Abstract

Vitamin (vit) E comprises 8 isoforms, of which only α-tocopherol (Toc) has been thoroughly investigated. Other vit E isoforms, particularly γ-Toc and δ-Toc, however are present in significant amounts in the North American diet. The effect of α-Toc, γ-Toc and δ-Toc in modulating oxidative status and inflammatory responses in adult-derived Caco-2 and fetal-derived FHs 74 Int intestinal cell lines were thus determined. Toc isoforms were effective antioxidants that protected against peroxyl radical-induced membrane oxidation in both cell lines in an isoform-dependent manner (δ-Toc>γ-To>α-Toc). Nevertheless, Toc isoforms exhibited differential modulation of inflammatory response in the two cell lines, in that Toc isoforms suppressed IFNγ/PMA-induced IL8 expression in Caco-2 cells, but promoted an inflammatory response in FHs 74 Int cells. Modulation of IL8 expression by Toc isoforms corresponded with an efficacy of Toc to modulate NfκB pro-inflammatory and Nrf-2 antioxidant enzyme signaling pathways. Non-α-Toc isoforms promoted Nrf-2 activation in both cell lines. Alpha-Toc and γ-Toc mitigated IFNγ/PMA-induced NfκB activation in Caco-2 cells while non-α-Toc isoforms promoted NfκB activation in FHs 74 Int cells. The pro-oxidant activity of δ-Toc corresponded to its lower ability to suppress IFNγ/PMA-induced IL8 expression and NfκB activation, but enhanced the Nrf-2 signal in Caco-2 cells. One key difference between the effect of Toc isoforms on modulation of NfκB and Nrf-2 signaling was that non-α-Toc isoforms down-regulated the gene expression of glutamate cysteine ligase, the rate-limiting enzyme in glutathione biosynthesis, in FHs 74 Int, but not in Caco-2 cells. This was supported by a reduced (P<0.05) glutathione content in FHs 74 Int cells after incubation with γ-Toc and δ-Toc that was not observed in Caco-2 cells. Downregulation of the glutathione content corresponded to the finding
that non-α-Toc isoforms can induce apoptosis-mediated cytotoxicity in FHs 74 Int, but not in Caco-2 cells. Taken together, Toc isoform-mediated modulation of the inflammatory response was not related to antioxidant activity, but rather was attributed to a cell-specific efficacy to modulate NfκB signaling that corresponded to depletion in glutathione content and apoptosis. Based on the current findings, non-α-Toc isoforms are biologically active forms of vit E and their effects on intestinal cells should not be overlooked.
Preface


Data from chapter 2.1 has been published in Elisia, I. and Kitts, D. D. Quantitation of hexanal as an index of lipid oxidation in human milk and association with antioxidant components. *Journal of Clinical Biochemistry and Nutrition* **2011**, *49* (3), 147-152. Ingrid Elisia conducted the experiments and wrote the manuscript. David D. Kitts critically read and revised the manuscript.

Presentation


Table of Contents

Abstract ........................................................................................................................................ ii
Preface ........................................................................................................................................ iv
Table of Contents ..................................................................................................................... vi
List of Tables ........................................................................................................................... xi
List of Figures ........................................................................................................................... xii
List of Abbreviations ................................................................................................................. xv
Acknowledgements .................................................................................................................. xx

1. General Introduction and Literature Review ................................................................. 1
   1.1. Introduction ..................................................................................................................... 1
   1.2. Literature Review .......................................................................................................... 2
       1.2.1. Chemistry of Tocopherol Isoforms ................................................................. 2
       1.2.2. Dietary Sources .................................................................................................. 5
           1.2.2.1. Food Sources .......................................................................................... 5
           1.2.2.2. Dietary Supplement ................................................................................. 5
           1.2.2.3. Effect of Vegetable Oil Processing ......................................................... 7
       1.2.3. Dietary Requirement ......................................................................................... 7
       1.2.4. Vitamin E in Infant Food .................................................................................... 8
       1.2.5. Absorption, Metabolism and Distribution of Tocopherol Isoforms ............... 8
           1.2.5.1. Absorption of Tocopherol ........................................................................ 8
           1.2.5.2. Distribution, Metabolism and Excretion of Tocopherol ...................... 9
           1.2.5.3. Cellular Uptake and Sub-cellular Localization ..................................... 10
       1.2.6. Oxidative Stress and Cellular Defense System .............................................. 11
           1.2.6.1. Reactive Species and Oxidative Stress .................................................. 11
           1.2.6.2. Reactive Species and Oxidative Damage .............................................. 12
           1.2.6.3. Reactive Species and Cellular Defense System .................................... 14
       1.2.7. Antioxidant Activity of Vitamin E .................................................................. 15
           1.2.7.1. Vitamin E as Antioxidants ..................................................................... 15
           1.2.7.2. Pro-oxidant Effect of Vitamin E .............................................................. 16
           1.2.7.3. Recycling of Vitamin E .......................................................................... 17
1.2.8. Biological Activity of Vitamin E .................................................................19
  1.2.8.1. Vitamin E and Cardiovascular Health .........................................................19
  1.2.8.2. Vitamin E and Cancer ..............................................................................21
  1.2.8.3. Why Vitamin E Fails ..............................................................................21
1.2.9. Inflammation ...............................................................................................22
  1.2.9.1. Inflammation and Oxidative Stress ............................................................23
  1.2.9.2. Intestinal Inflammatory Disease ................................................................24
  1.2.9.3. Interleukin 8 ............................................................................................25
  1.2.9.4. Nrf-2 Signaling Pathway ..........................................................................26
  1.2.9.5. NfκB Signaling Pathway ..........................................................................28
1.2.10. Apoptosis-mediated Cytotoxicity .............................................................30
1.2.11. Relative Bioactivity of Tocopherol Isoforms ...........................................32
  1.2.11.1. Antioxidant Activity of Tocopherol Isoforms .........................................32
  1.2.11.2. Antiproliferative Activity of Tocopherol Isoforms ...................................32
  1.2.11.3. Anti-inflammatory and Related Non-antioxidant Activity of Tocopherol
           Isoforms ..................................................................................................33
1.2.12. Cell Culture ...............................................................................................34
1.2.13. Review of Methods ..................................................................................35
  1.2.13.1. Measurement of Antioxidant Capacity In Vitro .......................................35
  1.2.13.2. Measurement of Oxidative Stress ............................................................36
  1.2.13.3. Measurement of Cell Viability .................................................................37
  1.2.13.4. Detection of Apoptosis .........................................................................37
  1.2.13.5. Detection of Nrf-2 and NfκB in Nucleus .................................................37
  1.2.13.6. Pathway-focused Gene Expression .........................................................38
  1.2.13.7. Inducer of Inflammation ......................................................................38
1.3. Research Hypothesis and Objectives .........................................................54
2. Characterization of Vitamin E in Foods and Their Antioxidant and Anti-inflammatory
   Activities .................................................................................................................60
  2.1. Characterization of Vitamin E Isoforms in Vegetable Oils and Infant Foods ......60
    2.1.1. Introduction ...............................................................................................60
    2.1.2. Materials and Methods ............................................................................61
3. Modulation of Nrf-2 and NfkB Signaling in Caco-2 and FHs 74 Int Cells by Tocopherol Isoforms ........................................................................................................................................88

3.1. Introduction ........................................................................................................................................................................88

3.2. Materials and Methods ..................................................................................................................................................................89
   3.2.1. Materials ................................................................................................................................................................................89
   3.2.2. Cell Culture ............................................................................................................................................................................90
   3.2.3. NfkB and Nrf-2 Activation .................................................................................................................................90
   3.2.4. Effect of Tocopherol Isoforms on Gene Transcription ..........................................................................................91
   3.2.5. Statistical Analysis .........................................................................................................................................................91

3.3. Results ..........................................................................................................................................................................................92
   3.3.1. Modulation of NfkB Activity .............................................................................................................................92
   3.3.2. Modulation of Nrf-2 Activity .................................................................................................................................93
   3.3.3. Tocopherol Modulation on Gene Transcription ..............................................................................................94

3.4. Discussion .......................................................................................................................................................................................96

4. Mechanistic Studies of Factors Contributing to Variable Inflammatory Responses Observed in Caco-2 and FHs 74 Int Cells ..................................................................................................................112

4.1. Introduction ................................................................................................................................................................................................112

4.2. Materials and Methods ..................................................................................................................................................................113
   4.2.1. Materials ................................................................................................................................................................................113
   4.2.2. Cell Culture ............................................................................................................................................................................114
   4.2.3. Cellular Uptake ....................................................................................................................................................................114
   4.2.4. Effect of Tocopherol on Oxidative Status of Cells Induced by IFNy/PMA ...............................................................114
   4.2.5. Effect of Tocopherol on Cell Viability .........................................................................................................................115
   4.2.6. Measurement of Apoptosis ........................................................................................................................................115
   4.2.7. Total Glutathione Content .......................................................................................................................................116
   4.2.8. Statistical Analysis.......................................................................................................................................................116

4.3. Results ..........................................................................................................................................................................................117
   4.3.1. Cellular Uptake ....................................................................................................................................................................117
   4.3.2. Effect of Tocopherol on Oxidative Status of Caco-2 Cells ..........................................................................................117
   4.3.3. Effect of Tocopherol on Oxidative Status of FHs 74 Int Cells ......................................................................................118
   4.3.4. Effect of Tocopherol on Glutathione Content ..............................................................................................................118
4.3.5. Effect of Tocopherol on Cell Viability ...............................................................118

4.4. Discussion ......................................................................................................................119

5. General Discussion and Conclusions ............................................................................144

5.1. Dietary Vitamin E Composition in Vegetable Oils ...................................................144

5.2. Vitamin E Isoforms Present in Infant Foods ............................................................144

5.3. Antioxidant and Anti-inflammatory Activities of Tocopherol Isoforms ..................145

5.4. Tocopherol Isoforms as Structural Elements of Cell Membrane ..............................146

5.5. Tocopherol Isoforms and IFNγ/PMA Cell Signaling Pathways .................................147

5.6. Membrane Composition and Pro-oxidant Activities of Tocopherol .........................148

5.7. Crosstalk between Nrf-2 and NfκB Isoforms ............................................................149

5.8. Antioxidant and Other Roles in Cellular Homeostasis .............................................150

5.9. Final Conclusion ........................................................................................................150

References ..........................................................................................................................152

Appendices .........................................................................................................................175

Appendix 1. Stability of Tocopherol Isoforms in Culture Media ................................. 175

Appendix 2. Fractionation Control Using Nuclear and Cytoplasmic Proteins ............. 177

Appendix 3. List of Genes Included in Custom Real Time PCR Array ..........................179

Appendix 4. Time Course of Activation of Nrf-2 and NfκB Signaling by IFNγ/PMA in Caco-2 and FHs 74 Int Cells ................................................................. 182
List of Tables

Table 1.1. Tocopherol Profile of Different Vegetable Oils. ........................................................... 6
Table 1.2. The Relative Antioxidant Activity of Tocopherol Isoforms........................................ 40
Table 1.3. The Relative Antiproliferative Activity of Tocopherol Isoforms................................. 44
Table 1.4. The Relative Anti-inflammatory Activity of Tocopherol Isoforms.............................. 48
Table 2.1. Uptake of Tocopherol Isoforms in Caco-2 and FHs 74 Int Cells. .............................. 87
Table 3.1. Effects of Tocopherol Isoforms on Modulation of Gene Transcription in Caco-2 cells
  Challenged with IFNγ/PMA.. ................................................................................................. 108
Table 3.2. Effects of Tocopherol Isoforms on Modulation of Gene Transcription in FHs 74 Int
  Cells Challenged with IFNγ/PMA.. ..................................................................................... 109
Table 3.3. Effects of Tocopherol Isoforms on Modulation of Gene Transcription in FHs 74 Int
  Cells Challenged without IFNγ/PMA.. ............................................................................... 111
Table 4.1. Highlight of the Effect of Toc Isoforms on Modulating Cellular Inflammatory
  Responses in Caco-2 and FHs 74 Int Cells................................................................. 143
List of Figures

Figure 1.1. Chemical Structure of Eight Vitamin E Isoforms ................................................................. 4
Figure 1.2. Recycling Mechanism of Tocopheroxyl Radical Involving Ascorbic Acid and Glutathione ............................................................................................................................................. 18
Figure 1.3. Basic Model of Nrf-2 Signaling Pathway in Cell System .......................................................... 27
Figure 1.4. Basic Model of NfκB Signaling Pathway in Cell System ........................................................... 30
Figure 2.1. Tocopherol Contents of Three Commercial Infant Formulae and Human Milk Samples Determined using HPLC with Fluorescence Detection ................................................. 67
Figure 2.2. Tocopherol Content of Commercially Available Vegetable Oils ........................................... 67
Figure 2.3. Antioxidant Activity of Tocopherol Isoforms Determined using Chemical-based ORAC Assay ......................................................................................................................................... 68
Figure 2.4. Ability of Tocopherol Isoforms to Prevent AAPH-induced Oxidative Stress Determined using DCFH-DA Probe in Caco-2 (A) and FHs 74 Int (B) Cells ....................... 83
Figure 2.5. Activity of Tocopherol Isoforms in Preventing AAPH-induced Oxidative Stress Determined using DPPP probe in Caco-2 (A) and FHs 74 Int (B) Cells ......................... 84
Figure 2.6. Ability of Tocopherol Isoforms to Modulate IL8 Secretion Stimulated by IFNγ/PMA in Caco-2 Cells ............................................................................................................................................. 85
Figure 2.7. Ability of Tocopherol Isoforms to Modulate IL8 Secretion Stimulated by IFNγ/PMA in FHs 74 Int Cells ............................................................................................................................................... 86
Figure 2.8. Ability of Tocopherol Isoforms to Modulate IL8 Secretion in FHs 74 Int Cells ................. 86
Figure 3.1. Tocopherol Isoforms Modulate NfκB Activation Induced by IFNγ/PMA in Caco-2 Cells ............................................................................................................................................. 104
Figure 3.2. Tocopherol Isoforms Modulate NfkB Activation Induced by IFNγ/PMA in FHs 74 Int Cells .......................................................... 105

Figure 3.3. Modulation of NfkB Activation by Tocopherol Isoforms in FHs 74 Int Cells ....... 105

Figure 3.4. Tocopherol Isoforms Modulate Nrf-2 Activation Induced by IFNγ/PMA in Caco-2 Cells at 24 Hours .......................................................... 106

Figure 3.5. Tocopherol Isoforms Modulate Nrf-2 Activation Induced by IFNγ/PMA Cocktail in FHs 74 Int Cells .......................................................... 107

Figure 3.6. Tocopherol Isoforms Modulate Nrf-2 Activation in FHs 74 Int Cells ............... 107

Figure 4.1. Cellular Uptake of Tocopherol Isoforms in Caco-2 Cells Stimulated with IFNγ/PMA and Cultured under Conditions Used to Evaluate Modulation of Inflammatory Responses .......................................................... 128

Figure 4.2. Cellular Uptake of Tocopherol Isoforms in FHs 74 Int Cells Cultured under Conditions Used to Evaluate Modulation of Inflammatory Response without (A) or with (B) IFNγ/PMA Stimulation .......................................................... 129

Figure 4.3. Effect of Tocopherol Isoforms on Oxidative Status of Caco-2 Cells Stimulated with IFNγ/PMA .......................................................... 130

Figure 4.4. Ability of Ascorbic Acid to Modulate Tocopherol-induced Changes in Oxidative Status of Caco-2 Cells Stimulated with IFNγ/PMA .......................................................... 131

Figure 4.5. Effects of Tocopherol Isoforms on Oxidative Status of FHs 74 Int Cells without (A) or with (B) IFNγ/PMA Stimulation .......................................................... 132

Figure 4.6. Ability of Ascorbic Acid to Modulate Tocopherol-induced Changes in Oxidative Status of FHs 74 Int Cells Stimulated with IFNγ/PMA .......................................................... 133
Figure 4.7. Ability of Ascorbic Acid to Modulate Tocopherol-induced Changes in Oxidative Status of FHs 74 Int Cells without IFNγ/PMA Stimulation ........................................ 134

Figure 4.8. Effect of 100 µM Tocopherol on IFNγ/PMA-treated Caco-2 Cells on Total (A), and Reduced (B) Glutathione Content After 24 Hours Incubation ................................. 135

Figure 4.9. Effect of 100 µM Tocopherol on Total (A) and Reduced (B) Glutathione Content of FHs 74 Int Cells Incubated for 24 Hours with or without IFNγ/PMA Stimulation.. 136

Figure 4.10. Effect of Tocopherol Isoforms on Caco-2 Cell Viability as Determined Using MTT Assay .............................................................................................................. 137

Figure 4.11. Effect of Tocopherol Isoforms on FHs 74 Int Cell Viability in the Absence (A) and Presence (B) of IFNγ/PMA as Determined Using MTT assay ................................. 138

Figure 4.12A. Effect of 100 µM Tocopherol on Apoptosis of FHs 74 Int Cells Cultured in the Absence of IFNγ/PMA .......................................................................................... 139

Figure 4.12B. Effect of 100 µM Tocopherol on Apoptosis of FHs 74 Int Cells Cultured in the Presence of IFNγ/PMA. ............................................................................................... 140

Figure 4.13. Proposed Mechanism for Non-α-Toc-induced Crosstalk Mechanism by which Activated NfkB Signaling Antagonizes Nrf-2 Signaling ........................................ 141

Figure 4.14. Alternative Proposed Mechanism for Non-α-Toc-induced Crosstalk Mechanism by which Activated NfkB Signaling Antagonizes Nrf-2 Signaling ................. 142
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>ascorbic acid</td>
</tr>
<tr>
<td>AAPH</td>
<td>2, 2' –azobis (2-amidinopropane) dihydrochloride</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>APAF-1</td>
<td>apoptosis protease activating factor-1</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
</tr>
<tr>
<td>ATBC</td>
<td>alpha tocopherol beta carotene cancer prevention</td>
</tr>
<tr>
<td>ATCC</td>
<td>american type culture collection</td>
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<tr>
<td>α-TTP</td>
<td>α-tocopherol transfer protein</td>
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<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>BID</td>
<td>BH3 interacting domain death agonist</td>
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<tr>
<td>β-PE</td>
<td>β-phycoerythrin</td>
</tr>
<tr>
<td>Caco-2</td>
<td>human intestinal adenocarcinoma cancer cell line</td>
</tr>
<tr>
<td>Caspase</td>
<td>cysteine-dependent aspartate-directed proteases</td>
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<tr>
<td>CBP</td>
<td>CREB binding protein</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<td>CEHC</td>
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<td>CHAOS</td>
<td>cambridge heart antioxidant study</td>
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<tr>
<td>cNOS</td>
<td>constitutive nitric oxide synthase</td>
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<td>COX2</td>
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<td>CRP</td>
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<td>CYP450</td>
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<td>DCFH-DA</td>
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<tr>
<td>DISC</td>
<td>death inducing signaling complex</td>
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<tr>
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<td>1, 1-diphenyl-2-picrylhydrazyl</td>
</tr>
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<td>DPPP</td>
<td>diphenyl-1-pyrenylphosphine</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ELISA</td>
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<td>eNOS</td>
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<td>extracellular signal-regulated kinases</td>
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<td>FADD</td>
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<td>glutathione</td>
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<td>IBD</td>
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<td>LDL</td>
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<td>minimum essential medium eagle</td>
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<td>Nrf2</td>
<td>nuclear factor -erythroid-derived 2-like 2</td>
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\(^1\text{O}_2\) singlet oxygen
\(\text{O}_2^-\) superoxide radicals
\(\text{OH}^-\) hydroxyl radicals
ORAC oxygen radical absorbance capacity
PARP poly (ADP-ribose) polymerase
PBS phosphate buffered saline
PGE2 prostaglandin E2
PI propidium iodide
PI3K phosphoinositide-3-kinase
PKB protein kinase B
PKC\(\alpha\) protein kinase C\(\alpha\)
PMA phorbol myristate acetate
PMN polymorphonuclear neutrophils
PP2A Protein phosphatase 2A
PPAR\(\gamma\) peroxisome proliferator activated receptor \(\gamma\)
PTGS2 prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase & cyclooxygenase)
PUFA polyunsaturated fatty acid
R• alkyl radical
RACK receptors for activated C-kinase
RDA recommended dietary allowance
RHD rel homology domain
RO2\(^-\) peroxyl radicals
ROS reactive oxygen species
SELECT selenium and vitamin E cancer prevention trial
<table>
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<tr>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
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<tr>
<td>TBST</td>
<td>tris-buffered saline-tween</td>
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<td>tumor necrosis factor alpha</td>
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<td>vitamin</td>
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<td>VLDL</td>
<td>very low density lipoprotein</td>
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</table>
Acknowledgements

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1 General Introduction and Literature Review

1.1 Introduction

Vitamin (vit) E refers to a group of 8 fat soluble compounds that consists of 4 tocopherol (α-, β-, γ-, δ-Toc) isoforms and 4 tocotrienol (α-, β-, γ-, δ-Tri) isoforms (Figure 1) (1). Since the discovery of vit E in 1922, of all isoforms α-Toc garnered the most interest as this isoform exhibits the greatest bioactivity in the rats gestation-resorption assay, and is the dominant circulating isoform in the body (2-4). For decades, the term vit E has been used interchangeably with α-Toc. In addition, the most established function of vit E is as a chain-breaking fat soluble antioxidant (5, 6). There has thus been intense research on the effect of α-Toc in providing health benefits in oxidative stress-related diseases, such as cardiovascular disease (CVD) and cancer (7-9). Results from many clinical trials however failed to demonstrate a protective effect of α-Toc supplementation, with some reporting instead harmful effects of high α-Toc intake (8, 10, 11).

As a consequence to the mainstream interest on α-Toc, the potential biological significance of non-α-Toc isoforms has been overlooked, resulting in a paucity of information on their bioactivity when compared to α-Toc in the literature. Gamma-Toc however is the primary vit E isoform that is consumed in North America attributable to the large consumption of soybean oil in the region (12, 13). In addition, an appreciable amount of δ-Toc is present in soybean oil, suggesting the potential relevance of both γ-Toc and δ-Toc in eliciting a biological response upon consumption (14). At the same time, other functions of vit E such as anti-inflammatory activity, antiproliferative and modulation of cell signaling that is independent of its antioxidant activity have been discovered in the past two decades (7, 15-17).
Although there is currently a growing interest on γ-Toc, there is still a paucity of information available on the bioactive function of non-α-Toc isoforms particularly δ-Toc, relative to α-Toc. Investigation on the potential effect of Toc isoforms on intestinal cells is particularly relevant since the intestine is exposed to dietary vit E prior to absorption, which leads to liver metabolism of non-α-Toc isoforms (18, 19).

The general objective of this work therefore is to determine the relative antioxidant and anti-inflammatory activities of vit E isoforms, as well as their efficacy in modulating stress-responsive cell signaling pathways, using cultured cell lines representing both adult and infant intestinal cells as the model system. Specifically, the questions raised were whether the antioxidant activity of vit E isoforms in a chemical assay translated to antioxidant protection against free radical scavenging activity in a cell system. Secondly, whether the antioxidant activity of the vitamers corresponded to anti-inflammatory activity, and thirdly whether vit E isoforms affected two major stress-responsive pathways, nuclear factor κB (NfκB) and nuclear factor -erythroid-derived 2-like 2 (Nrf-2) signaling, involved in inflammatory responses. Lastly, mechanisms elucidating the observed effect of vit E isoforms on cellular response were proposed.

1.2 Literature Review

1.2.1 Chemistry of Tocopherol Isoforms

The chemical structures of Toc isoforms is shown in Figure 1.1. Toc isoforms are distinct from tocotrienols in that Toc isoforms contain a saturated phytol ‘tail’, while tocotrienols are attached to an unsaturated phytol tail. The four Toc or tocotrienol isoforms are α-, β-, γ- and δ-
isoforms; which are distinguished from one another by the degree of methylation as well as the position of the methyl groups located at specific sites on the chromanol ‘head’. The α isoform contains three methyl groups attached to a chromanol ring; β isoform has one less methyl group at C7 position, while γ isoform is unmethylated at C5 position (Figure 1.1). On the other hand, δ isoform is methylated with only one methyl group at the C8 position (Figure 1.1; (20)). The structure of Toc isoforms also results in characteristic differences in polarity of the isoforms, as supported by different partition coefficients; δ-Toc is the most hydrophilic with α-Toc being the most hydrophobic (21, 22).
Figure 1.1. Chemical Structure of Eight Vitamin E Isoforms.
1.2.2 Dietary Sources

1.2.2.1 Food Sources

Vit E is present in a wide range of food sources that include nuts, grain and vegetables (23). The major dietary source of vit E however, is vegetable oil (24). Depending on the source of the vegetable oil, the profile of Toc isoforms may differ (Table 1.1). For example, soybean oil being the most commonly consumed vegetable oil in North America contains γ-Toc as the predominating Toc isoform (12-14).

1.2.2.2 Dietary Supplement

Another potentially significant source of vit E is dietary supplements. The frequently found vit E form in dietary supplements is all-rac-α-tocopheryl acetate, which comprises 8 stereoisoforms attributed to the three chiral centers on the phytyl chain of vit E (4, 25, 26). Since α-Toc acetate is esterified on the hydroxyl group in the C6 position of the chromanol ring that contributes to the antioxidant activity of vit E, esterified α-Toc such as all-rac-tocopheryl acetate does not exhibit free radical scavenging activity (25, 27). Nevertheless, the synthetic esterified form is more stable than the naturally occurring form and has thus been used extensively in dietary supplements and in many clinical trials (26, 28). It should be noted that the ester group on the vit E can be hydrolysed during absorption which consequently generates the free α-Toc form that exhibits antioxidant activity (25, 26).
Table 1.1. Vitamin E Profile of Different Vegetable Oils

<table>
<thead>
<tr>
<th>Oil</th>
<th>Vitamin E Isoform Concentration(^1)</th>
<th>(\alpha)-Toc</th>
<th>(\beta)-Toc</th>
<th>(\gamma)-Toc</th>
<th>(\delta)-Toc</th>
<th>(\alpha)-Tri</th>
<th>(\beta)-Tri</th>
<th>(\gamma)-Tri</th>
<th>(\delta)-Tri</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola</td>
<td></td>
<td>21.0</td>
<td>0.1</td>
<td>4.2</td>
<td>0.04</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Castor</td>
<td></td>
<td>2.8</td>
<td>2.9</td>
<td>11.1</td>
<td>31.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coconut</td>
<td></td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Corn</td>
<td></td>
<td>11.2</td>
<td>5.0</td>
<td>60.2</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cottonseed</td>
<td></td>
<td>38.9</td>
<td>-</td>
<td>38.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Olive</td>
<td></td>
<td>11.9</td>
<td>-</td>
<td>0.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Palm</td>
<td></td>
<td>25.6</td>
<td>-</td>
<td>31.6</td>
<td>7.0</td>
<td>14.6</td>
<td>3.2</td>
<td>28.6</td>
<td>6.9</td>
</tr>
<tr>
<td>Palm Kernel</td>
<td></td>
<td>6.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Peanut</td>
<td></td>
<td>13.0</td>
<td>-</td>
<td>21.4</td>
<td>2.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rice Bran</td>
<td></td>
<td>32.4</td>
<td>1.8</td>
<td>5.3</td>
<td>-</td>
<td>23.6</td>
<td>-</td>
<td>34.9</td>
<td>-</td>
</tr>
<tr>
<td>Safflower</td>
<td></td>
<td>34.2</td>
<td>-</td>
<td>7.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sesame</td>
<td></td>
<td>13.6</td>
<td>-</td>
<td>29.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Soybean</td>
<td></td>
<td>7.5</td>
<td>1.5</td>
<td>79.7</td>
<td>26.6</td>
<td>0.2</td>
<td>0.1</td>
<td>-</td>
<td>0.03</td>
</tr>
<tr>
<td>Sunflower</td>
<td></td>
<td>48.7</td>
<td>-</td>
<td>5.1</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Walnut</td>
<td></td>
<td>56.3</td>
<td>-</td>
<td>59.5</td>
<td>45.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wheat germ</td>
<td></td>
<td>133.0</td>
<td>71.0</td>
<td>26.0</td>
<td>27.1</td>
<td>2.6</td>
<td>18.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\)Values represent mg vit E isoforms/100 gram of product (14)
1.2.2.3 Effect of Vegetable Oil Processing

The vit E profile of vegetable oil is dependent on numerous factors that include the genotype of the plant, the environmental growth and harvest condition, as well as the processing and storage condition (29). Upon being harvested, crude oil is subjected to a refining process that may include degumming, neutralization, bleaching and deodorization aimed to produce an oxidatively stable and acceptable product for the consumer (29, 30). During vegetable oil processing, Toc isoforms can be lost to the greatest extent during the deodorization step where oil is subjected to steam distillation to remove volatile impurities (30). Nevertheless, the pattern of Toc found in the oil after refining remains similar to that of the crude oil (29).

