cysteine ligase (GCLC) primary antibody (ab41463) and mouse monoclonal anti-GAPDH primary antibody (ab8245) were obtained from Abcam (Cambridge, MA).

**Cell culture.** FHs 74 Int cells represent a primary cell line derived from the small intestine of a 3- to 4-mo-gestation human fetus. FHs 74 Int cells were maintained in Hybri-Care medium containing 10% FBS, 100 μg/ml streptomycin, 100 U of penicillin, and 30 ng/ml EGF. Initial seeding density for FHs 74 Int cells was 1.2 × 10⁶ cells/well in 2 ml of complete medium in six-well plates that were incubated for 2 days at 37°C. Cells were subsequently placed in serum-free medium for 18 h and then treated with Toc isoforms in reduced-serum (1%) medium. After 24 h, Toc-containing medium was removed, and the cells were challenged with a combination of IFN-γ-PMA (0.05 μg/ml) in 1.5 ml of medium cocultured with Toc isoforms. Toc isoforms were delivered in ethanol at a maximum concentration of 0.25% in culture medium.

**Cellular uptake of Toc.** The relative cellular uptake of different Toc isoforms in FHs 74 Int cells was determined using HPLC coupled with fluorescence detection based on previously established methods (19, 38, 42). At the end of each incubation period with different Toc isoforms, the culture medium was aspirated, and cells were washed once with 2% BSA in PBS on ice and then twice with PBS. The cells were lysed using a radioimmunoprecipitation assay (RIPA) buffer, and the protein content was measured by DC protein assay. The same cell extract was also analyzed for vitamin E content using HPLC (1100 series, Agilent Technology, Palo Alto, CA) (18). Briefly, separation of Toc isoforms was achieved using methanol as the mobile phase (1 ml/min) and a 3-μm SphereClone column (150 × 4.6 mm). Individual Toc isoforms were identified at an excitation wavelength of 292 nm and an emission wavelength of 330 nm.

**IL-8 measurement.** Cells were pretreated with IFN-γ-PMA cocktail for 24 h, the culture medium was recovered and centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was measured for IL-8 using an ELISA kit according to the manufacturer’s instructions (19). We repeated this experiment in the absence of IFN-γ-PMA to determine the effect of individual Toc isoforms on baseline IL-8 expression.

**NF-kB and Nrf2 activation.** To establish the effect of different Toc isoforms on the activation of NF-κB and Nrf2 signaling, we first established the effect of IFN-γ-PMA on NF-κB and Nrf2 activation. Cells pretreated with IFN-γ-PMA were harvested to obtain the nuclear fraction. The effect of Toc on modulation of NF-κB and Nrf2 signaling was determined after 90 min and 24 h of IFN-γ-PMA stimulation, respectively. We repeated this experiment in the absence of IFN-γ-PMA challenge to determine the role of different Toc isoforms on NF-κB and Nrf2 signaling in FHs 74 Int cells. Cells were then scraped into ice-cold PBS, and the nuclear pellet was obtained using the NE-PER kit. A nuclear extract was reconstituted in the lysis buffer supplied with the Nrf2 or NF-κB (p65) ELISA kit. Specifically, nuclei were lysed by vortexing once every 10 min for up to 40 min, and a final extract was recovered by centrifugation at 18,500 g for 10 min at 4°C. The relative presence of NF-κB and Nrf2 in the nuclear fraction was established using the TransAM ELISA kits according to the manufacturer’s instructions.

**Effects of Toc isoforms on gene transcription.** The ability of different Toc isoforms to modify expression of selected genes involved in Nrf2 and NF-κB signaling was determined using real-time PCR arrays. cDNA from 725 ng of mRNA obtained from FHs 74 Int cells as previously described (20). The custom PCR array consisted of 19 genes that transcribed proteins that regulate oxidative stress, inflammation, or cell proliferation; two housekeeping genes, one positive and one reverse transcription control, and a genomic contamination control were also present (Table 1). Each experiment consisted of three independent replicates of FHs 74 Int cells incubated with different Toc isoforms and challenged with IFN-γ-PMA for 4 or 24 h. Untreated cells served as the negative control, and the positive control consisted of cells that were incubated with IFN-γ-PMA. Reverse transcription, genomic DNA, and positive PCR controls were included for each sample. The experiment was also performed in the absence of IFN-γ-PMA challenge to determine the role of individual Toc isoforms in transcription of the select genes. PCR amplification proceeded as described using the ABI7500 fast real-time PCR system (Applied Biosystems, Foster City, CA), and the data were analyzed using a software tool downloaded from the Qiagen website (44).