1.2.3 Dietary Requirement

The occurrence of vit E deficiency in humans is very rare, and has never been described to occur due to a diet poor in vit E (31). Nevertheless, vit E deficiency is usually associated with other condition such as fat malabsorption syndromes or genetic abnormality in α-tocopherol transfer protein (α-TTP) (31). Similarly, there is no evidence for an adverse effect of vit E consumption from food. The recommended daily allowance (RDA) for adults between 19 to 50 years old is 15 mg per day, while an adequate intake for infants from birth to six months is 4 mg of vit E. The tolerable upper intake level for adults aged 19 years and older is 1000 mg per day. It should be noted that the current recommendation for vit E intake, as set by the Institute of Medicine, relies heavily on studies on α-Toc and does not include other Toc isoforms present in the diet (31).
1.2.4 Vitamin E in Infant Food

The main dietary source of vit E for infants is human milk and/or infant formula. The predominant vit E isoform in human milk is α-Toc with a minor amount of γ-Toc (32), which is reflective of that found in human plasma as a result of vit E metabolism. On the other hand, infant formula which is constituted of bovine-derived milk, typically consists of blended vegetable oil(s) as the source of fat (33, 34). Some of the most commonly listed vegetable oils as ingredients in infant formula include soybean oil, palm oil and high oleic safflower or sunflower, which can be sources of non-α-Toc isoforms. In addition to the naturally occurring vit E in vegetable oils, infant formulas are generally supplemented with α-tocopherol acetate, which is the synthetic, esterified form of vit E that exhibits no antioxidant activity (27). Lastly, with the growing awareness of ω-3 fatty acid requirements for infants, formula companies supplement their product with this long chain polyunsaturated fatty acid, which results in an increased susceptibility to oxidative rancidity. Associated with the polyunsaturated fatty acid (PUFA) supplementation is the addition of vit E that contributes to increased total Toc content (35).

1.2.5 Absorption, Metabolism and Distribution of Tocopherol Isoforms

1.2.5.1 Absorption of Tocopherol

Despite the high consumption of mixed Toc isoforms in the North American diet, α-Toc remains as the major Toc isoform identified in body fluids and tissues, owing to the presence of an α-TTP in the liver and the different metabolic fates of non α-Toc isoforms (1). Absorption of dietary Toc isoforms is facilitated by formation of biliary micelles in the intestine, where Toc isoforms largely enter the enterocytes through passive diffusion. However, specific transporters
have recently been identified in the transport of vit E across the enterocytes, including scavenger receptor class B type I and ATP-binding cassette transporter A1 (36, 37); the latter is involved in the secretion of Toc from the intestine to the High Density Lipoproteins (HDL) (37).

1.2.5.2 Distribution, Metabolism and Excretion of Tocopherol

Following absorption, Toc isoforms along with chylomicrons travel to the liver (38), where the metabolic fate of Toc isoforms is determined based on relative binding affinities to α-TTP. Liver α-TTP, having the greatest affinity for α-Toc, facilitates the repackaging of α-Toc into Very Low Density Lipoprotein (VLDL) for further distribution to peripheral tissues (19). For comparison, the relative affinity of liver α-TTP to β-, γ- and δ-Toc has been reported to be 38%, 9% and 2% of that for α-Toc, respectively (19). While α-Toc is preferentially retained in the circulation, other Toc isoforms including β-, γ- and δ-Toc are metabolized by cytochrome P450 (CYP450), an enzyme that is central in xenobiotic metabolism. The phytol chain of Toc are initially subjected to omega hydroxylation followed by sequential removal of two- or three-carbon moieties to yield the water soluble 2,2′-carboxyethyl hydroxychromans (CEHC) metabolites that are subsequently excreted in the urine or bile (1, 18). Consequently, the circulating level of α-Toc is reasonably dominant at 11-37 µM, while that of γ-Toc and δ-Toc are generally present in much lower concentration at 2-5 µM (39). The higher concentration of α-Toc is thus likely the contributing factor for the higher bioactivity of this isoform in the rat fetal resorption assay. It is important to note that the activity of CYP450 can also be regulated by Toc isoforms. An increased level of α-Toc, for example results in a decreased level of γ-Toc that was attributed to the up-regulation of the CYP450 pathway by α-Toc leading to the metabolism of γ-Toc to γ-CEHC (40).
Despite the higher plasma level of α-Toc, studies from in vivo experiments suggest that γ-Toc and α-Toc are absorbed comparably well at the intestinal level (41, 42). A similar conclusion was reached regarding the relative absorption of synthetic all rac-α-tocopheryl acetate compared to the natural RRR-α-Toc (25). Upon being absorbed, Toc isoforms were secreted from the intestinal cells with chylomicrons with no apparent biodiscrimination to the liver. Tissue distribution of α-Toc in rats showed the greatest accumulation in the liver, skeletal muscle, and adipose tissue (43). On the other hand, γ-Toc was shown to be present in the highest concentration in the adipose tissue, with intermediate concentrations in heart, kidney and muscle while liver and plasma contained the lowest concentration (42).

1.2.5.3 Cellular Uptake and Sub-cellular Localization

The cellular uptake, absorption and distribution of vit E have been investigated mostly for α-Toc. In human endothelial and mouse macrophages cell culture models exposed to equimolar concentration of Tocs, non-α-Toc isoforms were preferentially taken up by cells than α-Toc (44, 45). The higher cellular content of γ-Toc is supported by the finding that colonic intestinal cells collected from freshly passed human stools contained greater γ-Toc concentration than α-Toc (46).

The sub-localization of Toc isoforms in the cell has also been studied most extensively for α-Toc. Using cell culture models, α-Toc was shown to be present at the highest concentration at the microsomal membrane, followed by the mitochondrial fraction and nuclear and cytosolic fractions (47, 48). Interestingly, the cellular distribution of Toc appears to correlate with the lipid content of the subcellular fraction, suggesting that vit E localization is associated with lipid distribution in cells (47). The finding that vit E was most concentrated in microsomal and
mitochondrial fractions is consistent with previous observations showing high concentrations of vit E in the heart of miniature swine and liver of rat (49, 50). In adipocyte tissue recovered from humans, 99% of the Toc was shown to be accumulated in the bulk lipid instead (41). Although several Toc binding proteins have been reported (e.g. α-tocopherol transfer protein and cytosolic tocopherol associated protein), the exact mechanism by which Toc is transported intracellularly is still unclear (47, 51).

1.2.6 Oxidative Stress and Cellular Defense System

1.2.6.1 Reactive Species and Oxidative Stress

Reactive oxygen species (ROS) have been implicated in the pathogenesis of many diseases, including cardiovascular disease, cancer and diabetes (52). ROS is a term that is used to describe both non-radical oxygen species and oxygen-derived free radicals (53). A free radical is any species capable of independent existence that contains one or more unpaired electrons. Examples of oxygen-derived free radicals are hydroxyl radical (OH•), superoxide radical (O2•⁻) and peroxyl radical (RO2•). Non-radical reactive species, on the other hand are strong oxidants that may easily be converted to free radicals (53). Example of non-radical reactive oxygen species are hydrogen peroxide (H2O2) and singlet oxygen (¹O2). In addition, the importance of reactive nitrogen species (e.g. peroxynitrite) is increasingly acknowledged to be involved in inflammatory events that underlie disease progression (54). Other biologically relevant reactive species include carbon-, chlorine- and sulfur-centered radicals (55).

Free radical and non-radical-reactive species are highly reactive compounds that upon interaction with various biomolecules can initiate oxidative damage to protein, DNA and lipid
components of cells (56). This accumulation of oxidative damage is thought to alter the biochemical and physiological function of cells that may contribute to the development of the ailment (53). It should be noted, however, that reactive species are constantly generated as byproducts of cellular metabolism (57). In fact, specific reactive species such as H$_2$O$_2$ and nitric oxide are now recognized to play critical roles in cell signaling mechanisms that are important for proper functioning of the cell (58).

The level of reactive species in cells is managed by a number of antioxidant enzymes and low molecular mass antioxidants that constitute the cell defense system (59). An antioxidant is defined as ‘any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevent oxidation of that substrate’ (55). Antioxidant enzymes such as glutathione peroxidase (GPX), catalase and superoxide dismutase and low molecular mass antioxidants such as vit E and C neutralize the excess generation of reactive species (60). In the event redox signaling and control is disrupted, however, production of reactive species may overwhelm cell defense and repair systems, which in turn results in an imbalanced state commonly referred to as oxidative stress (61).

1.2.6.2 Reactive Species and Oxidative Damage

During oxidative stress, reactive species may inflict injury to major cellular key components such as DNA, protein and lipid molecules. Free radical attack to DNA, for example may lead to DNA strand breakage, base lesions as well as DNA-protein crosslinks and DNA adducts with reactive lipid oxidation products (62). These oxidative DNA alterations have been associated with DNA mutation that leads to carcinogenesis (63). Oxidative modification of protein includes oxidation of amino acid residues and cleavage of peptide bonds leading to the
formation of carbonyl derivatives and protein cross-linking (64). Carbonyl derivatives can also be generated from the interaction of the oxidized protein with lipid or carbohydrate oxidation products. The oxidized protein is generally dysfunctional which may disrupt cellular function (65). Free radical reactions with the lipid component of cells result in the formation of lipid hydroperoxide which may further be broken down to secondary oxidation products including reactive aldehydes (e.g. malondialdehyde (MDA)), alkanes, lipid epoxides and alcohols (66). Oxidative damage to membrane lipids may interfere with cell signaling, function and survival (67).

Of all radicals produced in a biological system, lipid radicals exhibit one of the longest half-lives (10^6 to 10^9 times compared to alkoxy and hydroxyl radical, respectively) (68). Lipid oxidation therefore has a greater potential to induce oxidative damage due to the prolonged exposure of lipid-derived radicals to biomolecules when compared to other reactive species (69).

Lipid oxidation is classically divided into three stages; initiation, propagation and termination. In the initiation stage, a free radical abstracts a hydrogen atom from the lipid molecule, resulting in the formation of an alkyl radical (L•). The alkyl radical then enters the propagation stage where more lipid radicals are generated. The alkyl radical is oxidized to form the peroxyl radical (LOO•), which may abstract a hydrogen atom from a nearby molecule resulting in the formation of another lipid radical. Alternatively, the peroxyl radical may dissociate to form an alkyl radical and the highly reactive hydroxyl radical. Lipid oxidation thus continues to propagate and only terminates when lipid radicals interact with each other to form non-radical products. A simplified description of lipid oxidation is summarized in Equation 1-6 (68, 70).
**Initiation**

\[ R^\bullet + LH \rightarrow RH + L^\bullet \text{ (Equation 1)} \]

**Propagation**

\[ L^\bullet + O_2 \rightarrow LOO^\bullet \text{ (Equation 2)} \]

\[ LOO^\bullet + LH \rightarrow LOOH + L^\bullet \text{ (Equation 3)} \]

**Termination**

\[ 2 LOO^\bullet \rightarrow \text{Non-radical products (Equation 4)} \]

\[ L^\bullet + LOO^\bullet \rightarrow \text{Non-radical products (Equation 5)} \]

\[ 2 L^\bullet \rightarrow \text{Non-radical products (Equation 6)} \]

1.2.6.3 *Reactive Species and Cellular Defense System*

To cope with chronic and elevated exposure of reactive species, cells are equipped with a host of antioxidant defense mechanisms that collectively protect against oxidative stress and the consequent oxidative damage-related injuries (55). Many of the antioxidants that constitute the cellular defense system, such as the antioxidant enzymes and other low molecular mass antioxidants such as glutathione and uric acid can be synthesized *de novo*. On the other hand, a range of low molecular mass antioxidants such as vit E and vit C need to be obtained from the diet (55). The primary defense against reactive species comprises antioxidants that prevent the
initiation of a free radical chain reaction thus maintaining the redox balance (59). This is achieved by suppressing the formation of reactive oxygen or nitrogen species, for example, by chelation of transition metals (e.g. iron and copper) that can be involved in free radical generation, or by breaking down hydrogen peroxide and lipid hydroperoxide to water and lipid hydroxides, respectively (59). Examples of antioxidants in this category are antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase, as well as ascorbic acid that can act as both reducing and metal chelating agents. The second line of defense includes scavenging antioxidants that neutralize reactive species and prevent free radical-induced oxidative damage to cellular components. Alpha-Toc is a classic example of a chain-breaking free radical scavenger. In the event that oxidative damage takes place, the third line of defense consists of enzymes that repair or remove damaged biomolecules in effect to restore the lost function (59).

1.2.7 Antioxidant Activity of Vitamin E

1.2.7.1 Vitamin E as Antioxidant

Vit E is antioxidant, the efficacy of which is attributed to a peroxyl radical scavenging activity that terminates the chain reaction of lipid oxidation (39). The antioxidant activity of vit E has been most extensively established for α-Toc. Alpha-Toc is an effective lipid soluble antioxidant as it reacts faster with a peroxyl radical than the reaction between a peroxyl radical and nearby lipid molecules (55). Upon acting as an antioxidant by donating a hydrogen atom to the peroxyl radical, it forms a resonance stabilized tocopheroxyl radical (71). The tocopheroxyl radical may further interact with another lipid peroxyl radical thus producing non-radical
products (Equation 7-8). One Toc molecule is thus technically capable of neutralizing two peroxidation reactions \((71, 72)\).

\[
\alpha\text{-TocH} + \text{LOO}^• \rightarrow \alpha\text{-Toc}^• + \text{LOOH} \quad \text{(Equation 7)}
\]

\[
\text{LOO}^• + \alpha\text{-Toc}^• \rightarrow \alpha\text{-TocOOL} \quad \text{(Equation 8)}
\]

### 1.2.7.2 Pro-oxidant Effect of Vitamin E

Despite the well established antioxidant activity, the pro-oxidant activity of vit E has also been reported to occur under certain conditions. The proposed mechanisms by which vit E turns into a pro-oxidant are related to its tocopheroxyl radical. One of the most frequently proposed mechanisms is that the \(\alpha\)-tocopheroxyl radical, when present at high concentration may abstract a hydrogen atom from an unoxidized lipid or lipid hydroperoxides to generate alkyl and peroxy radicals, respectively \((\text{Equation 9-10, }71)\).

\[
\alpha\text{-Toc}^• + \text{LH} \rightarrow \alpha\text{-TocH} + \text{L}^• \quad \text{(Equation 9)}
\]

\[
\alpha\text{-Toc}^• + \text{LOOH} \rightarrow \alpha\text{-TocH} + \text{LOO}^• \quad \text{(Equation 10)}
\]

The reactivity of these reactions of \(\alpha\)-tocopheroxyl radical is much lower than that for a peroxyl radical reaction with lipids or with unoxidized \(\alpha\)-Toc, suggesting that reactions of the tocopheroxyl radical with lipids or its hydroperoxide is unlikely when unoxidized \(\alpha\)-Toc is still available for oxidation reactions \((55, 71)\). Nevertheless, studies on low density lipoprotein (LDL) oxidation have suggested that \(\alpha\)-Toc may accelerate LDL oxidation under mild free radical conditions \((73)\). The explanation given for this observation is that the tocopheroxyl radical is unable to escape from the lipid fraction of LDL which forces it to oxidize the lipid within the LDL particle \((71)\).
Other mechanisms proposed for the pro-oxidant activity of α-Toc include the ability of Toc to reduce the oxidation state of transition metals (e.g. Cu$^{2+}$, Fe$^{3+}$ Equation 11-12), which can be involved in Fenton and Fenton-like reactions resulting in generation of reactive species and pro-oxidative state (Equation 13-14, (71)).

\[
\text{TocH} + M^{(n+1)^+} \rightarrow \text{Toc}^\prime + H^+ + M^{n^+} \quad \text{(Equation 11)}
\]

\[
\text{Toc}^\prime + M^{(n+1)^+} \rightarrow \text{Toc}^+ + M^{n^+} \quad \text{(Equation 12)}
\]

Fenton reaction:

\[
\text{H}_2\text{O}_2 + M^{n^+} \rightarrow \text{OH}^\prime + \text{OH}^- + M^{(n+1)^+} \quad \text{(Equation 13)}
\]

Fenton-like reaction:

\[
\text{LOOH} + M^{n^+} \rightarrow \text{LO}^\prime + \text{OH}^- + M^{(n+1)^+} \quad \text{(Equation 14)}
\]

1.2.7.3 Recycling of Vitamin E

The presence of co-antioxidants along with vit E may influence the antioxidant activity of the vitamin (71). The most efficient interaction demonstrated so far is the synergistic effect between α-Toc and ascorbic acid (59). Upon being oxidized to tocopheroxyl radical, ascorbic acid may reduce the oxidized form of α-Toc to its unoxidized form, thus restoring its antioxidant activity (Equation 15,(39)). Ascorbic acid for example was shown to prevent the pro-oxidant activity of α-Toc in LDL particles (73, 74).

\[
\text{Toc}^\prime + \text{AH} \rightarrow \text{TocH} + A^\prime \quad \text{(Equation 15, (39))}
\]
Other hydrogen donors such as glutathione or ubiquinol may also participate in the recycling mechanism of vit E (59, 71). Glutathione for example may reduce oxidized ascorbic acid which in turn is used in the regeneration of the tocopherol (Figure 1.2, (75)).

Figure 1.2. Recycling Mechanism of Tocopheroxyl Radical Involving Ascorbic Acid and Glutathione. LOO* = peroxyl radical, GSH = reduced glutathione, GSSG = oxidized glutathione (75).
The interaction between vit E and vit C was first pointed out by Tappel et al in 1968 (76). Although the synergistic effect between vit E and C in acting as antioxidants was later substantiated in various in vitro model systems, evidence for the interaction of the two antioxidants in vivo has been difficult to demonstrate (77-80).

1.2.8 Biological Activity of Vitamin E

Vit E was discovered as a vitamin essential for reproduction in rats (2). The effect of different Toc isoforms in the biological assay became the basis by which vit E activity was measured and expressed as International Units (IU) (3). One milligram of α-Toc is equivalent to 1.49 IU, while 1 mg of all rac-α-Toc acetate, a synthetic form of α-Toc consisting of 8 stereoisomers of esterified α-Toc, is defined as 1 IU (3). On the other hand, γ- and δ-Toc isoforms exhibit only 10% and 3% of α-Toc bioactivity, respectively (71).

The most well-established function of vit E is its property to act as a chain-breaking antioxidant in biological systems (5). Major interests in the potential ability of α-Toc to reduce the risk of many oxidative stress-related diseases, has thus resulted in numerous clinical trials designed to demonstrate the efficacy of α-Toc in mitigating the incidence of various major diseases (81-83). In particular, the relationship between α-Toc consumption and cardiovascular health and cancer prevention has been most investigated.

1.2.8.1 Vitamin E and Cardiovascular Health

Atherosclerosis involves the accumulation of oxidized LDL on the arterial wall that leads to inflammation of the artery resulting in the progression of atherosclerotic lesions (84). The
ability of a compound to prevent LDL oxidation has thus been related to the extent of atherosclerosis (8). Alpha-Toc was shown to dose-dependently suppress LDL oxidation in vitro, which provides a basis for further pursuing the possibility of vit E intake and mitigation of CVD risk (85). Additional support for the use of α-Toc in CVD was established in a cell culture model, where α-Toc was shown to be effective in mitigating endothelial activation attributed to high level of LDL and pro-inflammatory cytokines (86, 87). Also, there were evidences for a positive effect of α-Toc on endothelium vascular function in animal models of atherosclerosis (84, 88).

The evidence for a beneficial effect of α-Toc intake on prevention of heart disease in human studies has proven to be inconsistent (8). The relationship between vit E intake and reduced risk for heart disease is supported mostly by observational studies. On the other hand, intervention studies on the relationship between α-Toc intake and prevention of heart disease produced inconclusive findings (8, 89, 90). For example, supplementation of a high dose (800 IU/day) of α-Toc to chronic hemodialysis patients with pre-existing CVD for a median of 510 days reduced multiple cardiovascular end-points and myocardial infarctions (81). Similar reduced risk of non-fatal myocardial infarction was observed in the Cambridge Heart Antioxidant Study (CHAOS) with vit E supplementation at 400 or 800 IU/day to 2002 patients with coronary artery disease for 510 days (91). Nevertheless, other studies failed to demonstrate a relationship between α-Toc intake and prevention of heart disease end point measures (8, 92, 93). In fact, α-Toc supplementation at 400 IU/day to patients with high risk of CVD was associated with a significantly increased total number of heart failure events in the Heart Outcomes Prevention Evaluation (HOPE) study when compared to the placebo group (89, 93). In the follow-up study (HOPE-The Ongoing Outcomes), the number of patients in the vit E group hospitalized for heart failure was significantly higher than the placebo group (93).
1.2.8.2 *Vitamin E and Cancer*

There has also been a lack of evidence for the efficacy of α-Toc as an anti-cancer agent (7). For example, α-Toc acetate supplementation at 50 mg/day to 29,133 male smokers for five to eight years was shown to reduce prostate cancer by 32% while exhibiting no effect on lung cancer in the Alpha Tocopherol Beta Carotene Cancer Prevention (ATBC) trial (94). At the same time, serum α-Toc was found to be associated with reduced prostate cancer in current or recently former smokers (95). On the other hand, one of the largest clinical trials (the Selenium and Vitamin E Cancer Prevention Trial/SELECT) ensuing the ATBC study involves the supplementation of 400 IU/day all rac-α-Toc to 35,533 men for an intended period of 7 to 12 years (9, 11). The study was discontinued after 7 years due to negative findings and a non-significant ($P = 0.06$) increase in prostate cancer incidence in the vit E supplemented group (9, 11). Similarly, limited evidence is available on the potential cancer preventive effect of α-Toc on other types of cancer including colon and breast cancer from intervention studies (7). There was however increasing demonstration that γ-Toc or γ-Toc-enriched Toc mixtures were effective in suppressing cancer cell proliferation as demonstrated using cell culture and animal models (7, 96).

It should be noted that in the ATBC study, death from hemorrhagic stroke was increased by 50% among male smokers (94). In addition, a meta-analysis of 19 clinical trials on the relationship between vit E consumption and mortality suggests that α-Toc supplementation may increase the risk of all-cause mortality (10).

1.2.8.3 *Why Vitamin E Fails*
One of the hypotheses as to why α-Toc supplementation did not yield a generally favorable outcome in many clinical trials is related to the observation that increasing α-Toc intake reduces the concentration of circulating γ-Toc in vivo (97). Gamma-Toc is considered superior to α-Toc for antioxidant activity, primarily due to the lack of a methyl group at the C5 position of the chromanol ring. This structural difference results in the affinity of γ-Toc to be nitrated and to scavenge reactive nitrogen species in addition to reactive oxygen species (Figure 1.1) (20). The finding that increased α-Toc levels in vivo translates to a decreased γ-Toc concentration, further leads to the viewpoint that γ-Toc might be the most active vit E form. Consequently, there is now increased interest in the specific functionalities of non-α-Toc isoforms, particularly γ-Toc, or mixed Toc isoforms composed predominantly of γ-Toc.

Other possible explanations for the lack of efficacy of vit E in the prevention of oxidative stress-related diseases is that the dosage of the Toc used was either suboptimal or too high such that it would elicit a pro-oxidant effect instead (8). Also, there is a lack of clarity regarding the type of vit E used in the clinical trials, as the term vit E was not defined to specify whether the synthetic all-rac-α-tocopheryl acetate, or natural α-Toc were used (28, 98). The synthetic form of α-Toc demonstrates lower biological activity when compared to the natural α-Toc, which therefore may influence the overall efficacy of Toc (31).

1.2.9 Inflammation

Inflammation is a protective mechanism against a host of offending agents and tissue injuries which typically leads to regeneration of tissue structure and restoration of cell function (99). It can generally be classified into acute and chronic inflammatory response based on the
profile of inflammatory cells present in tissues. In the acute inflammatory response, a complex reversible biochemical event ensues relatively immediate after injury, which generally begins with activation of endothelial cells followed by the loosening of the endothelial tight junction. This results in protein and fluid leakage to the extravascular compartment. Activated endothelial cells express specific adhesion molecules and release pro-inflammatory cytokines and chemokines, combined effects of which result in the attachment of activated polymorphonuclear neutrophils (PMN) to the vascular endothelium. The opening of endothelial cell junctions facilitates the transmigration of the PMN to the extravascular compartment. Another event encountered during acute inflammation is activation of platelets and a clotting cascade that contributes to intravascular thrombosis. Lastly, hemorrhage may occur in the event of structural damage to the endothelial barrier. Inflammation is resolved when the trigger for the inflammatory response is removed (99).

The acute inflammatory response may turn into a chronic inflammatory response when the offending stimulus is persistent, resulting in a change in the types of inflammatory cells present in the tissue. Chronic inflammatory response is characterized by the presence of lymphocytes, monocytes that differentiate to macrophages and plasma cells in the tissue instead of granulocytes (PMN) present in acute inflammatory response. Lymphocytes and macrophages release pro-inflammatory cytokines that propagate the inflammatory response and stimulate fibroblasts to form collagenous scar tissue (99).

Although the inflammatory response is generally beneficial to the host, an excessive or dysregulated inflammatory response has been associated with the pathogenesis of various diseases such as atherosclerosis and inflammatory bowel disease. Neutrophils or the macrophages recruited to the injured tissue, for example, utilize reactive species and enzymes
that facilitate the digestion of cell debris and pathogens \((99, 100)\). A prolonged inflammatory response therefore could damage the host tissue that may otherwise not occur if inflammation is resolved appropriately.

1.2.9.1 Inflammation and Oxidative Stress

Oxidative stress and inflammation are often implicated together in the etiology of disease progression \((101-103)\). To date, no consensus has been reached whether a causal relationship between oxidative stress and inflammation exists. Some consider oxidative stress as an upstream component in signaling events leading to inflammatory response, while others view oxidative stress as a byproduct of inflammation \((102, 104)\). Despite the lack of clear mechanism linking oxidative stress and inflammation, there is evidence that specific components of inflammatory events are modulated by oxidative stress. For example, NfκB is a central regulator of inflammatory events that is also redox sensitive \((104, 105)\). At the same time, inflammatory cells such as macrophages and leukocytes release reactive species that may induce oxidative stress during inflammatory events \((99, 100)\). Oxidative stress therefore appears to be intertwined with inflammatory response.

1.2.9.2 Intestinal Inflammatory Disease

Inflammatory bowel disease (IBD) and necrotizing enterocolitis (NEC) are inflammatory conditions of the adult and infant intestine, respectively, where oxidative stress is implicated in the inflammatory response \((101, 106)\). Although the exact mechanism involved in the development of the conditions is currently unclear, both NEC and IBD are multifactorial diseases constituted of genetic and environmental factors associated with excessive inflammatory responses \((107)\). Both are also associated with altered immune response to commensal
microbiota, and compromised intestinal barrier integrity \((108, 109)\). In the case of NEC, immaturity in innate immune function of the intestine has been related to the hypersensitive inflammatory response \((110)\). NEC is also characterized by high levels of interleukin (IL) 8 that is attributable in part to under-expression of IKK, a regulator of the NFκB transcription factor that mediates the expression of pro-inflammatory cytokines \((108, 110)\). Nevertheless, the role of reactive species in intestinal inflammation has been suggested in both NEC and IBD \((101, 106, 111)\). Excessive inflammation may elicit free radical-induced oxidative damage to intestinal mucosa which leads to increased intestinal permeability that results in foreign antigens or pathogens entering mucosal tissues and invoking further inflammatory response \((109, 112)\). In addition, recruitment of activated neutrophil and macrophages in the inflammatory sites releases pro-inflammatory cytokines that exacerbate inflammation \((99, 109)\).

1.2.9.3 Interleukin 8

One of the cytokines expressed in high amount during inflammatory response is interleukin 8 (IL8), a chemoattractant that amplifies inflammation by recruitment of neutrophils to the injured site \((113)\). IL8 is barely secreted from non-stimulated cells but its expression can be triggered by a wide range of inducers that include oxidative stress and stimulation by pro-inflammatory cytokines \((114)\). There are numerous signaling pathways that may contribute to the synthesis of IL8. One mechanism is the de-repression of the IL8 gene promoter that initiates IL8 synthesis. Another indispensable factor involved in the regulation of IL8 production is the activation of the NFκB transcription factor and other signaling pathways such as the JUN-N-terminal protein kinase (JNK) pathway. Moreover, IL8 protein expression is enhanced via stabilization of IL8 mRNA by activation of the p38 Mitogen Activated Protein Kinase (MAPK) pathway \((114)\). In addition to the commonly reported signaling pathways involved in IL8
expression, activation of the nuclear factor -erythroid-derived 2-like 2 (Nrf-2) signaling pathway was found to increase the half-life of IL8 mRNA that was accompanied by only a weak increase in IL8 transcription (115). It should be noted that many of the signaling pathways involved in IL8 protein regulation, including NfkB and Nrf-2 pathways, are activated in response to oxidative stress, which becomes a factor that influences IL8 expression.

1.2.9.4 Nrf-2 Signaling Pathway

Nrf-2 is a transcription factor involved in the stress-response mechanism that up-regulates the expression of antioxidant enzymes and phase II detoxification enzymes (116). Nrf-2 is bound to a repressor protein, Kelch-like ECH-associated protein 1 (Keap-1) in the cytoplasm that keeps the transcription factor in the cytoplasm thus preventing it from binding to the antioxidant response element (ARE) and activating the Nrf-2 pathway. In the event of oxidative stress, the Nrf-2 translocates to the nucleus, to bind to the ARE and up-regulates the expression of antioxidant enzymes that in turn facilitates the cell’s coping mechanism against oxidative stress (117). Enzymes in which expression is mediated by Nrf-2 include glutamate-cysteine ligase (GCLC), glutathione-S-transferase, heme-oxygenase, and many others (116). A basic mechanism of Nrf-2 signaling pathway under basal and activated state is illustrated in Figure 1.3.
There are numerous proposed mechanisms that describe the activation of the Nrf-2 signaling pathway (117, 118). The ‘sequester and release’ model suggests that oxidants or electrophiles modify the conformation of cysteine-rich Keap-1 resulting in the release Nrf-2 from Keap-1 in the cytoplasm. Nrf-2 is then free to shuttle to the nucleus and up-regulate the expression of antioxidant enzymes. Another model of Nrf-2 activation proposes that the Nrf-2 transcription factor is expressed and degraded continuously resulting in a low basal level of Nrf-2 protein. Keap-1 is an adaptor protein to the Cullin 3 (Cul3)-based ubiquitin ligase that targets Nrf-2 for ubiquitination and subsequent proteosomal degradation. Pro-oxidants however are able to cause oxidative modification to Keap-1 resulting in the dissociation of Cul3 from Keap-1 and consequently preventing Nrf-2 from being degraded. As a result, the stability of Nrf-2 is increased and more Nrf-2 can bind to the ARE in the nucleus and activate Nrf-2 responsive
genes. One of the more recent models currently gaining recognition is the ‘hinge and latch’ model, where monomeric Nrf-2 binds with dimeric Keap-1 through the ETGE motif and DLG motif present on the structure of Nrf-2. The binding affinity of Keap-1 to the ETGE motif in Nrf-2 is approximately twice as strong as that of Keap-1 to the DLG motif, resulting in the two binding sites being referred to as the ‘hinge’ and ‘latch’, respectively. The presence of inducers leads to an oxidative modification of the cysteine residues in Keap-1 leading to the release of the weaker DLG interaction to Keap-1. Consequently, Nrf-2 is loosely bound to the Keap-1 through the ‘hinge’ binding site resulting in ineffective ubiquitination of Nrf-2 and degradation of the transcription factor. At the same time, newly synthesized Nrf-2 is unable to interact with the Keap-1 dimer as the binding site in Keap-1 is still occupied by latched Nrf-2. Taken together, a greater amount of free Nrf-2 leads to greater activation of the ARE-dependent genes transcription. It should be noted that in all the above proposed mechanisms, activation of the Nrf-2 signaling pathway involves an increased nuclear presence of Nrf- which results in Nrf-2 interaction with ARE and subsequent up-regulation of Nrf-2 mediated transcription of antioxidant enzymes (116-118).

1.2.9.5 NfkB Signaling Pathway

Another stress-responsive transcription factor modulating expression of pro-inflammatory responses is NfkB signaling (119). The NfkB pathway can be triggered by many stimuli, including pro-inflammatory compounds such as the lipopolysaccharide (LPS) and tumor necrosis factor- α (TNF-α), as well as an elevated level of oxidative stress (119, 120). The activation of NfkB results in the up-regulation of target genes encoding chemo- and cytokines, apoptotic regulatory proteins and those involved in promoting further pro-inflammatory responses. NfkB is a complex of structurally related proteins that consist of p50, p52, p65
(RelA), c-Rel, and RelB. Proteins of the NfκB family share a 300 amino acids sequence referred to as the Rel Homology Domain (RHD) that consist of a dimerization, DNA binding domain and nuclear localization signal (NLS). Only RelA, c-Rel and RelB contains transactivation domains. RelA and p50 can be found in a wide array of cell types while that of RelB is only expressed in highly specific sites. C-Rel in particular is only found in hematopoietic cells and lymphocytes \((120)\). In addition, p50 and p52 are synthesized as the inactive precursors NfκB1/ p105 and NfκB2/ p100, respectively. Upon activation, the precursors undergo proteolytic processing that cleaves the C-terminal inhibitory domains allowing the active transcription factors to translocate to the nucleus. Under basal conditions, the NfκB is present in homo- or hetero-dimers in the cytoplasm, bound to NfκB inhibitory proteins called the IκB. The IκB proteins possess 6-7 ankyrin repeats, consisting of 33 amino acid sequences that mediate the binding of IκB proteins to the NfκB dimers and mask their nuclear localization signals \((120)\). As a result, IκB proteins keep NfκB dimers in an inactive state in the cytoplasm. IκB proteins however can be phosphorylated by activated IκB kinase that leads to the ubiquitination and the subsequent proteasomal degradation of IκB. As a result, NfκB proteins are free to translocate to the nucleus and bind with the NfκB binding sites to up-regulate the genes it encodes \((120)\). A simplified representation of NfκB signaling pathway is illustrated in Figure 1.4.
1.2.10 Apoptosis-mediated Cytotoxicity

Another biological activity possibly modulated by Toc isoforms is cell viability. Two of the most prominent mechanisms of cell death are apoptosis or programmed cell death and necrosis. Apoptosis is a ‘self-destruct’ cell death mechanism characterized by membrane blebbing, cell shrinkage, chromatin condensation, and formation of apoptotic bodies (121). In addition, one of the hallmarks of apoptosis is the pre-lytic DNA fragmentation that leads to the formation of oligonucleosomes by endonucleases activated during apoptosis events (122). The process results in minimum inflammatory damage to surrounding tissue as macrophages recognize the apoptotic cells and engulf them out of the circulation (121). On the other hand, necrotic cells suffering from major insults swell followed by cellular rupture that leads to the release of cell contents and inflammatory events (121, 122).
Apoptosis can be induced by two main routes; the mitochondrial-mediated pathway (intrinsic) and death-receptor pathway (extrinsic). Common to apoptosis events is the activation of a series of cysteine-dependent aspartate-directed proteases (caspase) from their inactive form (pro-caspase) to its active cleaved form (caspase). There are initiator caspases (including caspase 2, 8, 9, and 10) which occurs early in apoptosis process and effector caspases (including caspase 3, 6, and 7) which trigger the apoptosis events (121).

In the mitochondrial-mediated pathway, apoptosis is initiated by alteration of membrane permeability that leads to the release of apoptogenic proteins such as cytochrome c, apoptosis-inducing factors and endonuclease G (121, 123). Often associated with the change in membrane permeability are the Bcl-2 family proteins which exhibit both pro-apoptotic (e.g. Bax, Bad, Bid) and anti-apoptotic (e.g. Bcl-2, Bcl-XL) activities (121). Upon being released, cytochrome c binds with apoptosis protease activating factor-1 (APAF-1) as well as pro-caspase 9, which results in the cleavage of pro-caspase 9 to caspase 9. In the death-receptor pathway, apoptosis begins upon the binding of a ligand to death receptors located on the cell surface, followed by association with an adaptor protein called the Fas-associated death domain (FADD). Pro-caspase 8 also interacts with the protein complex at the receptor resulting in the formation of the death inducing signaling complex (DISC) that leads to the activation of caspase 8. The active form of caspase 8 and caspase 9 cleaves pro-caspase 3 to caspase 3, an effector caspase which then execute the apoptosis events (121, 123). It should be noted that caspase 8 can also cleave Bid to truncated Bid (t-Bid), which may then translocate to mitochondria and initiate the mitochondria-mediated apoptosis route, therefore linking the two apoptosis pathways (121).
1.2.11 Relative Bioactivity of Tocopherol Isoforms

1.2.11.1 Antioxidant Activity of Tocopherol Isoforms

While all Toc isoforms exhibit free radical scavenging activities, the extent to which individual Toc isoforms act as antioxidants appears to be isoform-specific. Based on the chemical structure, the relative antioxidant activity of vit E isoforms is expected to follow the order of $\alpha$-Toc $>$ $\gamma$-Toc $>$ $\delta$-Toc. This is because substitution at ortho- and para- position to the hydroxyl function increases the electron density of the active center, which not only results in greater potency to donate hydrogen, but also greater stability of the tocopheroxyl radical as the substitution hinders further reaction of the phenoxy radical \((71)\). Nevertheless, the opposite trend of antioxidant activity of Toc isoforms ($\delta$-Toc $>$ $\gamma$-Toc $>$ $\alpha$-Toc) has also been reported in various in vitro model systems \((27, 71, 124, 125)\). The opposite behavior of Toc in different model systems is not entirely understood at this point, but it should be noted that the efficacy of antioxidants is dependent on many factors including the reactivity of the antioxidant to specific reactive species, the concentration, mobility and localization of the antioxidant, as well as the presence of other co-antioxidants in cells \((55, 59, 71)\). In biological systems, additional factors may influence the overall bioactive function of Toc isoforms, such as cellular uptake and subsequent metabolism. The results obtained from cell culture, animal and clinical trials on the comparative antioxidant activity of Toc analogs have thus been inconsistent \((Table 1.2)\).

1.2.11.2 Antiproliferative Activity of Tocopherol Isoforms

Amongst all of the newly discovered bioactive functions of Toc isoforms, the antiproliferative activity of vit E isoforms consistently shows notable relevance in a variety of cell culture models. The general trend observed with the antiproliferative activity of Toc
isoforms is that δ-Toc is the most cytotoxic, followed by γ-Toc, while α-Toc has the least or no effect, on cell proliferation (Table 1.3). Another consistent finding in the literature is that the cytotoxic effects of Toc isoforms are only observed in cancer cells, while primary cells derived from normal tissues are impervious to Toc isoform cytotoxicity. Current research findings on the antiproliferative activity of γ- and δ-Toc isoforms that are cytotoxic to cancer cells, but not to normal cells, suggest the possible use of Toc isoforms as an effective chemotherapeutic agent for targeting specific cancers. The antiproliferative activity of Toc isoforms was demonstrated by Tasinato et al (1995) to be independent of their antioxidant function. In the case of α-Toc and β-Toc with comparable antioxidant activities, only α-Toc was found to inhibit protein kinase C α (PKCα) activity (15); this was associated with growth inhibition of cultured smooth muscle cells (126).

1.2.11.3 Anti-inflammatory and Related Non-antioxidant Activity of Tocopherol Isoforms

Earlier reports demonstrating the anti-inflammatory activity of Toc isoforms were based on the observation that γ-Toc was superior to α-Toc in scavenging reactive nitrogen species (127, 128). The anti-nitrative effect of γ-Toc is postulated to be due to the lack of a methyl group at the C5 position that was otherwise occupied in α-Toc, thereby allowing γ-Toc to be nitrated at the C5 position (20). One of the major findings triggering interest in non-α-Toc isoform is that γ-Toc is more effective than α-Toc in suppressing prostaglandin E2 (PGE2) from LPS-activated macrophage cells (129). A recent finding however demonstrates that the anti-inflammatory activity of different Toc isoforms may be not only due to their relative ability to act as antioxidant and anti-nitrative agents, but also by modulation of cell signaling independently of an antioxidant action (130). The relative anti-inflammatory activity of different Toc isoforms reported in the literature thus far is highly inconsistent; this is largely due to the fact that
different model systems have been used, with differences in end-point measures tested, as well as concentrations and exposure times to different isoforms. There appears however to be a trend demonstrating the efficacy of γ-Toc enriched mixed Toc isoforms in acting as inflammatory agents \((131, 132)\). A summary of recent findings on the anti-inflammatory activities of Toc isoforms is summarized in Table 1.4.

Based on current literature on the bioactivity of different Toc isoforms, it is clear that the functionality of Toc isoforms is not limited to antioxidant activity as initially discovered. Rather, other non-antioxidant functions that include antiproliferative as well as anti-inflammatory activities are also relevant. By far, the antiproliferative effect of Toc isoforms is supported by compelling evidence that δ-Toc is generally mostly cytotoxic, followed by γ-Toc, while α-Toc is the least or not cytotoxic. It is also important to note that the cytotoxicity of Toc isoforms is mostly cancer-cell specific, which further supports its potential use as a chemo-preventive or chemotherapeutic agent. On the other hand, the relative antioxidant and anti-inflammatory activity of Toc isoforms reported in the literature is less consistent, albeit there is a trend exhibiting the potential efficacy of non-α-Toc isoforms individually or in mixtures as antioxidant or anti-inflammatory agents.

1.2.12 Cell Culture

Two cultured intestinal cell lines were used in this study. Caco-2 is a human adenocarcinoma cell line of adult origin, which undergoes spontaneous differentiation when cultured for 21 days \((133, 134)\). Differentiated Caco-2 cells are extensively used as an intestinal model system as they exhibit morphological characteristics that are similar to those of the small
intestine (135, 136). On the other hand, FHs 74 Int is a normal cell line that originates from the small intestine of a 3-4 month old fetus (137). Caco-2 and FHs 74 Int therefore are used as cell culture model system representing adult and infant intestinal cells, respectively.

1.2.13 Review of Methods

1.2.13.1 Measurement of Antioxidant Capacity In Vitro

Numerous assays measuring antioxidant capacity have been reported in the literature (59, 138). The antioxidant assays can be generalized based on the antioxidant mechanism that is measured by the assay. The two main antioxidant mechanisms commonly examined are the ability of a compound to donate hydrogen or to donate an electron (138). The efficacy of a compound to act as a hydrogen donor is related with peroxyl radical scavenging relevant to mitigation of lipid oxidation. On the other hand, electron donating ability is related with reducing activity that may prevent the initiation of free radical reactions (138).

One of the most extensively used assays that measures the antioxidant capacity of a compound is the Oxygen Radical Absorbance Capacity (ORAC) assay (59). ORAC employs a fluorescent probe and a free radical initiator to establish the peroxyl radical scavenging activity of a compound (139). The probe that was originally employed in the ORAC assay was beta phycoerythrin (β-PE), a large molecular weight fluoresceing protein which when oxidized loses fluorescence (140). The probe was then replaced with fluorescein, as the fluorescence of β-PE was reported to vary from lot-to-lot, was sensitive to photobleaching and the presence of phenolic compounds caused interference (139, 141). The free radical initiator 2, 2’ –azobis (2-amidinopropane) dihydrochloride (AAPH) is used as a source of peroxyl radical (139, 142).
AAPH is an azo compound that thermally decomposes to two nitrogen-centered radicals which upon reaction with oxygen form peroxyl radicals (143). Exposure of the fluorescein probe to 2, 2’–azobis (2-amidinopropane) dihydrochloride (AAPH) results in oxidation of the fluorescein and a loss of fluorescence intensity. Antioxidants capable of donating hydrogen atoms however may neutralize the peroxyl radical which in turn inhibits the fluorescence decay over time. The relative ability of a compound in preventing the loss of fluorescence intensity over a set period of time is expressed relative to Trolox, a water soluble analog of vit E, as a reference compound (139). The fact that ORAC measures the hydrogen donating ability to a peroxyl radical is considered relevant for measuring the antioxidant activity of vit E isoforms.

1.2.13.2 Measurement of Oxidative Stress

Intracellular oxidation can be measured primarily by a dichlorofluorescein diacetate (DCFH-DA) probe. DCFHDA is non-fluorescent water soluble probe that when taken up by cells is cleaved by an intracellular esterase to form the non-fluorescent dichlorofluorescein (DCFH) compound (144). Upon reacting with reactive species, DCFH is oxidized and converted into the fluorescent dichlorofluorescin (DCF). The generation of fluorescence can then be measured using a luminometer at an excitation wavelength of 485 nm and emission wavelength of 527 nm.

Another probe used to measure oxidative stress in this study was selected for its hydrophobic nature. Diphenyl-1-pyrenylphosphine (DPPP) is a membrane soluble probe that upon uptake is incorporated in the cell membrane rather than in the aqueous phase of the cell (145, 146). Lipid oxidation occurring in cell membrane generates lipid hydroperoxide which then oxidizes the non-fluorescent DPPP to DPP-oxide. The generation of fluorescence can then be measured at an excitation wavelength of 350 nm and emission wavelength of 380 nm (145).
1.2.13.3 Measurement of Cell Viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay measures cell viability based on the mitochondrial activity of live, metabolizing cells (147). MTT dye (yellow) is converted by mitochondrial succinate dehydrogenase to purple formazan crystal which can be quantified spectrophotometrically. Intensity of the purple color formation is a measure of cell viability while cells that do not convert the yellow MTT dye are not viable.

1.2.13.4 Detection of Apoptosis

To determine if apoptosis or programmed cell death is a possible mechanism mediating cell death, the TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay is used. The TUNEL assay detects apoptosis based on the presence of DNA strand breaks that are formed during the process of apoptosis (148). The 3’hydroxyl end is labeled with bromolated deoxyuridine triphosphates (BrdUTP), which is then recognized by fluoresceinated anti-BrdU antibodies that yield green fluorescent signals. Apoptotic cells therefore produce a positive TUNEL signal, while non-apoptotic cells remain unstained. In addition, propidium iodide (PI) that intercalates with DNA was used for DNA staining allowing cell cycle analysis to be performed at the same time (123).

1.2.13.5 Detection of Nrf-2 and NfkB in Nucleus

Activation of the Nrf-2 and NfkB pathways are measured by determining the relative presence of the transcription factors in the nuclear fraction of cells. Cells are initially fractionated to nuclear and cytoplasmic fractions. The abundance of the Nrf-2 or NfkB (p65 subunit) transcription factor in the nuclear fraction is then measured using an enzyme-linked immunosorbent assay (ELISA)-based assay (149). Nuclear extract is added to wells coated with
immobilized oligonucleotide containing the consensus binding site specific to the transcription factor. Primary antibody recognizing an epitope on the transcription factor is added, followed by the addition of horse radish peroxidase-conjugated secondary antibody which allows a colorimetric detection of bound transcription factor/DNA in the test wells (149).

1.2.13.6 Pathway-focused Gene Expression

To determine the effect of treatments at the transcription level, the mRNA levels of a specific protein of interest are determined. Isolated mRNA is converted to its complementary DNA, which is then added to a commercial 96 well plate pre-coated with primers of the gene of interest in its individual wells (150). A cocktail of mastermix containing DNA polymerase, free nucleotides and SYBR-green dye to detect the formation of DNA are added to the wells and real time PCR is performed. The level of mRNA transcript determines the number of cDNA copies, which then determines the minimum amount of heating cycles required to amplify the DNA.

1.2.13.7 Inducer of Inflammation

In this thesis, a cocktail of interferon gamma (IFNγ) and phorbol myristate acetate (PMA) is used to stimulate an inflammatory state in the cultured intestinal cell lines. Interferon gamma is a cytokine released by activated immune cells and functions to stimulate phagocytic cells (e.g. macrophage and natural killer cells) that facilitate the removal of intracellular pathogens (99, 151). The mechanism by which IFNγ elicits cellular response is by the JAK-STAT signaling pathway, which becomes activated upon binding of IFNγ to the IFNγ receptor on the cell membrane (152). JAK (Janus kinase) is associated with the IFNγ receptor, which then transphosphorylate each other and the cytoplasmic domain of the receptor creating a docking site for STAT1. The transcription factor becomes phosphorylated, forms a homodimer and is
released from the docking site. The STAT1 homodimer then becomes phosphorylated and travels to the nucleus to interact with DNA and initiate gene transcription (152). Recently however, IFNγ was reported to elicit its biological response through other pathways that work together or in parallel with the classic JAK-STAT signaling cascade (151). Other kinases such as phosphoinositide-3-kinase (PI3K), PKC isoforms, and mitogen activated protein kinase (MAPK) have also been proposed to be involved in IFNγ signaling (151, 152). In addition, other pathways such as NfkB activation have also been reported to mediate STAT1-independent signaling (151).

PMA is a tumor promoter that modulates a wide range of cellular responses including cell growth, immune response and programmed cell death through activation of PKC (153). Upon activation by PMA, PKC translocates to the membrane where it stimulates the various signaling cascades responsible for eliciting the cellular response (154, 155). A signaling pathway relevant to activation of inflammatory response is the up-regulation of NfkB pathway (153).

Despite demonstrating different primary mechanisms in eliciting a biological response, both IFNγ and PMA have been used to induce inflammation in cell culture models. Combined together, IFNγ and PMA were shown to be more effective in inducing inflammation than when individual compounds were used, as evidenced by increased nitric oxide secretion from differentiated Caco-2 cells (134).
Table 1.2. The Relative Antioxidant Activity of Tocopherol Isoforms *

<table>
<thead>
<tr>
<th>Model</th>
<th>End-point Measure</th>
<th>Concentration/Exposure Time</th>
<th>α</th>
<th>γ</th>
<th>δ</th>
<th>Mixed</th>
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<td><strong>Chemical Assay</strong></td>
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<tr>
<td>Bulk menhaden oil</td>
<td>Propanal</td>
<td>1.0 mmol/kg oil</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
<td>δ-Toc &gt; α-Toc in surface activity</td>
<td>(156)</td>
</tr>
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<td></td>
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<td></td>
<td>δ-Toc &gt; α-Toc in polarity</td>
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<tr>
<td>Menhaden oil in water</td>
<td>Propanal</td>
<td>0.5 mmol/kg oil</td>
<td>+</td>
<td>++</td>
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<td>DPPH assay</td>
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<td>+</td>
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<tr>
<td>ORAC assay</td>
<td></td>
<td></td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td></td>
<td></td>
<td>(27)</td>
</tr>
<tr>
<td>Human milk</td>
<td>Hexanal</td>
<td></td>
<td>+</td>
<td>0</td>
<td>0</td>
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<tr>
<td><strong>Cell Culture/Ex-Vivo</strong></td>
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<tr>
<td>Cerebral synaptosomes from rat + AAPH</td>
<td>Isoprostane Neuroprostane</td>
<td>1, 10, 100 μM 15 min</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>(157)</td>
</tr>
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<td>Liver hepatocytes from rat</td>
<td>MDA</td>
<td>20 μM 1h</td>
<td>+++</td>
<td>++</td>
<td>+</td>
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<td>antioxidant against cumene-OOH-induced LPO parallels↑ in cytotoxicity</td>
<td>(158)</td>
</tr>
<tr>
<td></td>
<td>MDA</td>
<td>100 μM 1h</td>
<td>-</td>
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<td>---</td>
<td></td>
<td>pro-oxidant against H₂O₂-induced LPO, γ- and δ-Toc &gt; α-Toc in causing cytotoxicity</td>
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<tr>
<td>Model</td>
<td>End-point Measure</td>
<td>Concentration/Exposure Time</td>
<td>α</td>
<td>γ</td>
<td>δ</td>
<td>Mixed</td>
<td>Comments</td>
<td>Ref.</td>
</tr>
<tr>
<td>--------------------------------------------</td>
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<td>--------------------------------------------------------------------------</td>
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<tr>
<td>Breast epithelial cells (MCF-10A) + 4-hydroxyestradiol</td>
<td>oxidative stress</td>
<td>30 μM</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td>while γ-Toc tends to be &gt; than α-Toc in ↓ oxidative stress,</td>
<td>(159)</td>
</tr>
<tr>
<td></td>
<td>GSH/GSSH</td>
<td>96 h</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td>α-Toc &gt; γ-Toc in ↑ DNA repair protein (BRCA1) and PARP</td>
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<tr>
<td></td>
<td>MnSOD/Actin</td>
<td>0/+</td>
<td></td>
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<td>+</td>
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<tr>
<td>Macrophage cells (RAW264.7) + LPS</td>
<td>oxidative stress</td>
<td>10 μM</td>
<td>+</td>
<td></td>
<td>+</td>
<td>++</td>
<td></td>
<td>(160)</td>
</tr>
<tr>
<td></td>
<td>Isoprostane</td>
<td>14h</td>
<td></td>
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<tr>
<td>Red blood cells from human + H₂O₂</td>
<td>MDA</td>
<td>30 μM - 480 μM 90 min</td>
<td>+</td>
<td></td>
<td></td>
<td>++</td>
<td>Mixed Toc: 64% γ-Toc, 24% δ-Toc, 12% α-Toc. Greater uptake of non-αToc in mixed Toc than α-Toc alone</td>
<td>(161)</td>
</tr>
<tr>
<td>Human monocytic U937 cells + oxysterol</td>
<td>cell counting</td>
<td>10 μM 48h</td>
<td>+</td>
<td>0</td>
<td></td>
<td></td>
<td>α-Toc but not γ-Toc prevented 7β-hydroxycholesterol-induced apoptosis</td>
<td>(162)</td>
</tr>
<tr>
<td>Myocytes from Sprague Dawley rats + hypoxia-reoxygenation</td>
<td>SOD activities</td>
<td>50 μM 30 min</td>
<td>+</td>
<td></td>
<td></td>
<td>++</td>
<td>Mixed Toc: 13% α-Toc, 62% γ-Toc, 25% δ-Toc.</td>
<td>(163)</td>
</tr>
</tbody>
</table>
Table 1.2. The Relative Antioxidant Activity of Tocopherol Isoforms Continued

<table>
<thead>
<tr>
<th>Model</th>
<th>End-point Measure</th>
<th>Concentration/Exposure Time</th>
<th>α</th>
<th>γ</th>
<th>δ</th>
<th>Mixed</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>Animal Trials</td>
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</tr>
<tr>
<td>male leptin deficient obese +LPS</td>
<td>liver MDA</td>
<td>500 mg/kg 5 weeks</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>(164)</td>
<td></td>
</tr>
<tr>
<td>Sprague Dawley insulin resistant rats vs. control + balloon catheter-induced vascular injury</td>
<td>plasma phosphatidylcholine Hydroperoxide</td>
<td>100 mg/kg/day 3 d prior injury + 2 weeks after</td>
<td>+</td>
<td>+</td>
<td></td>
<td>α-Toc supplementation ↓ γ-Toc in plasma and carotid artery</td>
<td>(165)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-nitrotyrosine</td>
<td></td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Carotid superoxide</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sprague Dawley Rats + FeCl₃ to induce arterial thrombosis</td>
<td>LDL-oxidation Arterial superoxide Plasma MDA SOD activity in aorta and plasma</td>
<td>100 mg/kg/day 10 days</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>LDL oxidation was induced by PMA-stimulated neutrophils</td>
<td>(166)</td>
<td></td>
</tr>
<tr>
<td>Male Wistar rats + carageenan-induced inflammation</td>
<td>8-isoprostane</td>
<td>33 mg/kg gavage 3 days</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>(16)</td>
<td></td>
</tr>
</tbody>
</table>
**Table 1.2. The Relative Antioxidant Activity of Tocopherol Isoforms Continued**

<table>
<thead>
<tr>
<th>Model</th>
<th>End-point Measure</th>
<th>Concentration/Exposure Time</th>
<th>α</th>
<th>γ</th>
<th>δ</th>
<th>Mixed</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 female Mice + LPS by intracerebro-ventricular injection</td>
<td>F4-neuroprostanes</td>
<td>100 mg/kg</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
<td>α-Toc &gt; γ-Toc in suppressing LPS-induced oxidative stress</td>
<td>(167)</td>
</tr>
<tr>
<td></td>
<td>Spine density</td>
<td>48h, 24h and 0h prior to LPS</td>
<td>+</td>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Dendrites degeneration</td>
<td></td>
<td>+</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprague Dawley Rats</td>
<td>LDL oxidation</td>
<td>100 mg/kg</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td>Placebo = rapeseed oil</td>
<td>(168)</td>
</tr>
<tr>
<td></td>
<td>SOD activity in plasma and aorta</td>
<td>7 to 10 days</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>SOD protein expression</td>
<td></td>
<td>+</td>
<td>++</td>
<td></td>
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<td></td>
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<tr>
<td>Human studies</td>
<td></td>
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<tr>
<td>Patients with type 2 diabetes</td>
<td>Plasma isoprostanes</td>
<td>500 mg/day</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Mixed Toc = 75 mg α-Toc, 315 mg γ-Toc, 110 mg δ-Toc</td>
<td>(169)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SOD and GPx from erythrocyte</td>
<td>6 weeks</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Subjects with metabolic syndrome</td>
<td>MDA +HNE</td>
<td>800 mg/day</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Mixed Toc = α-Toc + γ-Toc</td>
<td>(131)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipid peroxidase</td>
<td>6 weeks</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Urine nitrotyrosine</td>
<td></td>
<td>0</td>
<td>+</td>
<td></td>
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<tr>
<td>Normal/Ashmatic subjects</td>
<td>5-nitro-γ-Toc</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>Mixed Toc = 623 mg γ-Toc, 61.1 mg α-Toc, 11.1 mg β-Toc, and 231 mg δ-Toc</td>
<td>(170)</td>
<td></td>
</tr>
</tbody>
</table>