**GCLC protein and glutathione content.** To determine the relative effect of different Toc isoforms on GCLC protein expression, FHs 74 Int cells were rinsed twice with PBS and lysed with RIPA buffer containing protease inhibitor cocktail. The total protein content was normalized after measurement by DC protein assay. Proteins from cell lysates were separated in a 12% resolving SDS-polyacrylamide gel and subjected to Western blotting with anti-GCLC antibody (1:1,000 dilution); GAPDH was used as a loading control. The effect of different Toc isoforms on the glutathione content of FHs 74 Int cells was determined using a glutathione assay kit according to the manufacturer’s instructions.

**Effect of Toc on cell viability and apoptosis.** The effects of different Toc isoforms on FHs 74 Int cell viability were evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, as previously described (17). To determine whether the Toc-induced cell death was attributed to apoptosis, DNA fragmentation was examined using APO-BrdU TUNEL assay kits according to the manufacturer’s instructions. Flow cytometry analysis was performed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA), and flow cytometry data were analyzed using FlowJo (Tree Star, Ashland, OR) (19).

**Statistics.** All experiments were performed in triplicate, and data are expressed as means ± SD. Significant differences between Toc isoforms at specific concentrations were identified using a one-way ANOVA (P < 0.05) followed by Tukey’s post hoc analysis to determine differences between individual groups. In addition, the effect of different Toc isoforms on modulation of IL-8 expression, Nrf2 and NF-κB signaling, cell viability, and glutathione content relative to control cells was determined using a t-test (P < 0.05).
Table 1. *Genes tested in custom PCR array*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Function of Encoded Protein</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2 target genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPX1</td>
<td>Glutathione peroxidase 1</td>
<td>Classic GPX; detoxification of H₂O₂</td>
<td>27</td>
</tr>
<tr>
<td>GPX2</td>
<td>Glutathione peroxidase 2 (gastrointestinal)</td>
<td>Gastrointestinal GPX; detoxification of H₂O₂</td>
<td>3</td>
</tr>
<tr>
<td>GPX4</td>
<td>Glutathione peroxidase 4 (phospholipid hydroperoxidase)</td>
<td>Reduces H₂O₂, lipid hydroperoxides</td>
<td>27</td>
</tr>
<tr>
<td>GSTA1</td>
<td>Glutathione S-transferase-α₁</td>
<td>Detoxification mechanism of reactive xenobiotics</td>
<td>29</td>
</tr>
<tr>
<td>GCLC</td>
<td>Glutamate-cysteine ligase, catalytic subunit</td>
<td>Glutathione biosynthesis; rate-limiting enzyme</td>
<td>56</td>
</tr>
<tr>
<td>GSS</td>
<td>Glutathione synthetase</td>
<td>Glutathione biosynthesis</td>
<td>23</td>
</tr>
<tr>
<td>GSR</td>
<td>Glutathione reductase</td>
<td>Reduction of oxidized glutathione</td>
<td>54</td>
</tr>
<tr>
<td>NF-κB signaling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFKB1</td>
<td>Nuclear factor of κ-light polypeptide gene enhancer</td>
<td>Precursor to p50 subunit of NF-κB complex</td>
<td>45, 51</td>
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<tr>
<td>RELA</td>
<td>V-Rel reticuloendotheliosis viral oncogene homolog</td>
<td>Inactive p105 subunit acts as NF-κB inhibitor</td>
<td>41</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
<td>Proinflammatory chemokine; recruitment of neutrophils</td>
<td>34</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)</td>
<td>Synthesis of prostaglandin (PGE₂)</td>
<td>52</td>
</tr>
<tr>
<td>IRAK2</td>
<td>Interleukin-1 receptor-associated kinase 2</td>
<td>Component of Toll-like/IL-1 receptor signaling; positive regulator of NF-κB</td>
<td>14</td>
</tr>
<tr>
<td>BID</td>
<td>BH3-interacting domain death agonist</td>
<td>Proapoptotic protein</td>
<td>33</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
<td>Proapoptotic protein</td>
<td>33</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
<td>Transcription factor mediating cell death</td>
<td>43</td>
</tr>
<tr>
<td>Housekeeping Genes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RPL13A</td>
<td>Ribosomal protein L13a</td>
<td>Housekeeping gene</td>
<td>22</td>
</tr>
<tr>
<td>ACTB</td>
<td>β-Actin</td>
<td>Housekeeping gene</td>
<td>22</td>
</tr>
</tbody>
</table>