* + and - suggest positive and negative effect by the Toc isoform or mixtures, respectively, while 0 indicates no significant effect. The number of +/- sign denotes relative efficacy of to one another. Only treatments investigated were assigned symbols.
<table>
<thead>
<tr>
<th>Model</th>
<th>Endpoint Measure</th>
<th>Concentration/Exposure Time</th>
<th>α</th>
<th>γ</th>
<th>δ</th>
<th>Mixed</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cell Culture/Ex-Vivo</em></td>
<td>Colon &amp; liver cancer cells (Caco-2, HepG2)</td>
<td>Neutral Red</td>
<td>10, 100 µM</td>
<td>++</td>
<td>Toc alone were not cytotoxic, but γ-Toc &gt; α-Toc in exacerbating oxysterol-induced cytotoxicity in Caco-2, but not HepG2</td>
<td>(171)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>24h</td>
<td></td>
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<tr>
<td></td>
<td>Human mastocytoma (HMC-1)</td>
<td>Trypan Blue</td>
<td>50µM</td>
<td>++</td>
<td>γ-Toc &gt; δ-Toc in exacerbating oxysterol-induced cytotoxicity in Caco-2, but not HepG2</td>
<td>(172)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24h-48h</td>
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<tr>
<td></td>
<td>Normal mammary epithelial cells from BALB/c mice</td>
<td>MTT</td>
<td>100 µM, 250 µM</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>Cytotoxicity of δ-Toc associated with ↓ PKC membrane translocation from cytosol to membrane</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1-5days</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>BV-2 microglial cell line</td>
<td>Annexin V-PI</td>
<td>10, 30, 100, 300 µM</td>
<td>0</td>
<td>+</td>
<td></td>
<td>γ-Toc &amp; δ-Toc induces apoptosis indicated by phosphatidylserine exposition, chromatin condensation and blebbing cells</td>
</tr>
<tr>
<td></td>
<td>Primary microglial Cells</td>
<td></td>
<td>24h or 48h</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>Breast cancer cells (MCF7, MDAMB435)</td>
<td>Annexin V-PI</td>
<td>10, 20, 40 µM</td>
<td>0</td>
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<td></td>
<td>γ-Toc ↑ death receptor 5 expression (175)</td>
</tr>
<tr>
<td></td>
<td>Normal human mammary epithelial cells</td>
<td></td>
<td>1 - 3 days</td>
<td>0</td>
<td>0</td>
<td></td>
<td>caspase 8, bid and bax, caspase 9</td>
</tr>
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<td></td>
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<td>cytochrome c release with apoptosis</td>
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<tr>
<td>Model</td>
<td>Endpoint Measure</td>
<td>Concentration/Exposure Time</td>
<td>α</td>
<td>γ</td>
<td>δ</td>
<td>Mixed</td>
<td>Comments</td>
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<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Colon cancer lines (SW480, HCT-15, HCT-116, HT-29)</td>
<td>Trypan Blue</td>
<td>up to 100 µM</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
<td>γ-Toc &gt; α-Toc in cleavage of PARP, caspase 3, 7, 8 but not 9. Speculates PPARγ independent mechanism exist</td>
</tr>
<tr>
<td></td>
<td>Annexin V-PI</td>
<td>1 - 3 days</td>
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<tr>
<td>Primary colon cells (CCD-112CoN)</td>
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<tr>
<td>Prostate cancer lines LNCaP, androgen + PC3, androgen – Human lung cancer (A549)</td>
<td>MTT</td>
<td>10-100 µM</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
<td>γ-Toc induces apoptosis in LNCaP but not PC3. Apoptosis parallels cleavage of PARP, caspase 3 &amp; 9, &amp; caspase independent pathway. modulates de novo sphingolipids synthesis</td>
</tr>
<tr>
<td></td>
<td>DNA fragmentation</td>
<td>1 – 4 days</td>
<td>0</td>
<td>+</td>
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<tr>
<td>Mouse macrophage cell line (RAW264.7)</td>
<td>MTT</td>
<td>up to 80 µM</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td></td>
<td>δ-Toc cytotoxicity was due to apoptosis. D-Toc cytotoxicity was cell specific e.g. no effect on HepG2, &amp; bovine endothelial cells, but ↓ viability in MCF7, fibroblast &amp; primary macrophage.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12h - 48h</td>
<td></td>
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<tr>
<td>Prostate cancer (DU-145, LNCaP)</td>
<td>Trypan blue</td>
<td>up to 50 µM</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td>γ-Toc &gt; α-Toc in ↓ growth, DNA synthesis, no apoptosis but ↓ in cell cycle progression to S phase by down-regulation of cyclin D1 and E</td>
</tr>
<tr>
<td>Colon cancer (CaCo-2)</td>
<td></td>
<td>24h-48h</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>Osteosarcoma (SaOs-2)</td>
<td></td>
<td></td>
<td>0</td>
<td>+</td>
<td></td>
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</tr>
<tr>
<td>Model</td>
<td>Endpoint Measure</td>
<td>Concentration/ Exposure Time</td>
<td>α</td>
<td>γ</td>
<td>δ</td>
<td>Mixed</td>
<td>Comments</td>
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<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Mastocytoma cells</td>
<td>Cell Counter</td>
<td>50 µM 24, 48, 72h</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td></td>
<td>α-Toc only became cytotoxic after 72h(179) at 72h, δ-Toc&gt;γ-Toc&gt;α-Toc. Only δ-Toc affected PKB pathway.</td>
</tr>
<tr>
<td>Mouse melanoma cells B16</td>
<td>MTT</td>
<td>up to 500 µg/µL 72h</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td></td>
<td>γ-Toc &amp; δ-Toc ↓ melanin synthesis &amp; tyrosinase activity. The transcript of tyrosinase and its related protein (TRP-2) also ↓ by γ-Toc. (180)</td>
</tr>
<tr>
<td>Breast cancer cells</td>
<td>colony formation</td>
<td>up to 100 µM</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td>Cytotoxicity-mediated apoptosis, with (181) activation of caspase 8, 9, ↓ of c-FLIP and survivin protein expression, &amp; cleavage of PARP</td>
</tr>
<tr>
<td>Prostate cancer cells</td>
<td>Trypan blue</td>
<td>up to 50 µM</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td>PC3 more sensitive than HTB82 &amp; HECV</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td></td>
<td></td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td>γ-Toc &gt; α-Toc in ↓ cyclin D1, cdk4 p27 in PC3 cells, but no apoptosis</td>
</tr>
<tr>
<td>HTB82</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
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<tr>
<td>Human endothelial cord vein cells (HECV)</td>
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<td></td>
</tr>
<tr>
<td>Human glioma cells</td>
<td>cell counting</td>
<td>50µM 24h</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td>(183)</td>
</tr>
<tr>
<td>Human blood endothelial cells</td>
<td>Crystal violet/ MTT</td>
<td>up to 100 µM</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td></td>
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</tbody>
</table>
Table 1.3. The Relative Antiproliferative Activity of Tocopherol Isoforms Continued

<table>
<thead>
<tr>
<th>Model</th>
<th>Endpoint Measure</th>
<th>Concentration/Exposure Time</th>
<th>( \alpha )</th>
<th>( \gamma )</th>
<th>( \delta )</th>
<th>Mixed</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human lung carcinoma H129</td>
<td>MTT</td>
<td>up to 100µM 24h to 72h</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td></td>
<td>(77)</td>
<td></td>
</tr>
<tr>
<td>Hepatoma cells (AH109A)</td>
<td>(^3\text{H})thymidine incorporation</td>
<td>up to 50 µM 10 minutes</td>
<td>All Toc ↓ proliferation</td>
<td>No comparison between Toc were made</td>
<td>(185)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal Trials</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>transgenic rat for adenocarcinoma of prostate</td>
<td>adenocarcinoma formation</td>
<td>50 or 100 mg/kg 10 weeks</td>
<td>0</td>
<td>+</td>
<td></td>
<td>↓ in development of adenocarcinoma in ventral lobe, ↑ activation of caspase 3 and 7</td>
<td>(186)</td>
<td></td>
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<tr>
<td>BALB/c mouse 66clGFP mammary cancer model</td>
<td>tumor volume visible lung tumor</td>
<td>5 mg/day 3 weeks</td>
<td>0</td>
<td>+</td>
<td></td>
<td>But no effect of ( \alpha )-Toc or ( \gamma )-Toc on number of micrometastatic foci in lung or lymph node per mouse or in visible lymph node tumor</td>
<td>(181)</td>
<td></td>
</tr>
<tr>
<td>Male F344 rats</td>
<td>aberrant crypt cocci hydroxynonenal Nitrotyrosine Cyclin D1 PPAR( \gamma ) PGE2 Isoprostane</td>
<td>0.2% 8 weeks</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>a oxymethane-induced colon carcinogenesis</td>
<td>(187)</td>
</tr>
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</table>

* + and - suggest positive and negative effect by the Toc isoform or mixtures, respectively, while 0 indicates no significant effect. The number of +/- sign denotes relative efficacy of to one another. Only treatments investigated were assigned symbols.
Table 1.4. The Relative Anti-inflammatory Activity of Tocopherol Isoforms

<table>
<thead>
<tr>
<th>Model</th>
<th>Endpoint Measure</th>
<th>Concentration/Exposure Time</th>
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<th>δ</th>
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<th>Comments</th>
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<tr>
<td><strong>Chemical-Based</strong></td>
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<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Detoxification of NO₂</td>
<td>GC/thermal energy</td>
<td>1 mM</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nitric oxide formation</td>
<td>thermal energy Analyzer</td>
<td></td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Peroxynitrite-induced</td>
<td>hydroperoxide</td>
<td>5-20µM</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td>Suggests anti-nitrative activity of γ-Toc</td>
<td></td>
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<tr>
<td>lipid oxidation</td>
<td>from liposome</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cell Culture/Ex-Vivo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murine fibroblast</td>
<td>neoplastic transformation</td>
<td>30 µM</td>
<td>+</td>
<td>++</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>C3H/10T1/2 + 3-methylcholanthrene</td>
<td></td>
<td>4 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils from human +</td>
<td>↓O₂•</td>
<td>up to 50 µM</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td></td>
<td>Toc uptake follows the order: δ-Toc&gt;γ-Toc&gt;α-Toc.</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>↓Cytosol PKC activity</td>
<td>30 min</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td></td>
<td>Neutrophil from a subject w/ high level of α-Toc = promotion of superoxide generation = possible pro-oxidant activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↓NADPH oxidase activity</td>
<td>(cell free)</td>
<td>+</td>
<td>++</td>
<td>+++</td>
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<td></td>
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<tr>
<td>BV-2 microglial cell line</td>
<td>↑nitric oxide production</td>
<td>10, 30, 100, 300 µM</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td></td>
<td>Toc promotes nitrite production without affecting iNOS protein expression.</td>
<td></td>
</tr>
<tr>
<td>Primary microglial cells</td>
<td></td>
<td>24h or 48h</td>
<td>+</td>
<td>++</td>
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</table>
Table 1.4. The Relative Anti-inflammatory Activity of Tocopherol Isoforms Continued

<table>
<thead>
<tr>
<th>Model</th>
<th>Endpoint Measure</th>
<th>Concentration/Exposure Time</th>
<th>(\alpha)</th>
<th>(\gamma)</th>
<th>(\delta)</th>
<th>Mixed Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse macrophage cell line (RAW264.7) + LPS</td>
<td>↓ PGE2</td>
<td>10 µM 14h</td>
<td>0</td>
<td>+</td>
<td></td>
<td>Suppression of PGE2 was likely mediated from ↓ in COX2 activity. Suppression of nitric oxide in RAW 264.7 parallels ↓ in iNOS protein expression</td>
</tr>
<tr>
<td>A549 epithelial cells + IL1β</td>
<td>↓ Nitrite</td>
<td></td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549 epithelial cells + IL1β</td>
<td>COX2 activity</td>
<td>up to 40 µM 14h</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>↓ in PGE2 was related to ↓ in COX2 activity but not COX2 protein expression</td>
</tr>
<tr>
<td>Human blood endothelial cells</td>
<td>↓ Invasiveness</td>
<td>up to 100 µM</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>Toc ↓ TNFα-induced VCAM1 expression in BEC, but no effect on LEC.</td>
</tr>
<tr>
<td>Lymphatic endothelial cells</td>
<td>↑ Permeability</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Colon cancer cell (Caco-2)</td>
<td>↓ COX2 activity</td>
<td>0.1, 1, 10 µM 16h</td>
<td>+</td>
<td>++</td>
<td></td>
<td>No effect on COX2 mRNA</td>
</tr>
<tr>
<td>Splenocytes from rats</td>
<td>↑ IL2 production</td>
<td>0.25 -150 µM 4h</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>All Toc isoforms also ↓ PGE2 production associated with ↓ in COX2 activity</td>
</tr>
<tr>
<td></td>
<td>↑ Lymphocyte proliferation</td>
<td></td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Human keratinocytes cell line (NCTC 2544)</td>
<td>↑ PPARγ activation</td>
<td>50 µM 24h</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>↑ PPARγ also occurs with ↑ expression of transglutaminase-1</td>
</tr>
<tr>
<td></td>
<td>↑ PPARγ protein expression</td>
<td></td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>Endpoint Measure</td>
<td>Concentration/Exposure Time</td>
<td>α</td>
<td>γ</td>
<td>δ</td>
<td>Mixed</td>
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<tr>
<td>----------------------------------------------------------------------</td>
<td>--------------------------------</td>
<td>----------------------------</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>-------</td>
</tr>
<tr>
<td>Differentiated human blood neutrophils (HL-60) + ionophore</td>
<td>↓ LTB4</td>
<td>up to 200 µM</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Normal breast epithelial cells from BALB/c mice</td>
<td>↓ PKCα activity</td>
<td>up to 250 µM 5 d</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Monocytes from asthmatic subject + LPS</td>
<td>JNK phosphorylation</td>
<td>40 µM</td>
<td>0</td>
<td>+</td>
<td></td>
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<tr>
<td>EOC-20 mouse microglial cells + TNFα, or LPS/IFNγ or LPS</td>
<td>Nitrite production</td>
<td>up to 100 µM 30 min + coculture with stimulant</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human umbilical vein endothelial cells</td>
<td>Nitric oxide formation</td>
<td>10-200 µM 24h</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hepatoma cells (AH109A) + PMA</td>
<td>↓ invasiveness</td>
<td>up to 50 µM 10 minutes</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td></td>
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</tbody>
</table>
Table 1.4. The Relative Anti-inflammatory Activity of Tocopherol Isoforms Continued

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<tr>
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<th>δ</th>
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<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>Animal trials</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male Wistar rats + carageenan induced inflammation</td>
<td>↓ PGE2</td>
<td>33mg/kg</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
<td>γ-Toc&gt;α-Toc in ↓ TNFα (P = 0.07) (16) and ↓ total nitrate/nitrite (P = 0.1)</td>
</tr>
<tr>
<td>Male Wistar rats + carageenan induced inflammation</td>
<td>↓ PGE2</td>
<td>33mg/kg</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
<td>Combination of γ-Toc and aspirin (196) modestly ↓ PGE2 to lower than aspirin alone at p &lt;0.07 after 18h of carageenan injection.</td>
</tr>
<tr>
<td>Male leptin deficient ob/ob mice +LPS</td>
<td>↓ serum alanine aminotransferase</td>
<td>500 mg/kg</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>rats was model of steatohepatitis (164)</td>
</tr>
<tr>
<td></td>
<td>↓ TNFα</td>
<td>5 weeks</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum fatty acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum triglyceride</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>BALB/c mice + OVA</td>
<td>↓ Leukocyte number in lung</td>
<td>2 mg/d</td>
<td>-</td>
<td>+</td>
<td>0</td>
<td></td>
<td>Regulation of leukocyte migration (13) partly by modulating VCAM-1 activation of endothelial cells PKCα, without affecting secretion of chemokines, or lung tissue PGE2. Mixed Toc = α-Toc + 1/10 γ-Toc results in ablation of anti inflammatory effect of α-Toc.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 d</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Model</td>
<td>Endpoint Measure</td>
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<td>α</td>
<td>γ</td>
<td>δ</td>
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<td>----------</td>
</tr>
<tr>
<td>Sprague Dawley Rats + FeCl₃</td>
<td>↓ Platelet aggregation</td>
<td>100 mg/kg/day</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↓ thrombogenesis</td>
<td>10 d</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>α-TTP knockout mouse vs. wild type + MPTP</td>
<td>↓ Dopamine loss</td>
<td>1g/kg</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 weeks</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C57BL/6 female Mice + intracerebroventricular LPS injection</td>
<td>↓ Spine density loss</td>
<td>10 or 100 mg/kg</td>
<td>+</td>
<td>0</td>
<td></td>
<td></td>
<td>γ-Toc was found to ↓ PGE2</td>
</tr>
<tr>
<td></td>
<td>↓ Dendrites number loss</td>
<td>IP 48h, 24h &amp; prior to ICV injection</td>
<td>+</td>
<td>0</td>
<td></td>
<td></td>
<td>although no comparison to αToc was made</td>
</tr>
<tr>
<td>Male CD-1 mice + croton oil</td>
<td>↓ auricular thickness</td>
<td>topical treatment of 1.15 mg Toc</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td></td>
<td>Toc incorporated in nanoemulsion</td>
</tr>
<tr>
<td></td>
<td>↓ TNFα</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>was more effective than in suspension</td>
</tr>
<tr>
<td></td>
<td>↓ IL1α</td>
<td></td>
<td>0</td>
<td>+</td>
<td>0</td>
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<tr>
<td>Sprague Dawley insulin resistant rats vs. control + balloon catheter-induced vascular injury</td>
<td>neointima/media ratio</td>
<td>100 mg/kg/day</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
<td>γ-Toc &gt; α-Toc in ↓ neointima</td>
</tr>
<tr>
<td></td>
<td>TNFα or IL1β</td>
<td>3 d prior injury</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td>proliferation independent of its effect on superoxide production</td>
</tr>
<tr>
<td></td>
<td>↑ Nitric oxide</td>
<td>100 mg/kg</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprague Dawley Rats</td>
<td>↑ cNOS activity</td>
<td>7 to 10 days</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑ cNOS protein expression</td>
<td></td>
<td>0</td>
<td>+</td>
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### Table 1.4. The Relative Anti-inflammatory Activity of Tocopherol Isoforms Continued

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<tr>
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</tr>
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<td>Human studies</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Healthy subjects</td>
<td>Crp</td>
<td>300 mg</td>
<td>0</td>
<td></td>
<td></td>
<td>+</td>
<td>Mixed Toc = 60% γ-Toc, &amp; 28% δ-Toc, 10% α-Toc</td>
<td>(199)</td>
</tr>
<tr>
<td>patients with end stage renal disease</td>
<td>IL-6</td>
<td>14d</td>
<td>-</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Crp, PGE2</td>
<td>500 mg</td>
<td>0</td>
<td></td>
<td></td>
<td>0</td>
<td>Mixed Toc = 75 mg α-Toc, 315 mg γ-Toc, 110 mg δ-Toc</td>
<td>(169)</td>
</tr>
<tr>
<td>Patients with type 2 diabetes</td>
<td>TNFα, IL6</td>
<td>6 weeks</td>
<td>0</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCP1, MPO</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>LTB4</td>
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<tr>
<td></td>
<td>Crp</td>
<td>800 mg/d</td>
<td>0</td>
<td>0</td>
<td></td>
<td>+</td>
<td>IL1, IL6 and TNFα from LPS-activated whole blood release</td>
<td>(131)</td>
</tr>
<tr>
<td>Subjects with metabolic syndrome</td>
<td>IL1, IL6</td>
<td>6 weeks</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>TNFα</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Healthy subjects</td>
<td>↓ADP-induced platelet aggregation &amp; mixed Toc</td>
<td>100 mg</td>
<td>0</td>
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<td></td>
<td>+</td>
<td>Mixed Toc = 100 mg γ-Toc, 40 mg δ- and 20 mg α-Toc</td>
<td>(132)</td>
</tr>
<tr>
<td></td>
<td>↑NO release</td>
<td>8 weeks</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑eNOS activation</td>
<td></td>
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<td>++</td>
<td>Toc did not ↓ in PKC expression but ↓ activity</td>
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<td></td>
<td>↑PKC activation</td>
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<td>from PMA-activated platelets</td>
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* + and - suggest positive and negative effect by the Toc isoform or mixtures, respectively, while 0 indicates no significant effect. The number of +/- sign denotes relative efficacy of to one another. Only treatments investigated were assigned symbols.
1.3 Research Hypothesis and Objectives

Overall hypothesis

Non-α-Toc isoforms present in the North American diet can modulate oxidative status and inflammatory response to a comparable or greater extent than α-Toc in Caco-2 and FHs 74 Int cell lines.

Overall objective

To demonstrate the relative efficacy of Toc isoforms on oxidative and inflammatory responses associated with specific cell signaling mechanisms that occur in Caco-2 and FHs 74 Int cell lines.
EXPERIMENT 1. CHARACTERIZATION OF VITAMIN E ISOFORMS IN VEGETABLE OILS AND INFANT FOODS

HYPOTHESIS 1: Vegetable oils and infant formula represent rich sources of vitamin E (tocopherol) that varies in relative composition and absolute concentration of non-α-Toc isoforms.

OBJECTIVE: To characterize the major Toc isoforms present in vegetable oils and infant foods, specifically of human milk and infant formula.

HYPOTHESIS 2: Non-α-Toc isoforms exhibit equal or greater antioxidant activities as α-Toc.

OBJECTIVE: To establish the relative antioxidant activity of major Toc isoforms using the chemical-based free radical scavenging ORAC assay.
EXPERIMENT 2. ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF
TOC ISOFORMS ON CULTURED INTESTINAL CELL LINES

HYPOTHESIS 1: Antioxidant activity of Toc isoforms translates to an isoform-specific protection against free-radical induced oxidative stress in Caco-2 and FHs 74 Int cell lines.

OBJECTIVE: To determine the efficacy of Toc as antioxidants against peroxyl radical-induced oxidative stress using adult-derived Caco-2 intestinal cells and fetal-derived FHs 74 Int intestinal cells as model test systems.

HYPOTHESIS 2: The antioxidant activity of Toc isoforms corresponds to isoform specific anti-inflammatory activity in Caco-2 and FHs 74 Int intestinal cell lines.

OBJECTIVE: To determine the efficacy of Toc in modulating inflammatory response stimulated by IFNγ/PMA in both Caco-2 and FHs 74 Int cell lines by measuring secretion of IL8 as the end-point measure.
EXPERIMENT 3. MODULATION OF Nrf-2 AND NFκB SIGNALING IN CACO-2 AND FHS 74 INT CELLS BY TOC ISOFORMS

HYPOTHESIS: Variable IL8 responses of Caco-2 and FHs 74 Int cells to different Toc isoforms are associated with differential modulation of stress-activated Nrf-2 and NFκB signaling pathways.

OBJECTIVE 1a: To determine the effect of different Toc isoforms on IFNγ/PMA-induced NFκB and Nrf-2 activation in Caco-2 cells and FHs 74 Int cells by assessing the presence of the nuclear transcription factors.

OBJECTIVE 1b: To determine the effect of different Toc isoforms on NFκB and Nrf-2 nuclear translocation in FHs 74 Int cells in the absence of IFNγ/PMA challenge.

OBJECTIVE 2: To determine the effect of Toc isoforms on selected NFκB and Nrf-2 target genes in Caco-2 cells and FHs 74 Int cells.
EXPERIMENT 4. MECHANISTIC STUDIES OF FACTORS THAT CONTRIBUTE TO VARIABLE INFLAMMATORY RESPONSES OBSERVED IN CACO-2 AND FHS 74 INT CELLS

**HYPOTHESIS 1:** Efficacy of Toc in modulating inflammatory response is attributed to a pro-oxidant activity specific to non-α-Toc isoforms in Caco-2 and FHs 74 Int cells.

**OBJECTIVE:** To determine the effect of different Toc isoforms on oxidative status of IFNγ/PMA-treated cells. Further confirmation will be obtained by evaluating the effect of addition of ascorbic acid to Toc isoform on oxidative status of cells.

**HYPOTHESIS 2:** Efficacy of Toc in modulating inflammatory response is associated with isoform- and concentration-dependent cellular uptake of Toc.

**OBJECTIVE:** To quantify the Toc isoforms uptake into cells after treatment periods, measured using HPLC with fluorescence detection.
HYPOTHESIS 3: Toc differentially regulates glutathione synthesis in Caco-2 and FHs 74 Int cells, through specific down-regulation of glutathione content by non-\(\alpha\)-Toc in FHs 74 Int cells but not in Caco-2 cells.

OBJECTIVE: To measure glutathione content of Caco-2 and FHs 74 Int cells after exposure to different Toc isoforms.

HYPOTHESIS 4: Efficacy of Toc in modulating inflammatory response is associated with the cell-specific induction of apoptosis by non-\(\alpha\)-Toc isoforms.

OBJECTIVE 1: To measure the effect of different Toc on cell viability by MTT assay in both Caco-2 and FHs 74 Int cells.

OBJECTIVE 2: To identify and quantify the presence of apoptosis by TUNEL assay in both Caco-2 and FHs 74 Int cells after treatment with different Toc isoforms.
2. Characterization of Vitamin E in Foods and Their Antioxidant and Anti-inflammatory Activities

2.1 Characterization of Vitamin E Isoforms in Vegetable Oils and Infant Foods

2.1.1 Introduction

Vitamin E comprises four tocopherol (Toc) and four tocotrienol (Tri) isoforms (1). Among the Toc isoforms, \( \alpha \)-Toc has gained so much recognition that the terms vitamin E and \( \alpha \)-Toc have become interchangeable (10, 20, 81, 200, 201). The underlying rationale for this was the finding that \( \alpha \)-Toc is the most active isoform in rat gestation-resorption assays that have been traditionally used to classify the relative biological potency of Toc isoforms (20, 71). In addition, physiological levels of non-\( \alpha \)-Toc isoforms are low due to their being metabolized in the liver (18, 39) and thus their potential biological importance has been overlooked. Yet, other vitamin E isoforms in addition to \( \alpha \)-Toc might reach the intestine unchanged. Therefore, the effect of these isoforms on intestinal cells in particular is a relevant research question that has not been explored in detail.

The most established biological function of vitamin E is as a lipid soluble antioxidant (1) that could mitigate oxidative stress; an event that is often involved in inflammatory events (131, 201). However, differences in the chemical structures of vitamin E isoforms can influence the antioxidant activities, which in turn could translate to the isoform-specific modulation of oxidative stress and inflammatory responses observed in different cell systems (131, 138, 158). Thus, the vitamin E isoforms that are found in typical adult and infant diets should be identified as well as having a sound understanding of relative affinities to influence oxidative stress and inflammation in intestinal cells.
The objectives of this chapter were to characterize the relative composition and antioxidant capacity of vitamin E isoforms in vegetable oils as primary sources of vitamin E for adults, and also, in infant foods that include human milk and infant formula as typical sources of vitamin E for infants. The relative antioxidant activities of Toc isoforms were then characterized in cell-free chemical-based assays.

2.1.2 Materials and Methods

2.1.2.1 Materials

All materials and vegetable oils were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise described. HPLC grade methanol and RRR-α-Toc were purchased from Fisher Scientific (Fairlawn, NJ, USA). Randomly methylated β-cyclodextrin (Trappsol®) was obtained from Cyclodextrin Technologies Development, Inc. (High Springs, FL, USA). Wheat germ oil, flax seed oil and olive oil were obtained from a local supermarket.

2.1.2.2 Infant Formula Preparation

Three major commercial infant formula brands supplemented with ω-3 and ω-6 fatty acids were purchased from a local supermarket. Enfamil A+ (Mead-Johnson Nutrition, Ottawa, ON, Canada), GoodStart® with pro-biotic Stage 1 (Nestle®, North York, ON, Canada) and Similac Advance Step 1 (Abbott Nutrition, Saint-Laurent, Quebec, Canada) were selected as they are the leading infant formula brands for newborns aged up to six months. Infant formula powder was prepared according to the manufacturer’s instructions. In brief, 8.7 g of infant formula powder reconstituted in 60 mL of water was portioned and stored at -80°C until analysis.
2.1.2.3 Human Milk Collection

Mature human milk collected from three local mothers between one and three months post partum was immediately frozen at -20°C or chilled on ice for transport to the laboratory, where it was immediately portioned and stored at -80°C until analysis.