In regulating the Nrf2 signaling pathway, δ-Toc at 100 μM was shown to stimulate Nrf2 activation, as evidenced by the enhanced nuclear translocation that occurred regardless of IFNγ-PMA challenge (Fig. 4). In the absence of IFNγ-PMA challenge, this Nrf2 signaling was promoted to the greatest extent by δ-Toc, followed by γ- and α-Toc, which increased Nrf2 signaling to a similar extent.

Modulation of NF-κB and Nrf2 signaling. Incubation of FHs 74 Int cells with IFNγ-PMA stimulated a time-dependent translocation of Nrf2 and NF-κB (p65) to the nucleus. After 90 min of incubation with IFNγ-PMA, NF-κB was maximally translocated to the nucleus, while the Nrf2 transcription factor was translocated to the nucleus 4–8 h after IFNγ-PMA exposure (Fig. 3).

Tocs influenced NF-κB signaling in FHs 74 Int cells in an isoform-specific manner. In the absence of IFNγ-PMA stimulation, δ-Toc at 100 μM was the most effective activator of the NF-κB signaling pathway, followed by γ-Toc, while α-Toc had no effect (Fig. 4B). In the presence of IFNγ-PMA challenge, however, we did not observe further stimulation of NF-κB nuclear translocation beyond that elicited by IFNγ-PMA alone (Fig. 4A). It is possible that the effect of IFNγ-PMA had maximized NF-κB activity and, therefore, that an additional effect of Toc treatment on NF-κB signaling only extended the duration of NF-κB activation, rather than further upregulating nuclear NF-κB signaling.
Nevertheless, we observed that γ- and δ-Toc upregulated NF-κB target genes, such as IL-8 and PTGS2, and downregulated Nrf2 target genes, such as GCLC and GSTA1, despite the absence of the IFNγ-PMA challenge. In addition, the effect of δ-Toc was most pronounced, followed by γ-Toc, while α-Toc had no effect, in modulating the gene expression of IL-8 and GCLC, a finding consistent with that observed in FHs 74 Int cells challenged with IFNγ-PMA.

**Modulation of cellular glutathione content.** On the basis of the finding that γ- and δ-Toc modulated GCLC (the gene that encodes for γ-glutamylcysteine synthetase, a rate-limiting enzyme in glutathione biosynthesis) to the greatest extent, we examined the effect of different Toc isoforms on regulation of γ-glutamylcysteine synthetase protein expression and glutathione content in Toc-treated FHs 74 Int cells. Treatment of FHs 74 Int cells with IFNγ-PMA resulted in a significant (P < 0.05) decrease in the expression of GCLC protein (Fig. 5). γ- and δ-Toc significantly reduced GCLC expression in FHs 74 Int cells stimulated with IFNγ-PMA (P < 0.05; Fig. 6). In the absence of IFNγ-PMA challenge, however, only δ-Toc significantly reduced glutathione expression in FHs 74 Int cells (Fig. 5).

The reduction of GCLC expression in FHs 74 Int cells was found to correspond to significant changes in cellular glutathione content (Fig. 6). Specifically, γ- and δ-Toc were effective in reducing the glutathione content of IFNγ-PMA-challenged FHs 74 Int cells. Similar to the trend observed with the changes in GCLC protein expression, only δ-Toc suppressed (P < 0.05) the glutathione content of FHs 74 Int cells in the absence of IFNγ-PMA challenge (Fig. 6).