2.1.2.4 Vitamin E in Infant Formula and Human Milk

The vitamin E content of infant formula and human milk was determined as described by Korchazhkina et al (2006) with modifications (202). Milk (300 μL) was vortex mixed with 500 μL ethanol and 200 μL of 50% (w/v) KOH in amber microfuge tubes for 30 seconds and placed in a water bath at 70°C for 30 minutes with 30 seconds of vortex mixing at 15 minutes to induce saponification. Samples were cooled on ice and the pH of the milk mixture was acidified to ~5.0 with 6 N HCl. Hexane (700 μL) was added to the mixture and the tubes were vortex mixed for 30 seconds, followed by centrifugation at 9,500 g at 4°C for 10 minutes. The hexane upper layer was evaporated under a gentle stream of N2 gas and the solid residue was resuspended in 100 μL methanol. Samples were passed through 0.22-μM nylon filters and then Toc isoforms were separated by HPLC coupled with a fluorescence detector as previously described (32) and compared with external standards (α-Toc, γ-Toc, δ-Toc). Recovery was determined using δ-Toc and the % recovery of spiked δ-Toc was taken into account when measuring the Toc content of milk samples.

2.1.2.5 Vitamin E in Vegetable Oils

Toc isoforms were recovered from vegetable oils as described by Gimeno et al. (203). Mixtures of vegetable oils in hexane (200 μL; 10% w/v), ethanol (200 μL) and methanol (600 μL) were vortex-mixed for 30 seconds and then separated by centrifugation at 4,000 g for 5
minutes. The top layer (500 μL) was collected and passed through a 0.22 μM nylon filter before HPLC separation. This method yielded about 99.9% of the total δ-Toc added, indicating good recovery of the internal standard.

2.1.2.6 ORAC Antioxidant Assay

The free radical scavenging activity of Toc isoforms was determined using hydrophilic/lipophilic ORAC\textsubscript{FL} assays modified from a lipophilic-based ORAC assay (27). Toc isoforms were initially dissolved in acetone to a final concentration of 10 mM followed by further dilution in 7% randomly methylated cyclodextrin (RMCD) in 50% acetone. Mixtures were shaken at 200 rpm (Innova 4000, New Brunswick, NJ) for one hour in the dark. Toc isoforms (10 μL) in RMCD together with 90 μL of phosphate buffer were added to black 96 well plates. Standard curves were generated using Trolox, a water-soluble vitamin E analog in 90 μL of phosphate buffer and 10 μL of RMCD (7% in 50% acetone). The blank comprised 90 μL of phosphate buffer and 10 μL of RMCD. Fluorescein was then added at final concentration of 40 nM and plates were incubated at 37°C for 10 minutes (Fluoroskan Ascent FL, Labsystem, Helsinki, Finland). An AAPH-free radical generator was then added to a final concentration of 18 mM, and fluorescence intensity was measured for 100 minutes at 37°C. All results were analyzed as described for hydrophilic ORAC (204).

2.1.2.7 Statistical Analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation. Differences in ORAC values of Toc isoforms were identified using one-way ANOVA, followed by Tukey post-hoc analysis at P < 0.05 using GraphPad Prism 3.02 software (La Jolla, CA, USA).
2.1.3 Results

2.1.3.1 Vitamin E Content of Infant Food and Vegetable Oil

The total Toc isoforms in infant formula and human milk averaged 33.5 and 6.6 µM, respectively, and the proportions of individual isoforms differed. The dominant Toc isoforms in human milk were α-Toc, followed by γ-Toc and δ-Toc (Figure 2.1). In contrast, infant formula contained significantly more non-α-Toc isoforms (P < 0.05) than human milk, with γ-Toc and δ-Toc concentrations being 7 to 13-, and 19 to 33-fold higher, respectively, than in human milk. In addition, the concentration range of α-Toc acetate in infant formula was 21.6 – 24.6 µM.

The Toc isoform profiles of vegetable oil are shown in Figure 2.2. The dominant Toc isoform in nine of 15 tested vegetable oils was γ-Toc, and α-Toc in four. Although δ-Toc was the most abundant in soybean oil, it was also identified in eleven of fifteen evaluated vegetable oils.

2.1.3.2 Chemical-based Antioxidant Activity

The antioxidant activities of the isoforms determined by ORAC assays were isoform-dependent, with δ-Toc exhibiting the greatest (P < 0.05) ORAC value, followed by γ-Toc and α-Toc (Figure 2.3).

2.1.4 Discussion

The vit E profiles of commercially available vegetable oils were characterized to determine the vit E isoforms that are the most frequently consumed. Of all the vit E isoforms, γ-Toc was predominant in many of the tested vegetable oils. We also found δ-Toc in many vegetable oils, but it was the next most abundant to γ-Toc in soybean oil, which is a preferred
vegetable oil in North America (12, 13). Thus γ-Toc and δ-Toc are the main isoforms of vit E that are regularly consumed in North America.

To determine the relevance of non-α-Toc intake in infants, the vit E profile of infant foods that include human milk and infant formula were analyzed. The relative amounts and proportions of vit E isoforms found in human milk and infant formula are distinct. For example, whereas human milk contains mainly α-Toc with only a little γ-Toc and quantifiable levels of δ-Toc, infant formula contained up to 13-fold more γ-Toc and 33-fold more δ-Toc than human milk. Infant formula also contained a large amount of α-Toc acetate, which is the esterified form of α-Toc that lacks antioxidant activity and can be hydrolyzed to form the non-esterified α-Toc form that elicits antioxidant activity at the intestinal level (205). Therefore, when α-Toc acetate is taken into account, the amount of α-Toc in infant formula was 6 – 8-fold that in human milk. These findings indicate that large amounts of non-α-Toc isoforms are being consumed not only by adults, but also by infants fed with formula.

The finding that α-Toc is the dominant Toc isoform in human milk can be related to the fact that vit E consumed by the mother is metabolised by the liver that favors the retention of α-Toc in the circulation (1, 39). On the other hand, high levels of non-α-Toc isoforms in infant formula can be traced to a blend of vegetable oils as the primary source of fat in infant formula (33, 34). The addition of ω-3 fatty acids to infant formula also indirectly contributes to the higher levels of Toc isoforms in infant formula as mixed Toc is often added to stabilize polyunsaturated fatty acids that would otherwise readily become rancid (35).

The relative antioxidant activity of Toc isoforms in vegetable oil and infant foods was determined using cell free chemical-based ORAC antioxidant assays. The isoforms exhibiting
the most free radical scavenging activity were in the order of δ-Toc, γ-Toc and α-Toc. The structure-activity relationships observed with Toc isoforms could be attributed to the isoform-dependent hydrophobicity that is reflected by their partition coefficients (22). The least hydrophobic Toc isoform, δ-Toc therefore has greater affinity for the polar phase, whereas α-Toc with substitutions of three-methyl groups on the chromanol ring is the most hydrophobic. Thus δ-Toc is more likely to be readily available for peroxyl radical scavenging in the largely aqueous-based ORAC assay, and thus have greater free radical scavenging activity.

All of these findings indicate that the North American diet contains substantial amounts of non-α-Toc isoforms, specifically γ-Toc and δ-Toc. In addition, formula-fed infants are clearly consuming more γ-Toc and δ-Toc isoforms than breastfed infants. Considering the finding that the γ-Toc and δ-Toc isoforms have more free radical scavenging activity than α-Toc, these non-α-Toc isoforms could contribute to a greater protection against oxidative stress and inflammation than α-Toc. Investigation into the specific effects of Toc isoforms on modulating oxidative stress and inflammation on intestinal cells is thus warranted.
**Figure 2.1.** Tocopherol Contents of Three Commercial Infant Formula and Human Milk Samples Determined using HPLC with Fluorescence Detection. * denotes significant (P < 0.05) difference between means of specific Toc isoform of three commercial infant formulas and human milks analyzed in triplicate (n = 3) at P < 0.05

**Figure 2.2.** Tocopherol Content of Commercially Available Vegetable Oils (n = 3).
Figure 2.3. Antioxidant Activity of Tocopherol Isoforms Determined using Chemical-based ORAC Assay. Different letters denote significant differences between treatment means (n = 3) at P < 0.05.
2.2 Antioxidant and Anti-inflammatory Activities of Tocopherol Isoforms on Cultured Intestinal Cell Lines

2.2.1 Introduction

Having established that significant amounts of non-α-Toc isoforms are present in adult and infant diets, the question regarding the potential bioactivity of non-α-Toc isoforms relative to α-Toc on intestinal cells is justified. Of particular interest is the antioxidant and anti-inflammatory activity of dietary Toc isoforms on intestinal cells. This is relevant as oxidative stress has been implicated in adult and infant- inflammatory intestinal disorders such as IBD and NEC, respectively (101, 106, 206, 207). The potential ability of Toc isoforms to modulate oxidative stress and inflammation is therefore relevant to understanding the role of Toc in the prevention of IBD or NEC.

Investigations into the possible effects of non-α-Toc isoforms on infant intestinal cells is particularly important since infants fed with formula are consuming far more non-α-Toc than breast-fed infants. The case for feeding infants with formula has notably become a subject of a debate since a higher risk of developing various diseases was found among formula-fed, than breast-fed infants (208, 209). Newborns are exposed to elevated levels of oxidative stress associated with oxygen exposure after birth, and failure to cope with oxidative stress is considered to be a factor in the pathogenesis of ‘oxygen radical diseases’ such as necrotizing enterocolitis or inflammation of the intestine, retinopathy of prematurity and bronchopulmonary dysplasia (101, 210). Differences in Toc profiles between formula and breast milk could therefore contribute to the ability of infants to cope with oxidative stress and inflammation, and possibly to the higher rate of NEC in formula-fed infants. Oxidative stress is also implicated in
intestinal inflammatory disorders such as inflammatory bowel disease in adults (106, 207). Elucidating the role of Toc isoforms in modulating oxidative stress and inflammatory responses in adult and infant intestinal cells is therefore imperative.

The goal then was to determine the effect of Toc isoforms on modulating the oxidative status and inflammation of intestinal cells. Cultured intestinal cells derived from adults (Caco-2) and fetal intestine (FHs 74 Int) served as model test systems. The antioxidant effects of Toc were then related to its role in modulating inflammation in Caco-2 and FHs 74 Int cells.

2.2.2 Materials and Methods

2.2.2.1 Materials

Methanol (HPLC-grade) and RRR-α-Toc was from Fisher Scientific (Fairlawn, NJ, USA), whereas (+)-γ-Toc, (+)-δ-Toc, DCFH-DA and epidermal growth factor (EGF) were from Sigma-Aldrich (St. Louis, MO, USA). Caco-2, FHs 74 Int cells and Hybri-care medium were obtained from Cedarlane (Hornby, ON, Canada). Fetal bovine serum (FBS), penicillin/streptomycin and DPPP probe were from Gibco/Invitrogen (Grand Island, NY, USA). IL8 single analyte ELISA kits were from Qiagen (Valencia, CA, USA).

2.2.2.2 Cell Culture

Caco-2 cells were maintained in Minimum Essential Medium Eagle (MEM) culture media containing 10% FBS with 100 μg/mL streptomycin and 100 U penicillin. FHs 74 Int cells were maintained in Hybri-care medium containing 10% FBS, 100 μg/mL of streptomycin, 100 U of penicillin and 30 ng/mL of EGF. Caco-2 cells were differentiated by culture for 21 days with
media changes for 2-3 days (211) and then used for a maximum of 40 passages. The primary cell line was used within 8 passages.

2.2.2.3  DCFH-DA Assay

Caco-2 cells seeded at a density of $3.2 \times 10^5$/mL in 96-well plates were differentiated for 21 days with MEM media changes every 2-3 days. Similarly, FHs 74 Int cells were seeded at a density of $2.5 \times 10^4$/well in 96-well plates and cultured to confluence. Cells were incubated with $\alpha$-Toc, $\gamma$-Toc or $\delta$-Toc in ethanol for 24 hours in the respective complete media. Thereafter, media containing Toc was removed, and the cells were washed once with phosphate buffered saline (PBS). A DCFH-DA probe (5 µM) in PBS was added and the cells were incubated at 37°C for 30 minutes. Unincorporated probe was removed and AAPH was added at a final concentration of 1 mM to initiate oxidative stress. Fluorescence was measured using a luminometer (Fluoroskan Ascent FL, Labsystem, Helsinki, Finland) at excitation and emission wavelengths of 485 and 527 nm, respectively, at time zero or immediately after adding AAPH, and at one hour thereafter. The maximum ethanol concentration in all incubations was 0.25%.

The negative control comprised cells incubated with only the DCFH-DA probe. The positive control comprised cells incubated with the DCFH-DA probe and challenged with AAPH. Data are expressed according to the following equation:

$$\text{Fluorescence (\%)} = \frac{(F_i - F_{neg})}{(F_{pos} - F_{neg})} \times 100\%$$

Where $F_i$ is the fluorescence intensity of the sample, $F_{neg}$ is the fluorescence intensity of the negative control and $F_{pos}$ is the fluorescence intensity of the positive control.
2.2.2.4 **DPPP Assay**

We used a DPPP probe to determine protection against oxidative stress at the cellular membrane. Caco-2 cells were seeded at a density of $4.5 \times 10^5$ cells/mL in six well plates and were then differentiated for 21 days with MEM media (2 mL) changes every 2 - 3 days. FHs 74 Int cells were seeded in 100 × 20-mm culture dishes at a density of $6.25 \times 10^4$ cells per dish and grown to confluence in 4 mL of Hybri-Care media. The cells were then incubated with $\alpha$-Toc, $\gamma$-Toc or $\delta$-Toc for 24 hours at 37°C. Media containing Toc were removed and the cells were washed once with PBS before incubation with DPPP (5 µM) in PBS at 37°C for 30 minutes. The DPPP solution was removed and the cells were challenged with 5 mM AAPH in Hank’s Balanced Salt Solution (HBSS) for 4 hours. The cells were washed once with PBS, trypsinized and pelleted by centrifugation at 300 g for 5 minutes. The supernatant was removed and the cells were resuspended in PBS. The cells were pelleted again by centrifugation, resuspended in 3 mL of PBS and the fluorescence emission was measured at excitation and emission wavelengths of 350 and 380 nm, respectively, using an RF-5301 PC spectrofluorometer (Shimadzu, Kyoto, Japan). The negative control comprised cells incubated with only the DPPP probe. The positive control comprised cells incubated with the DPPP probe and challenged with AAPH. Data are expressed as described above for the DCFH-DA assays.

2.2.2.5 **Anti-inflammatory Activity of Tocopherol Isoforms**

We measured the ability of Toc to prevent IFN$\gamma$/PMA –induced IL8 secretion in Caco-2 and FHs 74 Int cells to determine whether Toc analogs have anti-inflammatory activity. Caco-2 cells were seeded at a density of $4.5 \times 10^5$ cells/mL in six-well plates and differentiated for 21 days. Thereafter, Toc analogs were added to 2 mL culture media then the cells were incubated
for 24 hours at 37°C. Media containing Toc (1.5 mL) was replenished after 24 hours and then the cells were challenged with IFNγ (8000 U/mL) and PMA (0.1 µg/mL) for 24 hours (134).

FHs 74 Int cells were seeded at a density of 1.2 × 10⁶/well in 2 mL of complete Hybrid care media containing 10% serum, 100 µg/mL of streptomycin, 100 U of penicillin and 30 ng/mL of EGF in six-well plates and incubated for two days at 37°C. The cells were then incubated in serum-free medium for 18 hours, followed by a 24-hours incubation with medium containing 1% serum, antibiotics and Toc isoforms in a maximal ethanol concentration of 0.25%. The medium containing Toc was removed and then the cells were challenged with IFNγ (4000 U/mL) and PMA (0.05 µg/mL) in 1.5 mL of medium containing 1% serum and replenished Toc isoforms for 24 hours. The culture medium was sampled, centrifuged at 1,000 g for 10 minutes at 4°C and IL8 in the supernatant was measured using ELISA kit according to the manufacturer’s instructions. The negative control comprised untreated cells, while the positive control consists of cells treated with IFNγ/PMA only. Data were expressed as percentages of IL8 secreted by cells treated with IFNγ/PMA relative to untreated cells as previously described for DCFHDA and DPPP assays. We repeated the experiment in the absence of IFNγ/PMA to determine the effect of individual Toc isoforms on IL8 expression.

2.2.2.6 Cellular Uptake of Tocopherol Isoforms

The relative uptake of Toc isoforms by Caco-2 and FHs 74 Int cells was determined by analyzing the cellular content of Toc using HPLC coupled with fluorescence detection based on the methods established by McCormick and Parker (2004) and Reboul et al. (2007) (177, 212). The culture conditions used to determine Toc uptake were similar to those used to determine the effect of Toc on the inflammatory responses of the cell lines. Differentiated Caco-2 cells were
incubated with Toc isoforms in 2 mL of medium containing 10% serum for 1, 2 or 3 days. FHs 74 
Int cells seeded in six-well plates were incubated in serum free media for 18 hours before 
exposure to Toc isoforms in 2 mL of medium containing 1% FBS.

The culture medium was aspirated and cells were washed once with 2% BSA in PBS on 
ice, followed by two more PBS washes. The cells were scraped into 100 μL of RIPA buffer, 
transferred to chilled microcentrifuge tubes, and placed on ice for 30-60 minutes. Distilled, 
deionised water (300 μL) was added, and the cells were centrifuged at 13,500 g for 20 minutes 
at 4°C. Protein content was measured in supernatant samples and then the cell pellet was re-
suspended in distilled, deionised water (100 μL) added to a final volume of 500 μL. Ethanol 
containing an internal standard was added and the cell suspension was vortex-mixed in 
microfuge tubes for 30 seconds. Delta-Toc served as the internal standard for samples containing 
α-Toc and γ-Toc, and α-Toc was the standard for those containing δ-Toc. Hexane (1.0 mL) was 
added and then the microfuge tubes were vortex-mixed for one minute. The organic layer was 
then separated from the aqueous phase by centrifugation at 9,500 g for 10 minutes at 4°C. The 
top hexane layer was recovered and evaporated under a stream of nitrogen gas. The remainder 
was reconstituted in methanol, filtered through 0.45 micron nylon syringe-filters and injected 
into the HPLC system. Toc isoforms were identified and quantified as described in Chapter 2.1.

2.2.2.7 Statistical Analysis

Experiments were performed in triplicate and results are expressed as means ± standard 
development. Significant differences between identical concentrations of Toc isoforms in the study 
of the effect of Toc on oxidative stress and inflammatory response were determined using one 
way ANOVA followed by Tukey’s test and significance was set at P < 0.05. In addition, 
modulation of oxidative stress and inflammatory response compared to control were determined
using t-test between treatment group and control. Differences in concentration and time-dependent effects in the cellular uptake study between cells exposed to various concentrations of the same isoform for different periods were determined using a two-way ANOVA.

2.2.3 Results

2.2.3.1. Antioxidant Activity Against Free Radical-induced Intracellular Oxidation

The antioxidant activity of Toc isoforms was determined in cell-based assays using non-fluorescent DCFH-DA probes to measure oxidative stress. The DCFH-DA probe is taken up by cells and cleaved by intracellular esterase to form non-fluorescent DCFH, which in turn generates fluorescent DCF after oxidization by intracellular free radicals, thus indicating levels of oxidative stress (213). Incubation with Toc isoforms protected Caco-2 cells against subsequent peroxyl radical-induced oxidative stress. A maximum of 20% inhibition was achieved at minimum concentrations of 1.25 µM for δ-Toc and 12.5 µM for α-Toc and γ-Toc (P < 0.05, Figure 2.4A). Increasing the concentrations of γ-Toc and δ-Toc resulted in diminished protection against oxidative stress. For example, the protective effect against oxidative stress induced by free radicals was not significant at > 25 µM γ-Toc and at > 50 µM δ-Toc when compared to control (Figure 2.4A). The results in FHs 74 Int cells were similar, in that all Toc isoforms conferred comparable and limited protection against free radical-induced oxidative stress (Figure 2.4B). At the minimum concentration of 3.2 µM, the inhibition of AAPH-induced oxidative stress (approximately 25%) was maximal. Increasing the Toc concentration to 100 µM did not significantly alter the degree of protection against oxidative stress induced by free radicals.
2.2.3.2 Membrane Oxidation Determined by DPPP Assay

The relative affinity of different Toc isoforms to protect against oxidative stress in the cellular membrane was also determined using the more hydrophobic DPPP probe. When oxidized by free radicals such as lipid hydroperoxide generated from oxidation of membrane lipid, DPPP is converted to fluorescent DPP-oxide, which signals oxidative stress in the membrane \((145, 146)\). Exposing Caco-2 cells to AAPH resulted in a significant increase in fluorescence intensity. Toc isoforms concentration-dependently suppressed AAPH-induced oxidative stress measured using DPPP in both Caco-2 and FHs 74 Int cells (Figure 2.5). In addition to being concentration-dependent, the ability of Toc isoforms to suppress oxidative stress induced by AAPH also seemed structure specific.

For example, 100 µM δ-Toc reduced fluorescence intensity in Caco-2 cells to 53% that of the negative control, whereas 100 µM α- and γ-Toc comparably inhibited 71 - 85% of AAPH-generated oxidative stress in the cell membrane (Figure 2.5A). Whereas α-Toc only suppressed AAPH-induced fluorescence at the highest concentration tested (100 µM), γ-Toc and δ-Toc started to mitigate \((P < 0.05)\) oxidative stress at 10 µM. Thus, α-Toc was the least, and δ-Toc was the most effective of all Toc isoforms in mitigating the generation of membrane-bound fluorescence in Caco-2 cells.

Membrane oxidation in FHs 74 Int cells was similarly diminished by Toc isoforms up to the highest concentration tested (10 µM), whereas γ-Toc and δ-Toc were more effective at the highest concentration tested (100 µM) than α-Toc (Figure 2.5B). At 100 µM, γ-Toc and δ-Toc suppressed fluorescence intensity to 34% - 43% below the negative control, whereas the
fluorescence intensity of cells incubated with α-Toc was comparable to that of the negative control.

2.2.3.3. Modulation of Inflammatory Response

The anti-inflammatory effects of Toc analogs were determined by measuring their ability to modulate the IFNγ/PMA-induced secretion of IL8 by Caco-2 and FHs 74 Int cells. Differentiated Caco-2 cells that were incubated with IFNγ/PMA for 24 hours expressed a significant (P < 0.05) amount of IL8 (Figure 2.6). Toc isoforms suppressed IL8 secretion to various degrees in IFNγ/PMA-challenged Caco-2 cells. The production of IFNγ/PMA-induced by IL8 was significantly more suppressed by γ-Toc (10-100 µM) than by α-Toc (P < 0.05) (Figure 2.6). In contrast, only δ-Toc - significantly suppressed IL8 production at the lowest concentration tested (1 µM) to levels similar to those of γ-Toc (10 µM). However, increasing the concentration of δ-Toc to 100 µM did not translate to the greater suppression induced by α-Toc and γ-Toc. In fact, δ-Toc caused the least (P < 0.05) suppression of IL8 production from IFNγ/PMA-induced Caco-2 cells.

The effects of Toc isoforms on IFNγ/PMA-challenged FHs 74 Int cells are described in Figure 2.7. Toc isoforms specifically and concentration-dependently promoted IL8 production by cells stimulated by IFNγ/PMA. Incubating cells with a minimum concentration of 10 µM γ-Toc isoforms resulted in more IL8 secretion from FHs 74 Int cells challenged with IFNγ/PMA than from cells incubated with comparable concentrations of Toc isoforms (P < 0.05). At 100 µM, γ-Toc significantly promoted IL8 production about 8-fold, whereas δ-Toc and α-Toc stimulated 3- and 2-fold increases, respectively, in IL8 production relative to control cells incubated with IFNγ/PMA alone.
We evaluated the effect of Toc isoforms on FHs 74 Int cells without IFNγ/PMA challenge. Toc isoforms at a minimum concentration of 10 µM promoted IL8 production when compared to untreated FHs 74 Int cells that produced a baseline IL8 concentration of 173 ± 4 pg/mL (Figure 2.8). In addition, 1 µM of γ-Toc was sufficient to significantly stimulate IL8 secretion from FHs 74 Int cells in the absence of IFNγ/PMA stimulation. At 100 µM in particular, Toc stimulated IL8 secretion in the order of δ-Toc > γ- Toc > α-Toc.

2.2.3.4. Uptake of Tocopherol Isoforms by Caco-2 and FHs 74 Int Cells Over Time

Toc isoforms were concentration- and time-dependently uptaken by Caco-2 and FHs 74 Int cells (Table 2.1). The amount of Toc taken up by cells incubated with the same concentration of Toc for three days steadily increased.

2.2.4 Discussion

The antioxidant activity of Toc isoforms was examined in Caco-2 and FHs 74 Int intestinal cells as biologically relevant models of the adult and infant intestines, respectively (133, 214). Although Toc demonstrated distinct isoform-dependent antioxidant activity in ORAC assays, their ability to suppress intracellular oxidation induced by free-radicals was comparably limited in Caco-2 and FHs 74 Int cells when measured using water-soluble DCFH-DA. In fact, the protective effect against AAPH-induced oxidative stress in Caco-2 cells was lost in the presence of increasing concentrations of γ-Toc and δ-Toc.

However, Toc isoforms prevented oxidative stress induced by free radicals in both Caco-2 and FHs 74 Int cells when measured using the membrane-soluble DPPP probe. This is
consistent with the affinity of Toc isoforms for the hydrophobic cellular membrane rather than
the aqueous cytosolic fraction, and indicated that Toc elicits antioxidant activity in the cellular
membrane rather than in the cytosol (215). Thus, the ability of the water-soluble DCFH-DA
probe to identify membrane-soluble antioxidant mechanisms such as that exhibited by Toc
isoforms is limited.

The relative antioxidant activity of Toc isoforms determined using the DPPP assay
corresponded to that identified in ORAC chemical-based assays, in that γ-Toc and δ-Toc were
more powerful antioxidants than α-Toc In particular, δ-Toc reduced oxidative stress to levels
below those in control Caco-2 and FHs 74 Int cells. These findings suggest that δ-Toc not only
neutralizes chemically-generated free radicals, but also reduces the oxidative burden of ordinary
cellular metabolism that results in a lower oxidative status. Taken together, these results indicate
that Toc isoform-dependently contribute to protection against oxidative stress induced by free
radicals in both Caco-2 and FHs 74 Int cells through free radical scavenging activity.

The possibility that Toc isoforms are degraded during culture at 37°C was considered.
However, Toc isoforms were relatively stable for up to three days at 37°C particularly in the
presence of a minimum concentration of 1% FBS in the culture medium (Appendix 1). Since the
culture conditions used in this study contained a minimum serum level of 1% FBS, the biological
effects can be attributed primarily to the Toc isoforms themselves rather than to their oxidative
products.

Incubating Caco-2 and FHs 74 Int cells with Toc isoforms resulted in concentration-
dependent cellular uptake that was increasingly evident for three days. The cellular uptake of
Toc continued to increase over the concentration range tested (1 – 100 μM) and over time. These
findings indicate that the concentrations of Toc applied herein produced an optimal concentration gradient with which Toc bio-functionality can be determined.

Having established the antioxidant activity of different Toc isoforms, a second focus to assess individual roles involved in modulating inflammatory status in Caco-2 and FHs 74 Int cells was determined. We induced an inflammatory state using the described specific mixture of IFNγ and PMA (134). Adding IFNγ/PMA to differentiated Caco-2 cells resulted in pronounced IL8 secretion that was modulated by Toc isoforms to various degrees. Minimal amounts of growth factors were added to the media in which FHs 74 Int cells were incubated because epidermal growth factor reportedly enhances IL8 expression in another primary fetal intestinal cell line (216). Here, FHs 74 Int cells cultured in media containing both EGF and serum produced ~16,600 pg/mL of IL8, whereas IL8 was barely detectable in Caco-2 cells cultured in medium containing serum. Challenging FHs 74 Int cells in medium containing growth factors with IFNγ/PMA did not increase IL8 production. On the other hand, withdrawing growth factors and depriving FHs 74 Int cells in serum-free medium before incubation with Toc reduced IL8 production in the absence of IFNγ/PMA challenge, thus establishing a baseline level of IL8. FHs 74 Int cells under these culture conditions became a useful model system for evaluating the inflammatory response, as challenge with IFNγ/PMA resulted in considerably more IL8 secretion than that produced by unstimulated FHs 74 Int cells.

IL8 is a chemoattractant that is minimally detectable under basal conditions, and its secretion is increased in response to pro-inflammatory stimuli or oxidative stress (113, 114). Although all Toc isoforms suppressed IL8 production from stimulated Caco-2 cells, γ-Toc was more effective than α-Toc. On the other hand, δ-Toc was most effective at the lowest, but least effective at the highest concentration tested compared with α-Toc and γ-Toc. When exposed to
Toc isoforms in the absence of IFNγ/PMA challenge, Caco-2 cells did not produce a significant amount of IL8.