**Modulation of apoptosis-mediated cytotoxicity.** Non-α-Toc isoforms exerted a cytotoxic effect on FHs 74 Int cells (Fig. 7) that was exacerbated by the addition of IFNγ-PMA. For example, whereas γ- and δ-Toc effectively reduced cell viability only at 100 µM, a minimum concentration of 10 µM was found to be sufficient to exert cytotoxic effects in FHs 74 Int cells that had been pretreated with IFNγ-PMA. In particular, δ-Toc reduced FHs 74 Int cell viability to the greatest extent, regardless of IFNγ-PMA stimulation. To determine whether programmed cell death or apoptosis was involved in the mechanism of cell death caused by different Toc isoforms, DNA fragmentation was assessed in FHs 74 Int cells using TUNEL assays (Fig. 8). Apoptosis was induced in IFNγ-PMA-challenged FHs 74 Int cells to the greatest extent by δ-Toc (Fig. 8), followed by γ-Toc, whereas α-Toc was ineffective. On the other hand, only δ-Toc elicited apoptosis-mediated cytotoxicity in FHs 74 Int cells in the absence of IFNγ-PMA stimulation.

**DISCUSSION**

Very recently, γ- and δ-Toc were shown to promote an inflammatory response in primary fetal-derived intestinal FHs 74 Int cells (19). In this study we relate the inflammatory response of FHs 74 Int cells exposed to equal concentration of Toc isoforms to the cellular concentration of Toc.

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Fig. 1. Cellular uptake of α-, γ-, and δ-tocopherol (Toc) in FHs 74 Int cells cultured under conditions used to evaluate Toc isoform modulation of the inflammatory response without (A) or with (B) IFNγ-PMA stimulation. Cells were treated with Toc isoforms for 24 h and then exposed for 24 h to replenished Toc isoforms in the presence or absence of IFNγ-PMA challenge. Different letters (a, b, c) indicate significant (P < 0.05) differences between Toc isoforms at the same concentration.

Fig. 2. Effect of different tocopherol isoforms on IL-8 secretion in FHs 74 Int cells in the presence (A) or absence (B) of IFNγ-PMA challenge. IL-8 was quantified from supernatants of cells that were treated with Toc isoforms prior to IFNγ-PMA challenge or no challenge. Different letters indicate significant (P < 0.05) differences between Toc isoforms at the same concentration. *P < 0.05 relative to control (19). In A, IL-8 in Toc-treated cells was compared with IL-8 in non-IFNγ-PMA-stimulated control cells (IL-8 = 1,067 pg/ml). In B, IL-8 in Toc-treated cells was compared with IL-8 in non-IFNγ-PMA-stimulated control cells (IL-8 = 173 pg/ml).
Fig. 3. Time-course activation of Nrf2 (A) and NF-κB (B) signaling by a mixture of IFNγ (4,000 U/ml) and PMA (0.05 µg/ml) in FHs 74 Int cells. Values are means ± SD (n = 3). *P < 0.05 vs. time 0.

Our approach enabled us to compare Toc isoform effects on inflammatory responses that occurred within a range of concentration-dependent uptakes for each isoform. The fact that Toc uptake by FHs 74 Int cells was isoform-dependent points to a specific structure-function activity for different Toc isoforms for cellular uptake. For example, cellular concentration-dependent uptakes for each isoform. The fact that Toc uptake by FHs 74 Int cells was isoform-dependent points to a specific structure-function activity for different Toc isoforms (e.g., δ-Toc > γ-Toc > α-Toc) promoted an inflammatory response in the primary fetal-derived intestinal FHs 74 Int cells. Thus it can be inferred that the relative uptake of different Toc isoforms contributed at least in part to the corresponding greater bioactivity of γ- and δ-Toc in FHs 74 Int cells.

In addition to the relative disparities in the extent to which different Toc isoforms were taken up by FHs 74 Int cells, we also observed specific Toc isoform-induced modification of the NF-κB signaling pathway in FHs 74 Int cells, NF-κB activation was promoted to the greatest extent by δ-Toc, followed by γ-Toc, while α-Toc was ineffective. This pattern of NF-κB activation corresponds to the stimulatory effect of Toc on IL-8 secretion in FHs 74 Int cells and confirms the indispensable role of the NF-κB signaling pathway in expression of the

Table 2. Effects of tocopherol isoforms on modulation of gene transcription in IFNγ-PMA-challenged FHs 74 Int cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>IFNγ-PMA</th>
<th>+ α-Toc</th>
<th>+ γ-Toc</th>
<th>+ δ-Toc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold Regulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRAK2</td>
<td>3.26</td>
<td>3.6a,b</td>
<td>4.0b</td>
<td>4.9c</td>
</tr>
<tr>
<td>NFKB1</td>
<td>4.66</td>
<td>5.4a,b</td>
<td>5.8a,b</td>
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<td>1.06</td>
<td>2.4b</td>
<td>2.9b</td>
<td>9.2c</td>
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<td>PTGS2</td>
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<td>10.3b</td>
<td>11.1b</td>
<td>27.3c</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td></td>
<td></td>
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<td>1.2a</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>1.2a</td>
<td>-1.8b</td>
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<tr>
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<td>1.2a</td>
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<tr>
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<td>-1.2a</td>
<td>-1.4a</td>
<td>-4.2b</td>
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<td>1.1a</td>
<td>-1.1b</td>
<td>-4.3c</td>
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<td>5.3b</td>
<td>5.6b</td>
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<td>3.1b</td>
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<tr>
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<td>28.9a</td>
<td>820.2b</td>
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<td>MAPK1</td>
<td>1.2a</td>
<td>1.2a</td>
<td>1.2a</td>
<td>-3.6b</td>
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*P < 0.05 differences between Toc isoforms; ND, not detectable.

Fig. 4. Modulation of NF-κB and Nrf2 activation by Toc isoforms in FHs 74 Int cells in the presence (A) or absence (B) of IFNγ-PMA challenge. Different letters indicate significant (P < 0.05) differences between Toc isoforms at the same concentration. NF-κB and Nrf2 were recovered from the nuclear fraction of cells that were treated with Toc isoforms and then challenged with IFNγ-PMA or left unchallenged for 90 min and 24 h, respectively. *P < 0.05; Toc-treated vs. IFNγ-PMA-treated cells. **P < 0.05, Toc-treated vs. untreated cells.
gene transcription in non-IFN

Effects of tocopherol isoforms on modulation of IL-8 secretion without promoting activation of NF-κB signaling and IL-8 secretion, our finding that was specific to the source of Toc isoform treatment. Analysis and, furthermore, that the duration of NF-κB activation reached a maximum nuclear translocation at the time of analysis and, furthermore, that the duration of NF-κB activation was specific to the source of Toc isoform treatment.

Despite the clear relationship between activation of NF-κB signaling and IL-8 secretion, our finding that α-Toc promoted IL-8 secretion without promoting activation of NF-κB signaling is difficult to explain. A possible role of Nrf2 signaling in proinflammatory cytokine IL-8. When we further challenged the cells using IFN-γ-PMA stimulation, NF-κB nuclear translocation was not further enhanced by Toc. Nevertheless, the finding that Toc modulates baseline IL-8 secretion regardless of IFN-γ-PMA challenge suggests that activation of NF-κB signaling did indeed occur. We suggest therefore that it is possible that NF-κB activation in IFN-γ-PMA-treated cells reached a maximum nuclear translocation at the time of analysis and, furthermore, that the duration of NF-κB activation was specific to the source of Toc isoform treatment.

Despite the clear relationship between activation of NF-κB signaling and IL-8 secretion, our finding that α-Toc promoted IL-8 secretion without promoting activation of NF-κB signaling is difficult to explain. A possible role of Nrf2 signaling in IL-8 expression in FHs 74 Int cells was thus determined. All Toc isoforms tested, including α-Toc, were found to enhance Nrf2 signaling in FHs 74 Int cells, with the most pronounced effect elicited by δ-Toc, followed by α- and γ-Toc, both of which were less effective. In the presence of an inflammatory challenge induced by IFN-γ-PMA treatment of FHs 74 Int cells, only δ-Toc enhanced Nrf2 activation. The current finding that γ- and δ-Toc promoted Nrf2 activation in FHs 74 Int cells while continuing to promote IL-8 expression was unexpected, since Nrf2 activation is largely believed to be associated with promotion of antioxidant enzyme expression. Our findings can be explained by results from an earlier study that reported an increase in Nrf2 expression associated with a concurrent increase in IL-8 expression, attributable to the stabilization of IL-8 mRNA (57). It is entirely possible, therefore, that the Nrf2 activation by different Toc isoforms in our study was due to the different abilities to stabilize IL-8 mRNA, which translated to a Toc isoform-dependent expression of IL-8 in FHs 74 Int cells.