Toc isoforms appeared to elicit the opposite effects in the primary FHs 74 Int cell model of the infant intestine. Incubating these cells with Toc isoforms exacerbated IFNγ/PMA-induced IL8 secretion, with γ-Toc being the most effective followed by δ-Toc and α-Toc. The effects of these isoforms were independent of IFNγ/PMA stimulation, because cells incubated with Toc isoforms continued to produce IL8 in the absence of the IFNγ/PMA challenge. In addition, the modulation of IL8 secretion by FHs 74 Int cells was distinctly isoform-dependent, with the most IL8 being generated in the order of δ-Toc > γ-Toc > α-Toc. Thus, Toc isoforms stimulated the inflammatory response in fetal-derived intestinal cells.

Toc isoforms protected against free radical-induced oxidative stress in both Caco-2 and FHs 74 Int cells as shown by the DPPP assay. However, Toc appeared to modulate inflammatory responses via different mechanisms in model systems representing adult and infant intestinal cells, which seemed to contradict the findings of a report indicating that IL8 secretion is associated with elevated oxidative stress (113). Since Toc protected against free radical-induced oxidative stress as shown in the DPPP assay, IL8 production in both cell lines should have been suppressed. The finding that Toc differently regulate the inflammatory response despite having similar antioxidant activities in Caco-2 and FHs 74 Int cells suggests that a mechanism(s) other than direct free radical scavenging activity is involved in modulating the inflammatory response in these cell lines. The following series of experiments was designed to elucidate this apparently conflicting ability of Toc isoforms to modulate IL8 expression in Caco-2 and FHS74Int cells. Some of the factors that might contribute to different mechanisms in adult and fetal-derived cells
are those associated with stress-responsive cell signaling pathways, cell viability and the cellular uptake of Toc.
**Figure 2.4.** Ability of Tocopherol Isoforms to Prevent AAPH-induced Oxidative Stress

Determined using DCFH-DA Probe in Caco-2 (A) and FHs 74 Int (B) Cells. Data are expressed as ratios (%) of differences between negative and positive controls.
Figure 2.5. Activity of Tocopherol Isoforms in Preventing AAPH-induced Oxidative Stress Determined using DPPP probe in Caco-2 (A) and FHs 74 Int (B) Cells. Data are expressed as ratios (%) of differences between negative and positive controls. Different letters indicate
significant (P < 0.05) differences observed between Toc isoforms at the same concentration (n=3).

Figure 2.6. Ability of Tocopherol Isoforms to Modulate IL8 Secretion Stimulated by IFNγ/PMA in Caco-2 Cells. Data are expressed as percentage of differences between negative and positive controls. Different letters indicates significant (P < 0.05) differences observed between Toc isoforms at the same concentration (n = 3).
Figure 2.7. Ability of Tocopherol Isoforms to Modulate IL8 Secretion Stimulated by IFNγ/PMA in FHs 74 Int Cells. Different letters indicates significant (P < 0.05) differences observed between Toc isoforms at the same concentration (n = 3).

Figure 2.8. Ability of Tocopherol Isoforms to Modulate IL8 Secretion in FHs 74 Int Cells. Different letters indicates significant (P < 0.05) differences observed between Toc isoforms at the same concentration.
### Table 2.1. Uptake of Tocopherol Isoforms in Caco-2 and FHs 74 Int cells*

| Day | Caco-2 | | | FHs 74 Int | | | | |
|-----|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|     | α-Toc (µM) | γ-Toc (µM) | δ-Toc (µM) | α-Toc (µM) | γ-Toc (µM) | δ-Toc (µM) | α-Toc (µM) | γ-Toc (µM) | δ-Toc (µM) |
| 1   | 0.07 ± 0.01 | 0.25 ± 0.02 | 1.15 ± 0.17 | 0.08 ± 0.01 | 0.59 ± 0.05 | 1.99 ± 0.25 | 0.12 ± 0.01 | 1.06 ± 0.14 | 3.36 ± 0.20 |
| 2   | 0.09 ± 0.00 | 0.53 ± 0.04 | 1.43 ± 0.10 | 0.10 ± 0.00 | 0.71 ± 0.07 | 2.93 ± 0.06 | 0.13 ± 0.01 | 1.01 ± 0.16 | 5.42 ± 0.16 |
| 3   | 0.12 ± 0.01 | 0.80 ± 0.11 | 2.68 ± 0.05 | 0.11 ± 0.00 | 1.01 ± 0.06 | 4.52 ± 0.31 | 0.14 ± 0.00 | 0.98 ± 0.03 | 6.96 ± 0.30 |

* Data are expressed as mean ± standard deviation (n = 3).
3 Modulation of Nrf-2 and NfkB Signaling in Caco-2 and FHs 74 Int Cells by Tocopherol Isoforms

3.1 Introduction

The effects of α-, γ- and δ-Toc isoforms in modulating oxidative stress and inflammation were established in Caco-2 and FHs 74 Int cells. Isoform-dependent free radical scavenging activities were comparable among the three isoforms in adult-derived Caco-2 cells and fetal-derived FHs 74 Int cells. However, the Toc isoforms mitigated and promoted IL8 pro-inflammatory cytokine secretion in IFNγ/PMA-induced Caco-2 cells and in FHs 74 Int cells, respectively.

Several factors might help to regulate cellular IL8 expression including activation of the cell signaling pathways that promote IL8 synthesis and stabilize IL8 transcripts (114, 115). Of all contributing factors, activation of the NfkB pathway is an indispensable mechanism of IL8 expression (114). Under non-stressed conditions, the transcription factor NfkB is bound to cytoplasmic inhibitors of NfkB proteins referred to as IkB (120). However, NfkB is released from the inhibitory protein complex during oxidative stress or in response to pro-inflammatory signals and translocated to the nucleus, where various pro-inflammatory cytokines, including IL8 are transcriptionally activated (109). IL8 expression may thus be triggered by oxidative stress and mitigated by antioxidants (113).

Another stress-responsive pathway that could modulate oxidative status in cells and consequently IL8 expression is the Nrf-2 signaling pathway (116, 217). Under basal conditions Nrf-2 transcription factor is associated with Keap1 protein in the cytoplasm (217). However,
Nrf-2 is released from Keap-1 during cellular stress and translocated to the nucleus where it binds to ARE and initiate the transcription of various antioxidant enzymes (117, 217).

The ability of Toc to modulate oxidative stress should then also influence stress-responsive signaling pathways such as NfκB and Nrf-2 with opposing effects. While the activation of NfκB signals promotes the expression of pro-inflammatory cytokines, the Nrf-2 signal should enhance the cellular defense system by stimulating the expression of antioxidant enzymes. The combined effect of the two pathways will determine the overall oxidative status of cells and consequently IL8 expression. This chapter therefore describes an investigation into whether Toc isoforms differentially modulate NfκB and Nrf-2 signaling pathways in Caco-2 and FHs 74 Int cells. The effects of Toc on Nrf-2 and NfκB signals were determined by assessing nuclear translocation of the transcription factors and the expression of several target genes of the pathways.

### 3.2 Materials and Methods

#### 3.2.1 Materials

RRR-α-Toc was obtained from Fisher (Fairlawn, NJ, USA) and (+)-γ-Toc, (+)-δ-Toc and the Bradford reagent were from Sigma-Aldrich (St. Louis, MO, USA). Caco-2 cells, FHs 74 Int cells and Hybri-care medium were obtained from Cedarlane (Hornby, ON, Canada). NfκB and Nrf-2 ELISA kits were from Active Motif (Carlsbad, CA, USA). IL8 single analyte ELISA kits and the Custom PCR array (CAPH10994) were from Qiagen (Valencia, CA, USA). The NE-PER nuclear protein extraction kit was from Pierce (Rockford, IL, USA). FBS, penicillin and streptomycin were from Gibco/Invitrogen (Grand Island, NY, USA).
3.2.2 Cell Culture

Caco-2 and FHs 74 Int cells were maintained according to American Type Culture Collection (ATCC) recommendations. The culture conditions under which we assessed the ability of Toc to modulate the inflammatory response were as described in Chapter 2.2. Briefly, differentiated Caco-2 cells were incubated with Toc isoforms in media containing serum, and FHs 74 Int cells were incubated in serum-free medium for 18 hours before incubation for 24 hours with Toc isoforms in medium containing 1% serum but without EGF. The medium containing Toc (1.5 mL) was replenished and IFNγ/PMA was added to induce inflammation.

3.2.3 NfκB and Nrf-2 Activation

To establish the effect of Toc isoforms on the activation of NfκB signaling, we determined the localization of p65 subunit of the NfκB complex in the nuclear fraction of Caco-2 and FHs 74 Int cells challenged with IFNγ/PMA for 90 minutes. The effect of Toc on modulating Nrf-2 signaling was determined after 24 hours of IFNγ/PMA stimulation in the presence of replenished Toc isoforms. The effects of Toc on NfκB and Nrf-2 signals in FHs 74 Int cells were also determined in the absence of IFNγ/PMA challenge after an incubation period specific to the respective signaling pathway.

Cells were scraped in ice-cold PBS and the nuclear pellet was obtained using the NE-PER kits. The nuclear extract was reconstituted in the lysis buffer supplied with Nrf-2 or NfκB Trans-Am ELISA kits and nuclei were lysed by vortex mixing at the maximum speed every 10 minutes for 40 minutes on ice. The final nuclear extract was obtained by centrifugation at 18,500 g for 10 minutes at 4°C. The protein content of the nuclear extract was adjusted to achieve equal concentration using the Bradford assay and subsequently analyzed using the Trans-Am ELISA.
kit according to the manufacturer’s instructions. Evidence supporting successful fractionation of the nuclear and cytoplasmic extract is attached in Appendix 2.

3.2.4 Effects of Tocopherol Isoforms on Gene Transcription

The effect of Toc isoforms on the modulation of selected genes involved in the Nrf-2 and NfkB signaling were determined using real time PCR arrays. cDNA from 1 µg of mRNA recovered from Caco-2 cells and 725 ng of mRNA from FHs 74 Int cells was prepared as previously described (218). The custom PCR array consisted of 19 genes of which the transcribed proteins modulate oxidative stress, inflammation or cell proliferation, two housekeeping genes (Appendix 3), one positive and one reverse transcription control, and a genomic contamination control. Each experiment comprised three independent replicates of Caco-2 cells incubated with different Toc isoforms and challenged with IFNγ/PMA for eight or 24 hours. FHs 74 Int cells were recovered after four- or 24-hour incubations with replenished Toc isoforms with or without IFNγ/PMA challenge. Untreated cells served as the negative control, and the positive control comprised cells incubated with IFNγ/PMA for the respective incubation periods. Reverse transcription, genomic DNA and positive PCR controls were included for each sample. PCR amplification proceeded as described using an ABI7500 (Applied Biosystems, Foster City, CA, USA) and data were analyzed using a software tool downloaded from the SABiosciences website (150).

3.2.5 Statistical Analysis

Experiments were performed in triplicate and results are expressed as means ± standard deviation. In the experiments investigating the effect of Toc isoforms on nuclear translocation of Nrf-2 and NfkB, significant differences at specific Toc isoforms concentrations were identified
using one way ANOVA at P < 0.05, followed by Tukey’s post-hoc analysis to determine differences between groups. In addition, modulation of Nrf-2 and NfκB response compared to control cells were determined using t-tests between treatment groups and controls. In the experiment investigating the effect of Toc isoforms on select gene transcription, one way ANOVA was performed between treatment groups to detect differences at P < 0.05 followed by Tukey’s post-hoc analysis to identify differences between treatment means.

3.3 Results

3.3.1 Modulation of NfκB Activity

Incubating Caco-2 cells and FHs 74 Int cells with IFNγ/PMA resulted in NfκB translocation to the nucleus. Activation was maximal at 90 minutes (Appendix 4). Among the tested Toc isoforms, only α-Toc exerted clear concentration-dependent inhibition of the NfκB activation induced by IFNγ/PMA in differentiated Caco-2 cells (Figure 3.1). At 10 µM, all Toc isoforms were shown to comparably suppress (P < 0.05) IFNγ/PMA-induced NfκB activation. Although γ-Toc suppressed (P < 0.05) NfκB activation at 10 µM, increasing the concentration 100 µM did not elicit further suppression. Furthermore, δ-Toc inhibited NfκB activation only at a concentration of 10 µM. The most effective Toc in terms of modulating NfκB activation and reducing IL8 secretion at a concentration of 100 µM was α-Toc, followed by γ-Toc whereas δ-Toc had no effect (Figure 3.1).

Toc isoforms also influenced NfκB signaling in FHs 74 Int cells. NfκB activation did not significantly differ among IFNγ/PMA-stimulated FHs 74 Int cells incubated with Toc (Figure 3.2). However, γ-Toc and δ-Toc isoform-dependently promoted NfκB activation in cells without
IFNγ/PMA challenge and δ-Toc at 100 µM was the most effective (Figure 3.3). On the other hand, α-Toc had no effect at all concentrations tested on NfκB signaling in the presence, or absence of IFNγ/PMA stimulation on FHs 74 Int cells.

3.3.2 Modulation of Nrf-2 Activity

Incubating cells with IFNγ/PMA resulted in not only activation of the NfκB pathway, but also the stress-activated Nrf-2 pathway in both Caco-2 and FHs 74 Int cells. The Nrf-2 transcription factor had obviously translocated to the nucleus in response to IFNγ/PMA after 4-8 hours in both cell lines (Appendix 4).

Incubating Caco-2 cells with α-Toc did not modulate Nrf-2 signaling at any tested concentrations, whereas γ-Toc significantly (P < 0.05) enhanced IFNγ/PMA-induced Nrf-2 activation at the highest concentration tested (100 µM; Figure 3.4). On the other hand, incubating IFNγ/PMA-treated cells with 10 µM δ-Toc significantly (P < 0.05) enhanced Nrf-2 translocation to the nucleus and 100 µM further augmented this response. This concentration of δ-Toc modulated IFNγ/PMA mediated Nrf-2 activation to a significantly greater degree than any of the other tested Toc isoforms (Figure 3.4).

Only 100 µM δ-Toc enhanced Nrf-2 activation by IFNγ/PMA challenge in FHs 74 Int cells (Figure 3.5). In the absence of IFNγ/PMA challenge, the modulation of Nrf-2 signaling was isoform-specific, although all Toc isoforms tested significantly (P < 0.05) increased Nrf-2 translocation to the nucleus (Figure 3.6). Nrf-2 signaling was promoted to a greater extent by δ-Toc than by α-Toc and γ-Toc, which comparably increased Nrf-2 signaling.
3.3.3 *Tocopherol Modulation on Gene Transcription*

The ability of Toc isoforms to modulate the expression of Nrf-2 and NfkB target genes was determined in Caco-2 and FHs 74 Int cells (Table 3.1-3.3) with and without IFNγ/PMA challenge for 8 and 24 hours, and 4 and 24 hours, respectively.

Stimulating Caco-2 cells with IFNγ/PMA for 8 hours resulted in the up-regulation of Nrf-2 and NfkB target genes, which became less pronounced after 24 hours of IFNγ/PMA challenge (Table 3.1). Incubating cells with Toc isoforms followed by IFNγ/PMA challenge in the presence of the isoforms differentially modulated the transcription of several NfkB- and Nrf-2-mediated genes. Among these, the effect on IL8 gene transcription was most distinct. At 8 hours of IFNγ/PMA challenge γ-Toc down-regulated the effect of IFNγ/PMA on IL8 gene transcription (P < 0.05), whereas α-Toc and δ-Toc were found to be ineffective. However, after 24 hours of IFNγ/PMA stimulation, α-Toc and γ-Toc equally modulated IL8, whereas δ-Toc enhanced (P <0.05) IFNγ/PMA-induced IL8 transcription (Table 3.1).

Other NfkB and Nrf-2 target genes were notably modulated by Toc isoforms in a similar manner as IL8 after both incubation periods. For example, γ-Toc maximally down-regulated IRAK2 gene expression in Caco-2 cells incubated with IFNγ/PMA for eight hours, whereas α-Toc and δ-Toc were comparably less effective. Both α-Toc and γ-Toc also down-regulated other genes involved in NfkB signaling such as NFKB1 and PTGS2 whereas δ-Toc produced comparable gene transcripts in IFNγ/PMA-challenged Caco-2 cells and control cells. NfkB-mediated gene transcription including that of IRAK2 and PTGS2 was similarly and significantly up-regulated by δ-Toc (P <0.05) in Caco-2 cells incubated with IFNγ/PMA for 24 hours compared with control cells (Table 3.1).
GPX2 appears to be the only Nrf-2–related gene that is altered by Toc isoforms in IFNγ/PMA-treated Caco-2 cells (Table 3.1). Although δ-Toc significantly (P < 0.05) up-regulated the GPX2 gene transcript at 8 hours of IFNγ/PMA challenge, all Toc isoforms similarly up-regulated GPX2 transcription after incubation with IFNγ/PMA for 24 hours (Table 3.1).

Toc modulated a more extensive set of Nrf-2 and NfκB target genes in FHs 74 Int cells than in IFNγ/PMA-treated Caco-2 cells (Table 3.2). At four hours of IFNγ/PMA challenge, NfκB-mediated gene transcription that was up-regulated >3-fold (P < 0.05) include IRAK2, NFKB1 and PTGS2. The relative expression of these genes in the NfκB signaling pathway was far less pronounced after a 24 hours incubation with IFNγ/PMA, except for IL8 which was up-regulated 5.4-fold (P < 0.05; Table 3.2).

Toc isoforms significantly (P < 0.05) increased the transcription of genes involved in NfκB signaling, including IRAK2, NFKB1, PTGS2 and IL8 (Table 3.2) in FHs 74 Int cells challenged with IFNγ/PMA for four hours. The effect was isoform-dependent, with δ-Toc enhancing the most IFNγ/PMA-induced gene transcription. After 24 hours of IFNγ/PMA challenge, the effect of Toc on IL8 and PTGS2 gene transcription became even more pronounced (Table 3.2). In particular, γ-Toc and δ-Toc isoform-dependently increased the levels of both IL8 and PTGS2 transcripts, whereas α-Toc exerted no significant effects compared with those in FHs 74 Int cells challenged with IFNγ/PMA. The down-regulated transcription of numerous Nrf-2-related genes accompanied the significant increase in that of NfκB target genes. The down-regulated genes included those involved in glutathione biosynthesis (glutatamate cysteine ligase (GCLC) and glutathione synthetase (GSS)), detoxification (glutathione-S-transferase (GSTA1)) and the antioxidant enzyme, glutathione peroxidase 1(GPX1). The effect was
particularly obvious for δ-Toc, which also down-regulated the gene expression of signaling molecules involved in cell proliferation (TP53, BAX) and other cell signaling mediators such as MAPK1.

The effect of Toc on FHs 74 Int cells was also evaluated in the absence of IFNγ/PMA challenge. Fewer genes were significantly regulated > 3 fold than when IFNγ/PMA was used to induce an inflammatory response in FHs 74 Int cells (Table 3.3). For example, only IL8 were significantly modulated by Toc isoforms at four hours of incubation with Toc isoforms. After 24 hours of incubation however, Toc induced a greater number of genes that include IL8 and PTGS2, although the magnitude of amplification was much lower than when FHs 74 Int cells were challenged with IFNγ/PMA. Although α-Toc did not modulate IL8 and PTGS2 transcript levels, γ-Toc and δ-Toc isoform-dependently increased IL8 and PTGS2 gene expression. Both γ-Toc and δ-Toc simultaneously and isoform-dependently were active at down-regulating the gene expression of GCLC and GSTA1; both of which are involved in glutathione biosynthesis and detoxification of electrophilic compounds. This finding was consistent with that observed in FHs 74 Int cells challenged with IFNγ/PMA.

3.4 Discussion

The effect of Toc isoforms on NfκB activation was determined based on the finding that IFNγ/PMA promotes IL8 secretion and that Toc alters IFNγ/PMA-induced IL8 in both Caco-2 and FHs 74 Int cells. Only α-Toc concentration-dependently suppressed IFNγ/PMA-induced NfκB activation in Caco-2 cells. NfκB activation was suppressed to the greatest extent by α-Toc, followed by γ-Toc, whereas δ-Toc was ineffective even at the highest concentration tested. This
outcome differed from Toc modulation of IL8 expression in Caco-2 cells challenged with IFNγ/PMA. Since oxidative stress activates NfκB signaling, Toc should mitigate IFNγ/PMA-induced NfκB signaling in the same order as antioxidant activity (119). However, the effect of Toc on NfκB signaling appeared to be the opposite considering that the relative antioxidant activity of Toc was δ-Toc > γ-Toc > α-Toc.

The finding that only α-Toc was active to produce a concentration-dependent suppression of NfκB activation suggests that Toc can modulate NfκB activity through non-antioxidant signaling. Chatelain et al. (1993) demonstrated that only α-Toc among all Toc isoforms inhibited the activity of PKCα, an upstream regulator of NfκB activity (219, 220). Thus, the suppression of NfκB by α-Toc in this case might have been mediated through suppressing PKCα that was otherwise not inhibited to a similar extent by other Toc isoforms.

The Nrf-2 stress-responsive pathway that signals the expression of antioxidant enzymes is also activated in both Caco-2 and FHs 74 Int cells in response to IFNγ/PMA (217). This pathway was enhanced in Caco-2 cells by δ-Toc and to a lesser extent by γ-Toc. On the other hand, α-Toc did not appear to be involved in regulating Nrf-2 signaling in stimulated Caco-2 cells. The finding that non-α-Toc isoforms promoted Nrf-2 signaling was notable because the activation of Nrf-2 signaling is generally associated with oxidative stress (217, 221). Therefore, Toc might act as pro-oxidants that in turn promote Nrf-2 activation.

The modulation of Nrf-2 and NfκB signaling by Toc in FHs 74 Int is distinct from that found in Caco-2 cells. For example, while Toc isoforms mitigated the IFNγ/PMA-induced NfκB signaling pathway in Caco-2 cells, they did not regulate IFNγ/PMA-induced NfκB activation in FHs 74 Int cells. However, the effect of Toc on the NfκB signaling pathway was evident in the
absence of IFNγ/PMA challenge. NfκB activation was promoted by δ-Toc and to a lesser extent by γ-Toc. The isoform-dependent activation of NfκB signaling by non-α-Toc isoforms therefore could explain why more IL8 was secreted from FHs 74 Int cells incubated with γ-Toc and δ-Toc. However, the finding that α-Toc also promoted IL8 secretion cannot be sufficiently explained only by the activation of NfκB signaling, because α-Toc did not promote NfκB activation. A possible role of Nrf-2 signaling on IL8 expression in FHs 74 Int cells was thus determined.

Only δ-Toc enhanced Nrf-2 activation in FHs 74 Int cells challenged with IFNγ/PMA. However, all Toc isoforms tested enhanced Nrf-2 signaling in the absence of IFNγ/PMA challenge, with δ-Toc eliciting the most pronounced effect, followed by α-Toc and γ-Toc, which were comparably less effective. Activation of the Nrf-2 signal should have induced the expression of antioxidant enzymes that reduce oxidative stress and consequently attenuate IL8 secretion by mitigating NfκB-induced IL8 gene expression. The current finding that Toc isoforms promoted Nrf-2 activation in FHs 74 Int cells was in fact the opposite of what should have occurred had the only mechanism triggered by Nrf-2 activation promoted antioxidant enzyme expression. Nevertheless, increased Nrf-2 expression in cultured human kidney mesangial and aortic endothelial cells increases IL8 expression that is attributed to the stabilization of IL8 mRNA (115). Here, Nrf-2 activation by Toc isoforms might have stabilized IL8 mRNA and consequently IL8 expression in FHs 74 Int cells. Therefore, activated Nrf-2 and NfκB signals can apparently work together in FHs 74 Int cells to contribute to the greater IL8 expression induced by Toc isoforms.

The involvement of Toc isoforms in the IFNγ/PMA-induced activation of Nrf-2 and NfκB was confirmed by determining the ability of Toc isoforms to modulate several Nrf-2 and NfκB target genes. Toc isoforms differentially modulate Nrf-2 and NfκB target genes in Caco-2
and FHs 74 Int cells. In Caco-2 cells challenged with IFNγ/PMA for 8 hours, γ-Toc down-regulated IL8 and other NfkB-mediated target genes more effectively than α-Toc and δ-Toc which were similarly more effective than untreated cells. This corresponded with the nature of IL8 secretion in IFNγ/PMA-treated Caco-2 cells, and confirmed that IL8 secretion is largely influenced by NfkB signaling. The fact that Toc isoforms modulated the Nrf-2 target gene GPX2 in a similar manner in which they modulated NfkB signaling suggests that NfkB expression could also influence GPX2 signaling (222). At 24 hours of IFNγ/PMA challenge, the transcript levels of NfkB target genes were higher in cells incubated with δ-Toc than with any other Toc or control cells, which suggests that δ-Toc prolonged the IFNγ/PMA-induced inflammatory response to a greater extent than other Toc isoforms. The finding that δ-Toc activated Nrf-2 signals together with NfkB further confirms the potential of δ-Toc to act as a pro-oxidant that modulates its ability to reduce the amount of IL8 secretion induced by IFNγ/PMA in Caco-2 cells. Taken together, Toc isoforms exerted effects on Nrf-2 and NfkB activation not only at the level of nuclear translocation, but also at that of transcription.

Toc antagonistically regulated NfkB target genes more effectively in the primary fetal-derived intestinal cell line than in adult-derived Caco-2 intestinal cells. While Toc isoforms mitigated the transcription of NfkB target genes in IFNγ/PMA-challenged Caco-2 cells, they exacerbated the expression of NfkB-related genes in FHs 74 Int cells. This was evidenced by the isoform-dependent increase in the transcription of NfkB target genes such as IRAK2 and NFKB1, along with IL8 and PTGS2 after inducing the inflammatory response with IFNγ/PMA (223-226). On the other hand, numerous Nrf-2 target genes were down-regulated only after challenging Toc-treated cells with IFNγ/PMA for 24 hours. In the absence of IFNγ/PMA challenge, non-α-Toc up-regulated IL8 and PTGS2 and expression, whereas the two Nrf-2 target
genes GCLC and GSTA1 remain down-regulated at 24 hours of incubation (227, 228). The finding that Toc isoforms regulate Nrf-2 and NfκB target genes in the absence of IFNγ/PMA challenge suggests that Toc themselves can effectively modulate the gene transcription involved in Nrf-2 and NfκB signaling, and that the extent can be augmented in the event of inflammation.

While most Nrf-2 target genes tested in this study were not affected by Toc isoforms in Caco-2 cells, many glutathione-related enzymes were down-regulated in IFNγ/PMA-treated FHs 74 Int cells. Nevertheless, only two Nrf-2 target genes (GSTA1 and GCLC) were down-regulated by δ-Toc in unchallenged FHs 74 Int cells. The GSTA1 gene encodes a glutathione S-transferase class A enzyme, which is involved in the cellular detoxification mechanism by conjugating xenobiotics or reactive species with reduced glutathione (229). The down-regulation of GSTA1 therefore could indicate compromised protection against reactive electrophilic compounds. However, the abundance of the GSTA1 gene is relatively low in FHs 74 Int cells since its expression can only be detected by PCR after 31-35 cycles. The low level of GSTA1 gene expression coupled with the further down-regulation of GSTA1 transcription by Toc isoforms suggests that changes in GSTA1 transcript level could have minimal biological significance. On the other hand, GCLC is quite abundant (Ct in control cells, 22 - 24) and its expression was down-regulated by γ-Toc and δ-Toc in FHs 74 Int cells regardless of IFNγ/PMA challenge. GCLC is the first and rate limiting enzyme in the two-step reactions of glutathione biosynthesis that catalyze the ligation of glutamate and cysteine to form g-glutamylcysteine (230). Addition of glycine to the dipeptide results in the formation of glutathione, a tripeptide, the amount of which is tightly regulated to maintain normal cellular function (230). Another hypothesis that can be derived from the current observation is that Toc differentially influence
glutathione biosynthesis in Caco-2 and FHs 74 Int cells, resulting in specific Tos isoforms lowering lower glutathione content in FHs 74 Int cells but not in Caco-2 cells.

Current investigations into the effect of Toc on Nrf-2 and NfκB signaling have provided novel insights regarding the relative potency of dietary Toc isoforms on modulating the two stress-responsive pathways particularly in intestinal cell lines. To date, information regarding the effect of Toc on Nrf-2 or NfκB pathways is only available for α-Toc or γ-Toc-enriched mixed Toc isoforms (231-234). To the author’s knowledge, little is known about the effect of γ-Toc at the time of writing, and a literature search did not reveal any reports about the effects of δ-Toc on either Nrf-2 or NfκB signaling.