The characteristic involvement of different Toc isoforms in modifying IFN-γ-PMA-induced activation of Nrf2 and NF-κB was confirmed by comparing the relative ability of Toc isoforms to modulate expression of several Nrf2 and NF-κB target genes. Consistent with the finding that γ- and δ-Toc promoted NF-κB translocation to the nucleus in FHs 74 Int cells was the observation that γ- and δ-Toc also enhanced the expression of NF-κB-related genes. We show evidence of a Toc isoform-dependent increase in the transcription of NF-κB target genes,
Int cells explains the observation that glutathione biosynthesis in FHs 74 Int cells. Isoforms tested in this study have the capacity to downregulate -Toc/H9251 FHs 74 Int cells. These findings confirm that non-expression, as well as lower the total glutathione content, of DNA fragmentation in FHs 74 Int cells when incubated with isoforms was mediated through apoptosis, as determined by its cytotoxic to FHs 74 Int cells. The cytotoxicity of these Toc treated with dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Cells were (A) or presence (B) of IFN-PMA challenge as determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Cells were treated with α-, γ-, and δ-Toc prior to challenge in the presence or absence of IFN-PMA. Different letters denote significant (P < 0.05) difference between α-, γ-, and δ-Toc at a given concentration. *P < 0.05, α-, γ-, and δ-Toc-treated vs. control cells.

such as IL-8 and PTGS2, that occurred regardless of IFNγ-PMA stimulation.

While the Nrf2 signaling pathway was activated in FHs 74 Int cells treated with different Toc isoforms, as shown by the increased translocation of Nrf2 to the nucleus, treatment of cells with γ- and δ-Toc resulted in downregulation of numerous Nrf2 target genes. The gene that was most prominently altered by Toc isoforms in our array was GCLC, a Nrf2 target gene (29, 30). GCLC is the first rate-limiting enzyme in the two-step reaction of glutathione biosynthesis, which catalyzes the ligation of glutamate and cysteine to form γ-glutamylcysteine (56). The addition of glycine to the dipeptide produces the tripeptide glutathione, which is regulated to maintain normal cellular antioxidant status (56).

Consistent with the effect of Toc isoforms on GCLC transcription, γ- and δ-Toc suppressed the expression of GCLC protein, which corresponded to a decreased glutathione content of IFNγ-PMA-challenged FHs 74 Int cells. In the absence of IFNγ-PMA, δ-Toc continued to reduce the GCLC protein expression, as well as lower the total glutathione content, of FHs 74 Int cells. These findings confirm that non-α-Toc isoforms tested in this study have the capacity to downregulate glutathione biosynthesis in FHs 74 Int cells.

Our finding that Toc induced glutathione depletion in FHs 74 Int cells explains the observation that γ- and δ-Toc were cytotoxic to FHs 74 Int cells. The cytotoxicity of these Toc isoforms was mediated through apoptosis, as determined by its DNA fragmentation in FHs 74 Int cells when incubated with γ- and δ-Toc, respectively (15). Glutathione, a low-molecular-weight thiol present at high concentrations in all mammalian cells, functions in the cellular defense system against the generation of reactive species and xenobiotic-derived electrophiles (26). Intracellular levels of glutathione represent a balance between synthesis and consumption, as well as extracellular transport (26). Glutathione depletion in cells is an early hallmark of apoptosis in response to various stimuli (24). Since glutathione reacts directly or catalytically with reactive species, a reduction of the intracellular glutathione content is associated with the level of oxidative stress, which induces apoptosis (24, 48).

A notable finding related to the downregulation of GCLC was the increase in Nrf2 translocation to the nucleus. One explanation for this finding concerns the fact that activation of NF-κB signaling could suppress the transcription of Nrf2 target genes, despite the increased translocation of Nrf2 to the nucleus. Activation of the NF-κB pathway was shown to antagonize Nrf2 signaling at the level of transcription through the p65 subunit of the NF-κB complex, which could deprive Nrf2 of cAMP response element-binding protein-binding protein (CBP; a common coactivator of NF-κB and Nrf2) (36) or promote recruitment of the corepressor histone deacetylase 3 to the CBP and Nrf2 dimerization partner MafK (36). Alternatively, if p65 interacts with Keap1, a repressor protein that facilitates ubiquitination of Nrf2 in the cytoplasm, then this interaction would lead to proteosomal degradation (55). During NF-κB activation, increased amounts of p65 transcription factor are translocated to the nucleus. Physical interaction between Keap1 and p65 could explain our finding of an increase in nuclear Keap1, which in turn facilitates the shutting of Keap1 from the nucleus to the cytoplasm to bind with Nrf2. The net effect would be repressed activity of antioxidant enzyme pathways.

Taken together, our findings show that γ- and δ-Toc, the dominant vitamin E isoforms found in infant formula, evoked a greater proinflammatory response in the fetal-derived intestinal cell line, than α-Toc, the dominant form of vitamin E in human milk. This may be of particular importance to formula-fed infants, who are at a relatively greater risk of developing neonatal necrotizing enterocolitis (37, 50), an inflammatory intestinal disorder.

The current finding that γ-Toc may promote an inflammatory response in the fetal-derived intestinal cell contradicts many studies that report the anti-inflammatory property of γ-Toc, including our finding in the adult-derived Caco-2 intestinal cell line (16, 19, 32). The anti-inflammatory activity of γ-Toc is touted to be superior to that of α-Toc, which is often attributed to the lack of a methyl group in the C-5 position in the chromanol ring (13, 28). Nevertheless, the ability of γ-Toc to oppose the anti-inflammatory effect of α-Toc has previously been reported (5, 12). In vitro, α-Toc was found to block, while γ-Toc was found to promote, VCAM-1-dependent lymphocyte transmigration. In combination, γ-Toc suppressed α-Toc inhibition of lymphocyte transmigration (5). The opposing effect of α- and γ-Toc was further confirmed in vivo, where α-Toc inhibits, while γ-Toc increases, leukocyte recruitment in the bronchoalveolar lavage and lung tissue during ovalbumin-induced lung inflammation in mice (5). An explanation for the apparent contradictory action of Toc to induce inflammation may involve Toc isoform-specific effects, as well as cell-specific responses. For example, Toc-induced lymphocyte mi-
Fig. 8. A: effect of 100 μM α-, γ-, and δ-Toc on apoptosis of FHs 74 Int cells as shown by flow cytometry dot plot (top) and summarized as percentage of total apoptotic cells (bottom). FHs 74 Int cells were treated with α-, γ-, and δ-Toc in the absence of IFNγ-PMA. Different letters denote significant difference (P < 0.05) between Toc isoforms. B: effect of 100 μM α-, γ-, and δ-Toc on apoptosis of FHs 74 Int cells as shown by flow cytometry dot plot (top) and summarized as percentage of total apoptotic cells. FHs 74 Int cells were treated with α-, γ-, and δ-Toc and then challenged with IFNγ-PMA. Different letters denote significant (P < 0.05) difference between Toc isoforms.
tration has only been observed in endothelial cells, but not lymphocytes, when exposed to α- and γ-Toc (5). Thus there is a clear need for more research on the specific roles of γ- and δ-Toc in regulating inflammatory events.

Conclusion. We have shown that γ- and δ-Toc evoke a greater inflammatory response in fetal-derived intestinal cell lines than α-Toc. Modification of the inflammatory response was attributed, at least in part, to the isoform-dependent uptake of Toc in FHS 74 Int cells and also to their characteristic affinities to alter Nr2f and NF-κB cell signaling pathways. γ- and δ-Toc specifically triggered NF-κB activation, which antagonized Nr2f-mediated transcription and resulted in a downregulation of glutathione biosynthesis. The depletion of glutathione content corresponded to apoptosis-mediated downregulation of glutathione biosynthesis. The depletion of glutathione content corresponded to apoptosis-mediated cytotoxicity, which was induced to a greater extent by γ-Toc and δ-Toc. γ- and δ-Toc, therefore, represent biologically active vitamin E forms in fetal-derived intestinal cells that can induce a proinflammatory effect. This finding should prompt further investigation into the safety of these vitamin E forms, which are present at high levels in infant formula compared with mother’s milk.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

I.E. and D.D.K. are responsible for conception and design of the research; I.E. and D.D.K. edited and revised the manuscript; I.E. and D.D.K. approved the final version of the manuscript.

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