Findings from the few published reports on the effects of δ-Toc-enriched Toc mixtures on the Nrf-2 pathway suggest that mixed Toc isoforms promote Nrf-2 signaling and the expression of antioxidant enzymes in animal models (231, 232). The present finding that non-α-Toc isoforms, particularly γ-Toc and δ-Toc can independently promote Nrf-2 activation could thus explain why mixed isoforms enriched with γ-Toc up-regulated the Nrf-2 pathway and the expression of its associated antioxidant enzymes.

One report indicates that α-Toc can activate Nrf-2 signaling and protect human retinal pigment epithelial cells from acrolein-induced oxidative stress (233). We found here that α-Toc did not modulate Nrf-2 signaling in IFNγ/PMA-induced Caco-2 or FHs 74 Int cells, but promoted Nrf-2 nuclear translocation in unchallenged FHs 74 Int cells. Thus, the notion that α-Toc has the potential to up-regulate Nrf-2 pathway is somewhat consistent. The discrepancy between cell lines might be attributable not only to differences in cell types, but also to varying culture conditions and inducers.
Along with its ester derivatives, α-Toc has consistently mitigated NfκB signaling in various model systems (235-240). One of the earliest demonstrations of the effect of Toc on NfκB activation used α-Toc acetate and α-Toc succinate with TNFα-induced Jurkat T cells (239). The outcome of a more recent investigation into the effects of α-Toc on lung epithelial cells was similar, in that α-Toc mitigated TNFα-induced NfκB activation (236). The present finding that α-Toc mitigated IFNγ/PMA-induced NfκB activation in Caco-2 cells is therefore consistent with other reports. The finding that α-Toc promoted NfκB activation in FHs 74 Int cells hence appears contradictory to the commonly observed effect of α-Toc on NfκB signaling. Nevertheless, Nakamura and Omaye (2008) reported greater NfκB (p50)-DNA binding activity in human umbilical vein endothelial cells (HUVEC) incubated with α-Toc (241). The authors attributed the greater NfκB activation to pro-oxidant activity of α-Toc at the tested concentration (241). The possibility that Toc isoforms modulate oxidative status in both Caco-2 and FHs 74 Int cells is outlined in the next chapter.

While information about the effects of α-Toc on NfκB signaling is relatively abundant, the relative order of Toc isoforms that include α-Toc, γ-Toc and δ-Toc in modulating NfκB activation has not been established. One study compared α-Toc and γ-Toc mitigation of LPS-induced NfκB activation in monocytes recovered from volunteers with asthma. The results however suggested that γ-Toc, and not α-Toc reduces IkBa degradation. Despite the opposing regulation in Caco-2 and FHs 74 Int cells, the present findings are consistent with the notion that γ-Toc tends to be more effective than α-Toc in regulating NfκB signaling.

Taken together, differential modulation of the level of IL8 transcripts by Toc isoforms in the two cell lines is relatively consistent with the expression of IL8 in the two cell lines. That is, Toc isoforms ameliorated IL8 expression in IFNγ/PMA-treated Caco-2 cells, but enhanced IL8
expression in FHs 74 Int cells regardless of IFNγ/PMA challenge. At the same time, the general
trend that Toc repressed NfκB signaling induced by IFNγ/PMA in Caco-2 cells but isoform-
dependently stimulated NfκB signaling in FHs 74 Int cells corresponds with the opposing nature
of IL8 regulation by Toc isoforms in the two cell lines. On the other hand, Toc promoted Nrf-2
signaling in both Caco-2 and FHs 74 Int cells. Therefore, NfκB activation is a key factor in IL8
expression. Furthermore, IL8 expression appears to be regulated at the level of cell signaling that
is probably not mediated through the direct antioxidant activity of Toc isoforms.
Figure 3.1. Tocopherol Isoforms Modulate NfkB Activation Induced by IFNγ/PMA in Caco-2 Cells. Different letters indicates significant (P < 0.05) differences observed among Toc isoforms at the same concentration.
Figure 3.2. Tocopherol Isoforms Modulate NfκB Activation Induced by IFNγ/PMA in FHs 74 Int Cells.

Figure 3.3. Modulation of NfκB Activation by Tocopherol Isoforms in FHs 74 Int Cells.

Different letters indicates significant (P < 0.05) differences observed among Toc isoforms at the same concentration.
Figure 3.4. Tocopherol Isoforms Modulate Nrf-2 Activation Induced by IFNγ/PMA in Caco-2 Cells at 24 Hours. Different letters indicates significant (P < 0.05) differences observed between Toc isoforms at the same concentration.
Figure 3.5. Tocopherol Isoforms Modulate Nrf-2 Activation Induced by IFNγ/PMA Cocktail in FHs 74 Int Cells. Different letters indicates significant (P < 0.05) differences observed between Toc isoforms at the same concentration.

Figure 3.6. Tocopherol Isoforms Modulate Nrf-2 Activation in FHs 74 Int Cells. Different letters indicates significant (P < 0.05) differences observed among Toc isoforms at the same concentration.
Table 3.1. Effects of Tocopherol Isoforms on Modulation of Gene Transcription in Caco-2 cells Challenged with IFNγ/PMA

<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><strong>8 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPX2</td>
<td>6.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GCLC</td>
<td>4.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IRAK2</td>
<td>13.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NFκB1</td>
<td>4.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL8</td>
<td>542.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>439.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>276.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>557.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PTGS2</td>
<td>5.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>24 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPX2</td>
<td>4.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>IRAK2</td>
<td>3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL8</td>
<td>12.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>5.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different letters indicates significant (P < 0.05) differences observed between treatment groups. GCLC, Glutamate-cysteine ligase catalytic subunit; GPX2, glutathione peroxidase; IL8, Interleukin 8; 2IRAK2, Interleukin-1 receptor-associated kinase 2; NFκB1, Nuclear factor of kappa light polypeptide gene; PTGS2, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase). Fold regulation is expressed relative to untreated control cells. Only genes that were significantly regulated > 3-fold are listed.
Table 3.2. Effects of Tocopherol Isoforms on Modulation of Gene Transcription in FHs 74 Int cells Challenged with IFNγ/PMA

<table>
<thead>
<tr>
<th>Gene</th>
<th>IFNγ/PMA + α-Toc</th>
<th>+ γ-Toc</th>
<th>+ δ-Toc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4 h</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRAK2</td>
<td>3.2a</td>
<td>3.6ab</td>
<td>4.0b</td>
</tr>
<tr>
<td>NFKB1</td>
<td>4.6a</td>
<td>5.4ab</td>
<td>5.8ab</td>
</tr>
<tr>
<td>IL8</td>
<td>1.0a</td>
<td>2.4b</td>
<td>2.9b</td>
</tr>
<tr>
<td>PTGS2</td>
<td>5.5a</td>
<td>10.3b</td>
<td>11.1b</td>
</tr>
<tr>
<td><strong>24 h</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPX1</td>
<td>1.3a</td>
<td>1.2a</td>
<td>1.3a</td>
</tr>
<tr>
<td>GSTA1</td>
<td>-5.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GCLC</td>
<td>-1.2b</td>
<td>1.2a</td>
<td>-1.8c</td>
</tr>
<tr>
<td>GSS</td>
<td>1.2a</td>
<td>1.3a</td>
<td>1.2a</td>
</tr>
<tr>
<td>BAX</td>
<td>-1.1a</td>
<td>-1.2a</td>
<td>-1.4a</td>
</tr>
<tr>
<td>TP53</td>
<td>1.1a</td>
<td>1.1a</td>
<td>-1.1b</td>
</tr>
<tr>
<td>IRAK2</td>
<td>-1.7a</td>
<td>1.2a</td>
<td>5.3b</td>
</tr>
<tr>
<td>NFKB1</td>
<td>1.3a</td>
<td>1.6a</td>
<td>3.1b</td>
</tr>
<tr>
<td>IL8</td>
<td>5.4a</td>
<td>6.2a</td>
<td>169.5b</td>
</tr>
<tr>
<td>PTGS2</td>
<td>1.8a</td>
<td>28.9a</td>
<td>820.2b</td>
</tr>
<tr>
<td>MAPK1</td>
<td>1.2a</td>
<td>1.2a</td>
<td>1.2a</td>
</tr>
</tbody>
</table>

Different letters indicates significant (P < 0.05) differences observed between treatments. BAX, BCL2-associated X protein; GPX1, glutathione peroxidase 1; GPX2, glutathione peroxidase 2; GSTA1, glutathione S-transferase alpha 1; GCLC, glutamate-cysteine ligase catalytic subunit; GSS, Glutathione synthetase; IL8, Interleukin 8; IRAK2, Interleukin-1 receptor-associated kinase 2; NFKB1, Nuclear factor of kappa light polypeptide gene; MAPK1, Mitogen-activated protein kinase 1; PTGS2, Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase.
and cyclooxygenase). TP53, Tumor protein p53. Fold regulation is expressed relative to untreated control cells. Only genes that were significantly regulated > 3-fold are listed. ND, not detectable.
Table 3.3. Effects of Tocopherol Isoforms on Modulation of Gene Transcription in FHs 74 Int Cells Challenged without IFNγ/PMA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>+ α-Toc</th>
<th>+ γ-Toc</th>
<th>+ δ-Toc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL8</td>
<td>1.0(^a)</td>
<td>3.0(^b)</td>
<td>1.6(^a)</td>
<td>1.8(^a)</td>
</tr>
<tr>
<td><strong>24 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTA1</td>
<td>1.0(^a)</td>
<td>1.1(^a)</td>
<td>-1.1(^a)</td>
<td>-10.6(^b)</td>
</tr>
<tr>
<td>GCLC</td>
<td>1.0(^a)</td>
<td>1.1(^a)</td>
<td>-1.5(^b)</td>
<td>-6.4(^c)</td>
</tr>
<tr>
<td>IL8</td>
<td>1.0(^a)</td>
<td>1.3(^a)</td>
<td>21.5(^b)</td>
<td>28.6(^c)</td>
</tr>
<tr>
<td>PTGS2</td>
<td>1.0(^a)</td>
<td>2.2(^a)</td>
<td>9.4(^a)</td>
<td>147.8(^b)</td>
</tr>
</tbody>
</table>

Different letters indicates significant (P < 0.05) differences observed between treatment groups. Control consists of untreated cells. GSTA1, Glutathione S-transferase alpha 1; GCLC, Glutamate-cysteine ligase, catalytic subunit; IL8, Interleukin 8; PTGS2, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase). Fold regulation is expressed relative to untreated control cells. Only genes that were significantly regulated > 3-fold are listed.
4 Mechanistic Studies of Factors Contributing to Variable Inflammatory Responses Observed in Caco-2 and FHs 74 Int Cells

4.1 Introduction

Toc isoforms (Toc) exerted isoform-specific anti-inflammatory effects in Caco-2 cells but elicited an inflammatory response in FHs 74 Int cells. Toc also differentially modulated the NfκB and Nrf-2 stress-responsive signaling pathways that functionally produced opposing effects. The activation of NfκB leads to the production of pro-inflammatory cytokines while the Nrf-2 signal activates the expression of antioxidant enzymes that might prevent an inflammatory response \((242, 243)\). Several mechanisms are proposed to be involved in the Toc-specific differences in inflammatory responses between Caco-2 and FHs 74 Int cells based on the effect of Toc on Nrf-2 and NfκB signaling.

Firstly, the relative cellular uptake of Toc isoforms in Caco-2 and FHs 74 Int cells was investigated to determine whether changes in the inflammatory response could be attributed to the cell- or isoform-specific uptake of Toc isoforms. Secondly, the collective outcomes of NfκB and Nrf-2 signaling resulted in the modulation of cellular oxidative status that consequently determines the cellular inflammatory response. Thus, it is proposed that dominant NfκB activation might result in a more pro-oxidant environment while antioxidant enzymes mediated by increased Nrf-2 expression could protect against the generation of oxidative stress that might trigger IL8 expression.

A third mechanism to be investigated is that Toc isoforms reduce the glutathione content in fetal-derived FHs 74 Int, but not in adult-derived Caco-2 cells. This is based on the finding that non-α-Toc isoforms down-regulated the gene expression of GCLC, a rate limiting enzyme in
glutathione biosynthesis, in FHs 74 Int, but not in Caco-2 cells. Glutathione is a low molecular weight non-protein thiol that conjugates reactive electrophiles and acts as a substrate for enzymes that neutralize peroxides. Glutathione therefore serves as an intracellular antioxidant that directly or indirectly protects against reactive species (244). Depleting cells of glutathione might therefore compromise the cellular defense system that could in turn affect the inflammatory response. Lastly, Toc might influence cell viability within the concentration ranges tested, which in turn could influence the interpretation of their biochemical effect in both cell lines.

Thus, the objective of the experiments described in this chapter was to determine the effects of Toc on the overall oxidative status, glutathione content and cell viability of Caco-2 and FHs 74 Int cells stimulated with IFNγ/PMA. The effects of Toc in the absence of an inflammatory stimulant were also tested in FHs 74 Int cells to determine if the bioactivity is attributable to Toc alone. The uptake of experimental concentrations of Toc isoforms by both cell lines was also determined.

### 4.2 Materials and Methods

#### 4.2.1 Materials

RRR-α-Toc and HPLC-grade methanol were from Fisher (Fairlawn, NJ, USA); (+)-γ-Toc, (+)-δ-Toc, DCFH-DA and EGF were from Sigma-Aldrich (St. Louis, MO, USA); Caco-2 cells, FHs 74 Int cells and Hybri-care medium were from Cedarlane (Hornby, ON, Canada); FBS and penicillin/ streptomycin were Gibco/Invitrogen (Grand Island, NY, USA); Apo-BrdU
TUNEL and glutathione assay kits were purchased from Phoenix Flow Systems (San Diego, CA, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively.

4.2.2 Cell Culture

Caco-2 cells were differentiated by media changes every 2-3 days and incubated with Toc isoforms for 24 hours in complete media. The Toc isoforms were replenished and the cells were incubated for a further 24 hours with or without IFNγ/PMA (8000 U/mL/0.1 µg/mL). FHs 74 Int cells were incubated for two days in complete media and then placed in serum free-media for 18 hours before incubation for 24 hours in medium containing 1% serum and Toc isoforms. The Toc isoforms were then replenished in medium containing 1% serum and then the cells were incubated for a further additional 24 hours with or without IFNγ/PMA.

4.2.3 Cellular Uptake

The relative uptake of Toc isoforms was identified and quantified in both cell lines after incubation with IFNγ/PMA and in FHs 74 Int cells incubated without IFNγ/PMA using HPLC with fluorescence detection as described in Chapter 2.2.2.6.

4.2.4 Effect of Tocopherol on Oxidative Status of Cells Induced by IFNγ/PMA

The effects of Toc on the oxidative status of Caco-2 and FHs 74 Int cells induced by IFNγ/PMA for 24 hours were examined as follows. The medium containing Toc was removed from 96-well plates and after rinsing once with PBS, the cells were incubated with the DCFH-DA probe (5 µM) in PBS for 30 minutes. The probe was removed and then the cells were incubated for 60 min in HBSS at 37°C. Fluorescence intensity at excitation and emission wavelengths of 485 and 527 nm was quantified using a luminometer (Fluoroskan, Vantaa,
Finland). The positive control comprised cells incubated with DCFH-DA and IFN\(\gamma\)/PMA, and the negative control comprised cells incubated only with the DCFH-DA probe. To determine whether ascorbic acid affects the activities of Toc isoforms, the experiment was repeated in the presence of equimolar ascorbic acid during incubations with Toc isoforms and co-incubation with IFN\(\gamma\)/PMA.

4.2.5  **Effect of Tocopherol on Cell Viability**

The effects of Toc isoforms on Caco-2 and FHs 74 Int, cell viability were evaluated using the MTT assay as previously described \((245)\). Briefly, medium containing Toc in 96 well plates was removed after processing the cells to measure inflammatory responses to Toc isoforms. Cells were washed with PBS and MTT (0.5 mg/mL) was added and then the plates were incubated at 37°C for 4 hours in the dark. Sodium dodecyl sulfate (10% in 0.01 N HCl) was added and formazan crystals were solubilized overnight at 37°C in complete darkness.

4.2.6  **Measurement of Apoptosis**

To determine whether Toc-induced cell death can be attributed to apoptosis, DNA fragmentation was examined using Apo-BrdU TUNEL assay kits, according to the manufacturer’s instructions. At the end of the incubation period, supernatants were collected from six-well plates and cells were rinsed once with PBS before trypsinization. Harvested cells were then combined with the supernatant followed by two PBS washes. Pelleted cells re-suspended in 1% paraformaldehyde in PBS (pH 7.4) were fixed for one hour on ice, rinsed twice with PBS and then ice cold 70% ethanol was added dropwise onto cell pellets. Samples were stored at -20°C overnight.
Briefly, cell suspensions in ethanol were separated by centrifugation and the supernatant was discarded. Cells were washed twice, DNA fragmentation sites were labeled with a cocktail of labeling solutions for one hour at 37°C with shaking every 15 minutes. The reaction was then terminated by adding rinse buffer. The cell suspension was separated by centrifugation and after one additional rinse, antibody was added and the samples were further incubated at room temperature for 30 minutes. Lastly, 300 µL propidium iodide solution (50 µg/mL) and RNase A (100 µg/mL) in PBS was added to the mixture. After incubation at room temperature in total darkness for at least 15 minutes, cells were analyzed using FACscan flow cytometry (Beckton-Dickinson, Mountain View, CA). Flow cytometry data were analyzed with FlowJo (Tree Star, Inc., Ashland, OR).

4.2.7 Total Glutathione Content

The effects of Toc isoforms on the glutathione content of Caco-2 and FHs 74 Int cells were determined using a glutathione assay kit according to manufacturer’s instructions. Cells in six-well plates were scraped at the end of the incubation into 100 µL of 50 mM MES buffer, pH 6-7 containing 1 mM EDTA. Cells were freeze-thawed three times between -80°C and room temperature and deproteinized with an equal volume of 10% metaphosphoric acid. Cell lysates were placed for five minutes at room temperature and then separated by centrifugation at 9,500 g for five minutes. The supernatant was recovered for total glutathione analysis.

4.2.8 Statistical Analysis

Experiments were performed in triplicate and results are expressed as means ± standard deviation. Significant differences between Toc isoforms at specific concentrations were identified using one way ANOVA at P < 0.05, followed by Tukey’s post-hoc analysis to
determine differences between groups. In addition, the effect of Toc on glutathione content was
determined by t-test at $P < 0.05$ against control cells. The effect of adding ascorbic acid at
specific Toc isoform concentrations was determined using a t-test with significant differences
established at $P < 0.05$.

4.3 Results

4.3.1 Cellular Uptake

The Toc contents of Caco-2 and FHs 74 Int cells at the end of incubation period were
analyzed to further establish whether their bioactive function could be attributed to the extent to
which they are taken up by intestinal cells. Cellular uptake of Toc isoforms (Figure 4.1 and
Figure 4.2) increased in a concentration and isoform-dependent manner, with $\delta$-Toc being taken
up to the maximal extent, followed by $\gamma$-Toc and $\alpha$-Toc in Caco-2 cells. The findings in FHs 74
Int cells were similar in the absence of IFN$\gamma$/PMA. However, no isoform-specific differences in
cellular uptake were observed in FHs 74 Int cells treated with the highest concentration of Toc
isoforms (100 µM) when cells were challenged with IFN$\gamma$/PMA, even though isoform-specific
differences persisted at lower concentrations (Figure 4.2B).

4.3.2 Effect of Tocopherol on Oxidative Status of Caco-2 Cells

The effects of Toc on the oxidative status of Caco-2 cells stimulated with IFN$\gamma$/PMA for
24 hours were determined using DCFH-DA assays. Increasing the concentration of $\delta$-Toc
promoted oxidative stress and did not exert an antioxidant activity, observed for $\alpha$-Toc and $\gamma$-
Toc, respectively (Figure 4.3). There was no significant difference in the levels of oxidative
stress between cells stimulated with IFN$\gamma$/PMA and control cells at 24 hours of incubation.
Ascorbic acid did not significantly alter the oxidative status of cells treated with either α-Toc or γ-Toc (Figure 4.4A and B). On the other hand, an equimolar ratio of ascorbic acid to δ-Toc reversed the pro-oxidant effect observed for δ-Toc in Caco-2 cells (Figure 4.4C). Whereas δ-Toc alone caused a concentration-dependent increase in oxidative stress, combining δ-Toc with ascorbic acid produced a concentration-dependent suppression of oxidative stress.

**4.3.3 Effect of Tocopherol on Oxidative Status of FHs 74 Int Cells**

Fluorescence intensity generally decreased in a concentration-dependent manner when FHs 74 Int cells with or without, IFNγ/PMA were incubated with Toc. One exception was γ-Toc at 10 µM, which significantly enhanced the fluorescence intensity of IFNγ/PMA-stimulated cells although all Toc isoforms at 100 µM comparably decreased fluorescence intensity (Figure 4.5B). In the absence of IFNγ/PMA however, Toc isoform-dependently reduced fluorescence intensity from FHs 74 Int cells (Figure 4.5A), with δ-Toc being more effective than γ-Toc, while α-Toc had no effect on oxidative status. At lower concentration, only δ-Toc at 10 µM significantly reduced oxidative status of FHs 74 Int cells challenged with IFNγ/PMA, while γ-Toc and δ-Toc were comparable in suppressing oxidative stress in the absence of IFNγ/PMA.

Adding an equimolar amount of ascorbic acid to FHs 74 Int cells incubated with 1 or 10 µM Toc did not significantly change fluorescence (Figure 4.6 and Figure 4.7), but did however, significantly increase oxidative status when added along with 100 µM Toc to FHs 74 Int cells that were stimulated with (Figure 4.6) or without (Figure 4.7) IFNγ/PMA.

**4.3.4 Effect of Tocopherol on Glutathione Content**

Treatment of Caco-2 and FHs 74 Int cells with IFNγ/PMA resulted in a significant (P < 0.05) decrease in total and reduced glutathione content. Toc isoforms however did not further
reduce the glutathione content of Caco-2 cells (Figure 4.8) whereas γ-Toc or δ-Toc significantly reduced the glutathione content of FHs 74 Int cells particularly with the stimulation with IFNγ/PMA (P < 0.05; Figure 4.9). In the absence of IFNγ/PMA challenge, only δ-Toc significantly reduced the glutathione content in FHs 74 Int cells.

**Effect of Tocopherol on Cell Viability**

Under the culture conditions used to determine the effect of Toc on the inflammatory response, Toc exerted no cytotoxic effects on differentiated Caco-2 cells but had a cytotoxic effect on FHs 74 Int cells (Figure 4.10 and 4.11) according to MTT assays. The effects of Toc on cell viability were exacerbated by the addition of IFNγ/PMA. For example, whereas Toc effectively reduced cell viability only at 100 µM, a minimum concentration of 10 µM was sufficient to exert cytotoxic effects in FHs 74 Int cells that were stimulated 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 Toc isoforms, DNA fragmentation was assessed in FHs 74 Int cells using TUNEL assays (Figure 4.12). Apoptosis was isoform-dependently induced in FHs 74 Int cells by δ-Toc (Figure 4.12) followed by γ-Toc, whereas α-Toc was ineffective.

### 4.4 Discussion

Uptake of Toc by both Caco-2 and FHs 74 Int cells was concentration and isoform-dependent, with δ-Toc being taken up to the greatest extent, followed by γ-Toc and α-Toc. Based on the finding that concentrations of δ Toc and γ-Toc were higher than those of α-Toc in cells incubated with equimolar concentrations of the isoforms, it can be postulated that the greater Toc
content was an important factor in the observed greater bioactivity in these cell lines to the
different Toc. Nevertheless, the opposite regulation of the inflammatory response and NfkB
signaling by Toc isoforms in Caco-2 and FHs 74 Int cells cannot simply be attributed to isoform-
dependent cellular uptake because the uptake was similar in both cell lines. Thus, Toc
modulation of the inflammatory response in these adult- and fetal-derived cell lines is not related
to the amount of cellular Toc uptake, but is rather isoform-specific and furthermore, cell-type
dependent.

In addition to being concentration-dependent, Toc isoforms were taken up in an isoform-
dependent manner that followed the relative order of δ-Toc > γ-Toc > α-Toc. Greater uptake of
Toc other than α-Toc has been demonstrated in a different cell model system (44, 45, 191). For
example, both Gao et al. (2002) and Tran et al. (1992) demonstrated greater cellular uptake of γ-
Toc than α-Toc in the RAW 264.7 mouse macrophage cell line and in human endothelial cells,
respectively (44, 45). Wu et al. (2000) not only demonstrated greater uptake of γ-Toc and δ-Toc
in mouse splenocytes, but also found corresponding isoform-dependent cytotoxicity (δ-Toc > γ-
Toc > α-Toc) (191). The mechanism of the greater uptake of non-α-Toc than α-Toc remains
unclear. Nevertheless, Toc uptake might be receptor-mediated, which would influence the extent
of cellular uptake (36, 191).

In the previous chapter, it was noted that Toc isoform-specific relationships involved
modulation of both Nrf-2 and NfkB signaling. In particular, α-Toc and γ-Toc mitigated
IFNγ/PMA-induced NfkB activation in Caco-2 cells, whereas δ-Toc had no effect at the highest
concentration tested. On the other hand, γ-Toc and δ-Toc at the corresponding concentration
enhanced Nrf-2 signaling in Caco-2 cells stimulated with IFNγ/PMA. One mechanism that might
contribute to the effect of Toc on both Nrf-2 and NfkB activation is the possibility that specific
Toc isoforms function as pro-oxidants at high concentrations. When acting as antioxidants in the membrane, Toc are converted to more water-soluble Toc-derived radicals which if not neutralized, might act as pro-oxidants in the cytoplasmic fraction (246). The isoform-specific pro-oxidant activity of Toc can be attributed to the different degree of hydrophobicity and free radical scavenging activity. The most hydrophilic Toc isoform, δ-Toc, also exhibits the greatest antioxidant activity and it is probably partitioned closer to the cytoplasm and oxidized to Toc radicals more effectively than other Toc isoforms (21). Greater free-radical scavenging activity translates to more formation of Toc-derived radicals and thus greater pro-oxidant activity. The possibility that specific Toc isoforms act as pro-oxidants is consistent with the finding that γ-Toc and δ-Toc promoted the Nrf-2 response and that δ-Toc suppressed NfκB activation to a lesser extent. In addition, non-α-Toc isoforms up-regulated NfκB target genes in Caco-2 cells stimulated with IFNγ/PMA.

The pro-oxidant activity of δ-Toc was confirmed by the finding that δ-Toc promoted oxidative stress in Caco-2 cells stimulated with IFNγ/PMA in a concentration dependent manner. Thus, modulation of the Nrf-2 and NfκB pathways by δ-Toc is associated with its pro-oxidant activity. This was further supported by the finding that ascorbic acid neutralized the oxidative-stress promoted by δ-Toc. Vitamin C, a water soluble antioxidant, might reduce the formation of Toc-derived radicals that contributed to the generation of oxidative stress in cells incubated with δ-Toc.

Oxidative stress was notably induced by δ-Toc- in Caco-2 cells although GPX2 and GCLC gene transcripts were up-regulated compared with IFNγ-/PMA stimulated control cells. This finding suggests that the increased levels of antioxidant enzyme transcripts did not
sufficiently enhance the intracellular defense system that could ultimately neutralize oxidative stress.

Toc isoforms, particularly γ-Toc and δ-Toc, promoted both NfkB and Nrf-2 signals in FHs 74 Int cells, which suggests that Toc isoforms have pro-oxidant activity in this primary fetal cell line. However, an investigation into the effect of Toc on the oxidative status of FHs 74 Int cells with or without IFNγ/PMA stimulation, uncovered no indication that Toc produced oxidative stress such as that observed in Caco-2 cells. In fact, the highest concentration of Toc (particularly δ-Toc) decreased oxidative status in FHs 74 Int cells. This result suggested that Toc act as antioxidants even though δ-Toc down-regulated the gene that encodes GPX1, a classic antioxidant enzyme and other glutathione-synthesizing enzymes such as GSS and GCLC. Adding ascorbic acid in the presence of Toc did not further decrease oxidative status, which was found in Caco-2 cells. On the contrary, ascorbic acid promoted oxidative stress when combined with higher concentrations of different Toc isoforms. Nevertheless, the reduction in oxidative status by Toc isoforms appeared to correspond with a capacity to reduce cell viability. A more likely explanation for the lower oxidative status in FHs 74 Int cells is thus that non-α-Toc elicit a cytotoxic effect in FHs 74 Int cells that results in fewer cells emitting fluorescence which may have created an apparently reduced oxidative status.

The finding that Toc isoforms promoted NfkB and Nrf-2 signaling in the absence of increased oxidative stress suggests that changes in intracellular oxidative status are not the primary contributing factor to the Toc-induced inflammatory response of FHs 74 Int cells. The mechanism through which Toc modulate cell signaling during inflammation in Caco-2 and FHs 74 Int cells might therefore be specific to Caco-2 versus FHs 74 Int cells.
Another hypothesis derived from the different manner in which Toc modulate Nrf-2 and NfκB target genes in Caco-2 and FHs 74 Int cells is that Toc isoforms were found to affect glutathione biosynthesis differently in these cell lines. This was based on the finding that levels of GCLC transcripts were down-regulated particularly in response to non-α-Toc in FHs 74 Int cells stimulated with IFNγ/PMA, but unaffected by any Toc in Caco-2 cells. A differential effect of Toc on the glutathione content of Caco-2 and FHs 74 Int cells stimulated with IFNγ/PMA was thus determined.

Consistent with the effect of Toc on GCLC transcription, Toc did not alter the total glutathione content of Caco-2 cells stimulated with IFNγ/PMA, but did suppress it in an isoform-specific manner in FHs 74 Int cells stimulated with IFNγ/PMA. In the absence of IFNγ/PMA, δ-Toc continued to reduce the total glutathione content of FHs 74 Int cells. These findings suggest that specific Toc isoforms down-regulate GCLC transcription, which in turn decreases the glutathione content of FHs 74 Int cells. Therefore, one of the underlying mechanisms that could contribute to differences in the inflammatory responses between FHs 74 Int and Caco-2 cells is the ability of Toc to reduce glutathione content in the former, but not in the latter.

Under the culture conditions used to test the effect of Toc on the inflammatory responses of intestinal cells, γ-Toc and δ-Toc were found to be cytotoxic in FHs 74 Int cells regardless of IFNγ/PMA stimulation, whereas none of the Toc isoforms were cytotoxic against differentiated Caco-2 cells. The cytotoxicity of Toc isoforms was also mediated through apoptosis, as determined by its DNA fragmentation hallmark in FHs 74 Int cells incubated with γ- and δ-Toc, respectively (148).
The effect of Toc on inducing apoptosis in numerous cancer cell lines has been reported (17, 174, 175, 247). Of the three Toc isoforms commonly described in the literature, α-Toc is generally regarded the least cytotoxic, whereas γ-Toc and δ-Toc are increasingly cytotoxic against various cancer cell lines (177, 179, 180, 248). The Toc studied herein, were not cytotoxic against adenocarcinoma-derived Caco-2 cells. The current finding that neither α-Toc not γ-Toc were cytotoxic against Caco-2 cells supports the findings of O’Sullivan et al. (2003) (171). On the other hand, Gysin et al. (2002) showed that α-Toc and γ-Toc decreased cell proliferation by 50% and 64%, respectively (178), possibly through inhibiting cell cycle progression in the absence of apoptosis. However, both of these studies used undifferentiated, instead of the differentiated Caco-2 cells used herein. Caco-2 cell differentiation requires 21 days of continuous proliferation, which results in up to a five-fold increase over the seeded cell density (249). The lack of cytotoxicity observed under the culture conditions reported herein therefore, is likely due to a greater cell density that resulted in increased resistance to cytotoxic agents compared with the number of seeded cells.

Toc-induced glutathione depletion in FHs 74 Int, but not in Caco-2 cells corresponds with the finding that the primary fetal cell line is more susceptible to apoptosis-mediated cytotoxicity than Caco-2 cells. Glutathione, a low molecular weight thiol, is found at high concentrations in all mammalian cells, where it functions in the cellular defense system against the generation of reactive species and xenobiotic-derived electrophiles (244). Intracellular levels of glutathione represent a balance between synthesis and consumption, as well as extracellular transport (244). Depleted glutathione content in cells is an early hallmark of apoptosis in response to various stimuli (250). Since it might react directly or catalytically with reactive species, a reduction in the intracellular glutathione content has been associated with levels of oxidative stress that are
generally sufficient to induce apoptosis (250, 251). Nevertheless, glutathione depletion might induce apoptosis in the absence of ROS generation, suggesting a more central role of glutathione in the progression of cell death that is independent of oxidative stress (250). The current finding of a reduced glutathione content corresponding to the induction of apoptosis therefore supports the latter notion that glutathione depletion is a trigger for apoptosis in the absence of increased oxidative stress.

A notable finding related to GCLC down-regulation is that the same procedure applied to FHs 74 Int cells resulted in an increased Nrf-2 translocation to the nucleus which theoretically signals the transcription of Nrf-2 target genes such as GCLC. Such increase in Nrf-2 presence in the nucleus was accompanied by the down-regulation of several other Nrf-2 target genes, including GSTA1 and GSS. One explanation for this event is that activation of the NfκB signal repressed the transcription of Nrf-2 target genes, despite the increased translocation of Nrf-2 to the nucleus. The potential for cross-talk between the Nrf-2 and NfκB signaling pathways has often been proposed, but very few mechanisms of interaction between them have been described in the literature (252, 253). Liu et al. (2008) comprehensively demonstrated that NfκB signaling inhibits the Nrf-2 pathway in cultured cell lines (252). Activation of the NfκB pathway antagonized Nrf-2 signaling at the level of transcription through the p65 subunit of the NfκB transcription factor complex depriving Nrf-2 of CREB binding protein (CBP; a common co-activator of NfκB and Nrf-2). Another plausible mechanism for this finding is that p65 transcription factor might promote the recruitment of the co-repressor histone deacetylase 3 (HDAC3) to the CBP and Nrf-2 dimerization partner MafK. Interaction between HDAC3 and CBP link the co-repressor to ARE while deacetylating CBP that results in the loss of co-activator activity. The simultaneous deacetylation of MafK by HDAC3 results in the repression of ARE-
driven transcription (252). A simplified figure describing this proposed mechanism is included in Figure 4.13.

A later study by Yu et al. (2011) suggests that p65 directly interacts with Keap-1, a repressor protein that facilitates the ubiquitination of Nrf-2 and leads to its proteosomal degradation (253). The association between Nrf-2 and Keap-1 as well as its constant cytoplasmic degradation are mechanisms through which basal Nrf-2/ARE expression is maintained (117). However, oxidative stress causes Keap-1 release from Nrf-2, which can then translocate to the nucleus, bind to the ARE and thus activate the expression of cytoprotective enzymes. Nrf-2 simultaneously escapes ubiquitination that results in increased stability of the transcription factor and an extended half-life, which also contributes to activation of the Nrf-2 pathway. However, Keap-1 not only represses Nrf-2 in the cytoplasm, but also translocates to the nucleus, where it then associates with Nrf-2 and shuttles back to the cytoplasm (117). Nrf-2 is then vulnerable to cytoplasmic degradation and thus its activation is suppressed. During NfκB activation, increased amounts of p65 transcription factor are translocated to the nucleus. Physical interaction between Keap-1 and p65 might thus result in increased nuclear Keap-1 which in turn facilitates the shuttling of Keap1 to the cytoplasm to bind with Nrf-2 and represses the antioxidant enzymes pathway (Figure 4.14).

The finding that the transcription of several other cell-signaling pathways (e.g. p53, MAPK) was concurrently down-regulated suggests that NfκB activation also diminished the transcriptional activity of these pathways. Repression of the glucocorticoid receptor, PPARγ, and p53 pathways by activated NfκB/p65 signaling has previously been reported (252, 254-256).
A summary of the effect of Toc isoforms on modulating the key cellular events involved in the observed inflammatory response is presented in Table 4.1. The present findings on the differential effects of Toc on Nrf-2 and NfκB signaling pathways in FHs 74 Int and Caco-2 cells are consistent with a mechanism through which NfκB activation antagonizes Nrf-2 mediated transcription. Levels of nuclear NfκB and of Nrf-2 that signal the activation of the stress-responsive pathways were found to be increased particularly in FHs 74 Int cells, and target genes of NfκB were significantly up-regulated, whereas those of Nrf-2 were down-regulated. The absence of down-regulated Nrf-2 target genes in Caco-2 cells could be due to the fact that Toc isoforms mitigated NfκB signals instead. These findings imply that the differential effect of Toc on the inflammatory response, including IL8 expression, glutathione content and the associated apoptosis-mediated cytotoxicity between Caco-2 and FHs 74 Int cells are attributed to differences in the relative affinity of which Toc modulate NfκB signaling.
**Figure 4.1.** Cellular Uptake of Tocopherol Isoforms in Caco-2 Cells Stimulated with IFNγ/PMA and Cultured under Conditions Used to Evaluate Modulation of Inflammatory Responses.

Different letters indicate significant (P < 0.05) between the same concentrations of Toc isoforms.
Figure 4.2. Cellular Uptake of Tocopherol Isoforms in FHs 74 Int Cells Cultured under Conditions Used to Evaluate Modulation of Inflammatory Response without (A) or with (B) IFNγ/PMA Stimulation. Different letters indicates significant ($P < 0.05$) differences observed between Toc isoforms at the same concentration.
Figure 4.3. Effect of Tocopherol Isoforms on Oxidative Status of Caco-2 Cells Stimulated with IFNγ/PMA. Oxidative status was measured using a DCFH-DA probe in Caco-2 cells incubated with Toc-isoforms and then co-cultured with IFNγ/PMA. Control cells were only stimulated with IFNγ/PMA. Different letters denote significant difference at P < 0.05 between Toc isoforms at a given concentration.
Figure 4.4. Ability of Ascorbic Acid to Modulate Tocopherol-induced Changes in Oxidative Status of Caco-2 Cells Stimulated with IFNγ/PMA. * denotes significant $P < 0.05$ difference.
between Toc-treated cells in the presence or absence of ascorbic acid at specified Toc concentrations.

Figure 4.5. Effects of Tocopherol Isoforms on Oxidative Status of FHs 74 Int Cells without (A) or with (B) IFNγ/PMA Stimulation. Control cells were incubated with either PBS (A) or IFNγ/PMA (B). Different letters denote significant difference at P < 0.05 at a given concentration.
Figure 4.6. Ability of Ascorbic Acid to Modulate Tocopherol-induced Changes in Oxidative Status of FHs 74 Int Cells Stimulated with IFNγ/PMA. a.a., Ascorbic acid. * denotes significant P < 0.05 difference between Toc-treated cells in the presence or absence of equimolar ascorbic acid at specified Toc concentrations.
Figure 4.7. Ability of Ascorbic Acid to Modulate Tocopherol-induced Changes in Oxidative Status of FHs 74 Int Cells without IFNγ/PMA Stimulation. a.a., Ascorbic acid.* P < 0.05 between Toc and Toc + equimolar ascorbic acid at specified concentration.
Figure 4.8. Effect of 100 μM Tocopherol on IFNγ/PMA-treated Caco-2 Cells on Total (A), and Reduced (B) Glutathione Content After 24 Hours Incubation. No significant difference was observed between Toc-treated cells and IFNγ/PMA treated cells.
Figure 4.9. Effect of 100 µM Tocopherol on Total (A) and Reduced (B) Glutathione Content of FHs 74 Int Cells Incubated for 24 Hours with or without IFNγ/PMA Stimulation. * denotes significant $P < 0.05$ difference of Toc-treated cells to control cells.
Figure 4.10. Effect of Tocopherol Isoforms on Caco-2 Cell Viability as Determined Using MTT Assay.
Figure 4.11. Effect of Tocopherol Isoforms on FHs 74 Int Cell Viability in the Absence (A) and Presence (B) of IFNγ/PMA as Determined Using MTT assay. Different letters denote significant difference at $P < 0.05$ between Toc isoforms at a given concentration.
Figure 4.12A. Effect of 100 µM Tocopherol on Apoptosis of FHs 74 Int Cells Cultured in the Absence of IFNγ/PMA. Different letters denote significant difference at P < 0.05 between Toc isoforms.
Figure 4.12B. Effect of 100 µM Tocopherol on Apoptosis of FHs 74 Int Cells Cultured in the Presence of IFNγ/PMA. Different letters denote significant difference at P < 0.05 between Toc isoforms.
Figure 4.13. Proposed Mechanism for Non-α-Toc-induced Crosstalk Mechanism by which Activated NfkB Signaling Antagonizes Nrf-2 Signaling. Increased presence of NfkB p65 subunit in the nucleus competes for binding with CBP, a common coactivator of Nrf-2 and NfkB transcription factor, depriving Nrf-2 from its coactivator. In addition, NfkB p65 subunit also recruits co-repressor HDAC3 to CBP/Nrf-2 complex, suppressing Nrf-2 mediated gene transcription.
**Figure 4.14.** Alternative Proposed Mechanism for Non-α-Toc-induced Crosstalk Mechanism by which Activated NfkB Signaling Antagonizes Nrf-2 Signaling. Keap1 nucleocytoplasmic shuttling system removes Nrf-2 from the nucleus to the cytoplasm for degradation under basal condition. In activated NfkB signaling, p65 subunit is associated with Keap1 and facilitates the translocation of Keap1 to the nucleus and enhances the removal of Nrf-2 from the nucleus, repressing the Nrf-2-mediated gene transcription. Figures were adapted from Baird et al (117).
Table 4.1. Highlight of the Effect of Toc Isoforms on Modulating Cellular Inflammatory Responses in Caco-2 and FHs 74 Int Cells*

<table>
<thead>
<tr>
<th>End Point Measures</th>
<th>Caco-2</th>
<th>FHs 74 Int</th>
<th>FHs 74 Int</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL8</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>NfkB</td>
<td>↓ for α-Toc and γ-Toc</td>
<td>0</td>
<td>↑ for non-α-Toc</td>
</tr>
<tr>
<td>Nrf-2</td>
<td>↑ for non-α-Toc</td>
<td>↑ for δ-Toc</td>
<td>↑</td>
</tr>
<tr>
<td>GCLC</td>
<td>0</td>
<td>↓ for non-α-Toc</td>
<td>↓ for non-α-Toc</td>
</tr>
<tr>
<td>Glutathione Content</td>
<td>0</td>
<td>↓ for δ-Toc</td>
<td>↓ for non-α-Toc</td>
</tr>
<tr>
<td>Cell Viability</td>
<td>0</td>
<td>↓ for δ-Toc</td>
<td>↓ for non-α-Toc</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>0</td>
<td>↑ for δ-Toc</td>
<td>↑ for non-α-Toc</td>
</tr>
</tbody>
</table>

* Observed effects of Toc isoforms at 100 μM. ↑ and ↓ suggest promotion and suppression effect by the Toc, respectively, while 0 indicates no significant effect.
5 General Discussion and Conclusions

5.1 Dietary Vitamin E Composition in Vegetable Oils

Vegetable oil represents a primary source of dietary vitamin E, with the three major Toc isoforms being α-Toc, γ-Toc and δ-Toc. The most popular vegetable oil in North America is soybean oil and its vitamin E profile indicates that γ-Toc is the most frequently consumed Toc isoform (12, 13). However, δ-Toc is also abundant in soybean oil, suggesting the potential to elicit significant biological function at the intestinal level exists. Whereas the bioactivity of α-Toc has been exhaustively investigated, γ-Toc has only recently attracted interest and even less is understood about the role of δ-Toc in modulating cellular responses. This situation provides justification for investigating the roles of γ-Toc and δ-Toc relative to α-Toc in modulating specific biological responses in intestinal cells.

5.2 Vitamin E Isoforms Present in Infant Foods

The significance of non-α-Toc isoforms in modulating intestinal cell response should be of particular interest when considering infant nutrition. This is because the concentrations of γ-Toc and δ-Toc in infant formula are 7 to 13- and 19 to 33-fold higher than that commonly found in breast milk because vegetable oil is the source of fat in infant formula (33, 34). Furthermore, the recent supplementation of infant formula with omega 3 fatty acids has led to the further addition of mixed vitamin E isoforms as stabilizers (35). Consequently, the relative abundance of total vitamin E in infant formula is substantially higher than in human milk. Infants that consume formula containing large amounts of non-α-Toc isoforms might be at greater risk of developing NEC than infants fed with human milk. This conclusion is made on the basis that non-
antioxidant properties of Toc isoforms can exist, albeit our findings indicate that this is a concentration dependent reaction.

However, the possible implications of exposing infants to large amounts of non-α-Toc isoforms have not been reported. The bioactivities of Toc have not been elucidated in biological systems and although one group has noted the significantly higher levels of γ-Toc in infant formula compared with human milk, the possible biological significance of this finding for infants was not discussed (257). This is perhaps because α-Toc has received intense focus for almost a century whereas the importance of other Toc isoforms has been generally overlooked. Research interest in γ-Toc is a relatively recent burgeoning area that has probably not yet reached the infant nutrition field. The research findings reported herein, therefore provides novel information about the potential significance of biological activities of other Toc isoforms relative to α-Toc in model systems comprising adult and fetal-derived intestinal cell lines.

5.3 Antioxidant and Anti-inflammatory Activities of Tocopherol Isoforms

Results herein showed that Toc isoforms conferred isoform-dependent protection in the relative order of δ-Toc > γ-Toc > α-Toc against oxidative stress induced by peroxyl radicals in adult- and fetal-derived intestinal Caco-2 and FHs 74 Int cells, respectively. Despite having similar antioxidant activities, Toc isoforms elicited converse effects on the inflammatory responses of the two cell lines. Specifically, Toc exerted anti-inflammatory activities, and after stimulation with IFNγ/PMA, mitigated IL8 secretion from Caco-2 cells, whereas they isoform-dependently promoted IL8 secretion from FHs 74 Int cells regardless of the presence of IFNγ/PMA stimulation.
The differential regulation of Toc isoforms on inflammatory responses in Caco-2 and FHs 74 Int cells could be related to their ability to modulate the cell signaling pathways that regulate IL8 expression. While non-α-Toc enhanced Nrf-2 signaling in both Caco-2 and FHs 74 Int cells, our results also showed that Toc mitigated IFNγ/PMA-induced NfkB activation in Caco-2 cells but promoted NfkB signaling in FHs 74 Int cells. The converse regulation of IL8 in Caco-2 and FHs 74 Int cells therefore corresponded with Toc modulation of NfkB signaling rather than the Nrf-2 response. The current findings do not rule out the possibility that other signaling pathways that contribute to IL8 expression such as the JNK p38, MAPKinase pathway, could also be modulated by Toc isoforms (114).

5.4 Tocopherol Isoforms as Structural Elements of Cell Membrane

Our results show that NfkB signaling was activated much earlier than Nrf-2 signaling, which corresponds to the converse trend of IL8 expression and indicates that the differential effect of Toc on the two cell lines is elicited upstream of NfkB signaling. Toc might elicit contrasting responses before NfkB signaling via several possible mechanisms. First, it is possible that the structure-function relationships of Toc determined herein were derived from Toc acting as structural elements in the cellular membrane. Atkinson et al. (2008) described the following main features of α-Toc in the lipid bilayer: its position and orientation in membrane phospholipid, and the depth of penetration by the head chroman group responsible for the antioxidant activity, the dynamics of Toc movement and Toc associations with specific lipids or lipid domains (258).

The cell membrane is stabilized by α-Toc associating with polyunsaturated fatty acid (258). In addition, phospholipid composition might vary across the cell membrane, suggesting
that specific lipid domains are enriched in various types of phospholipid. A difference in the chromanol structure is considered important to influence the location of Toc in the cell membrane, determine Toc association with PUFA, and alter membrane fluidity. In addition, since proteins associate with particular lipid rafts, Toc isoforms are likely to partition to the membrane domain only with specific proteins (258). Changes in membrane microviscosity could alter the mobility of Toc isoforms and proteins associated with the membrane, thus potentially influencing the rate of reactions involved in the modulation of cell signaling and downstream IL8 expression. Taken together, these findings indicate that a complex interplay between Toc and the local membrane results in the structural effects of Toc on the cell membrane which could indeed be associated with isoform-dependent activities in Caco-2 and FHs 74 Int cells redox homeostasis.

The signaling mechanism through which IFNγ and PMA induce inflammation notably includes IFNγ and PMA interaction with membrane-associated protein. For example, IFNγ initiates inflammation through interaction with IFNγ receptors embedded in the membrane (151). Similarly, PMA activates PKC which then translocates to the membrane to interact with membrane-bound receptors for activated C-kinase (RACK) (155). Thus, a mechanism through which Toc interferes with IFNγ/PMA signaling by isoform-dependently modifying the structure and dynamics of the cell membrane could plausibly explain the differential biological activities of Toc.

5.5 Tocopherol Isoforms and IFNγ/PMA Cell Signaling Pathways

Another possible mechanism through which Toc might elicit isoform-specific responses is that of modulating components of the IFNγ/PMA-induced cell signaling pathways that
influence NfkB signaling. The classical JAK/STAT signaling pathway is activated by IFNγ, while the established signaling pathway of PMA is via PKC activation (152, 155). Components of both cell signaling pathways might overlap with NfkB signaling. For example, PI3K/Akt and PKC kinase isoforms are involved in phosphorylating STAT1 in the JAK/STAT signaling pathway of IFNγ (151) and are upstream molecular signals for NfkB activation (151, 153). Therefore, Toc isoforms are likely involved in these upstream molecular components of IFNγ/PMA signaling pathways in an isoform-dependent way that leads to NfkB signaling. Nevertheless, the possibility that Toc also differentially stimulate cellular responses through an NfkB-independent mechanism(s) cannot be excluded. The effects of Toc on cell signaling pathways that influence or are independent of NfkB signaling and modulate IL8 expression requires further investigation.

5.6 Membrane Composition and Pro-oxidant Activities of Tocopherol Isoforms

Based on the notion that a membrane-mediated mechanism plays a role in the inflammatory response, the converse effect of Toc in modulating the cellular responses of Caco-2 and FHs 74 Int cells might be attributed to differences in the membrane composition of these cell lines. Differences in membrane composition could therefore also explain why specific Toc isoforms elicit pro-oxidant activity in Caco-2, but not in FHs 74 Int cells. In addition, ascorbic acid in the presence with Toc isoforms neutralized the pro-oxidant effects of Toc in Caco-2 cells, but did not offer the same effect with FHs 74 Int cells. Differences in membrane composition mean that Toc isoforms might reside at different depths of penetration from the surface of the lipid bilayer.
The pro-oxidant activity of δ-Toc could be attributed to the reactivity of its tocopheroxyl radical; that is, δ-tocopheroxyl radical is more reactive than γ-Toc and α-Toc (258). While acting as an antioxidant, δ-Toc is able to form tocopheroxyl radicals with high reactivity, which if not neutralized by a reducing agent such as ascorbic acid, would propagate nearby lipid oxidation reactions and thus elicit the pro-oxidant effect that was evident in Caco-2 cells in this experiment. On the other hand, differences in membrane composition could also have lead to the tocopheroxyl radical in FHs 74 Int cells, thus becoming so deeply embedded into the membrane that access to oxidize the DCFH-DA probe did not occur and as a result compromised this method to monitor oxidative stress at the membrane surface. The notion that the tocopheroxyl radical is embedded more deeply within the bilayer could also explain why water-soluble ascorbic acid did not alter Toc-induced changes in oxidative status, as the radical would have been inaccessible. This hypothesis should be tested in a study of the dynamics of Toc isoforms and their radicals in the cell membrane.

5.7 Crosstalk between Nrf-2 and NfkB

Incubating FHs 74 Int cells particularly with δ-Toc resulted in the activation of NfkB signaling that is associated with the down-regulation of several Nrf-2 target genes, such as GCLC, decreased glutathione content and the induction of apoptosis. Thus, the activation of NfkB signaling in FHs 74 Int cells might have antagonized Nrf-2 signaling in FHs 74 Int cells, which in turn would reduce glutathione synthesis and lead to a trigger for apoptosis induction. (252) Crosstalk between Nrf-2 and NfkB is one mechanism through which Toc isoforms could modulate oxidative status and the inflammatory response. Crosstalk between the NfkB and Nrf-2 signaling pathways in FHs 74 Int cells could be substantiated by knocking down endogenous p65.
using RNA interference and assessing whether selected Nrf-2 target genes can still be down-regulated by δ-Toc (252). This constitutes a topic for future research.

5.8 Antioxidant and Other Roles in Cellular Homeostasis

The findings in this study showed that Toc isoforms conversely regulate the inflammatory response despite having similar trends in cellular uptake and antioxidant activities in Caco-2 and FHs 74 Int cells. The concentration-dependent increase of Toc isoforms did not correspond with the anti-inflammatory activities of Toc in Caco-2 cells. Thus, Toc cannot modulate the inflammatory response solely by scavenging peroxyl radicals. The current findings therefore point to a non-antioxidant mechanism that might involve the ability of Toc to function as structural elements of the cell membrane or to modulate cell signaling components involved in the inflammatory response.

5.9 Final Conclusion

Bioactive dietary Toc isoforms (α-Toc, γ-Toc and δ-Toc) modulated oxidative status and inflammatory responses in adult- and fetal-derived intestinal cell lines. In addition, non-α-Toc isoforms tended to be more biopotent than α-Toc. The effect of Toc on modulation of inflammatory response was not only isoform-specific, but also cell-specific. Notably, Toc could elicit simultaneous antioxidant and non-antioxidant mechanisms of modulating inflammatory responses in Caco-2 and FHs 74 Int cells. The fact that γ-Toc and δ-Toc are typically consumed as part of the North American diet suggests that they also are involved in mitigating or promoting the pathogenesis of diseases in which oxidative stress and inflammation are implicated. Although γ-Toc has attracted increasing attention as a potentially more bioactive
Toc isoform, additional studies should investigate the biological activities of γ-Toc and δ-Toc in detail.

In terms of infant nutrition, the present findings suggest that non-α-Toc isoforms which are present in large amount in infant formulas promote inflammation in fetal-derived intestinal cells, indicating an important future goal to investigate the safety of these isoforms in these cells. Since non-α-Toc isoforms could alter the oxidative and inflammatory status of fetal-derived intestinal cells, the rationale for including large amounts of these isoforms in infant formula should also be re-evaluated.
References


127. Christen, S.; Woodall, A. A.; Shigenaga, M. K.; Southwell-Keely, P. T.; Duncan, M. W.; Ames, B. N. γ-tocopherol traps mutagenic electrophiles such as NOx and


137. American Tissue Culture Collection  Product Description FHs 74 Int. 2012.


173. Sylvester, P. W.; McIntyre, B. S.; Gapor, A.; Briski, K. P. Vitamin E inhibition of normal mammary epithelial cell growth is associated with a reduction in protein kinase C\( \alpha \) activation. *Cell Prolif* 2001, 34 (6), 347-357.


252. Liu, G. H.; Qu, J.; Shen, X. NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochim Biophys Acta* **2008**, *1783* (5), 713-727.


Appendices

Appendix 1. Stability of Tocopherol Isoforms in Culture Media

The stability of Toc isoforms in culture media was determined by incubating 600 µL of cell-free MEM or Hybri-Care medium containing 100 µM Toc isoforms at 37°C for various periods in 24-well culture plates. Thereafter, Toc isoforms were extracted from 500 µL samples and quantified using HPLC with fluorescence detection as described in Chapter 2.2.

The stability of Toc isoforms in cell-free culture media was dependent on the type of culture media, serum concentration and incubation period (Appendix Table A1). Adding Toc isoforms to MEM culture media without serum resulted in their degradation after three days of incubation. This time-dependent decrease in Toc isoforms was not evident in MEM culture media containing at least 1% serum. No clear trend in Toc degradation was uncovered in FHs 74 Int culture media at any serum concentration tested over a period of three days. In MEM containing at least 1% serum and FHs 74 Int culture media, a maximum of 20% -25% of Toc isoforms was lost, whereas up to 90% of Toc isoforms were degraded after three days of incubation in serum-free MEM at 37°C.
Appendix Table A1. Stability of Toc Isoforms in Cell-free Culture Media.

<table>
<thead>
<tr>
<th>Serum content</th>
<th>Tocopherol content (%)*</th>
<th>Days</th>
<th>0%</th>
<th>1%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>MEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Toc</td>
<td>36.7 ± 6.3</td>
<td>20.5 ± 2.7</td>
<td>9.4 ± 1.5</td>
<td>92.8 ± 3.7</td>
<td>94.4 ± 0.6</td>
</tr>
<tr>
<td>γ-Toc</td>
<td>27.4 ± 1.9</td>
<td>15.9 ± 0.6</td>
<td>13.0 ± 0.7</td>
<td>89.3 ± 1.3</td>
<td>84.1 ± 2.3</td>
</tr>
<tr>
<td>δ-Toc</td>
<td>29.3 ± 3.1</td>
<td>16.9 ± 2.8</td>
<td>18.3 ± 0.8</td>
<td>88.5 ± 2.3</td>
<td>87.4 ± 1.6</td>
</tr>
<tr>
<td>Hybricare medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Toc</td>
<td>116.0 ± 3.7</td>
<td>103.4 ± 6.7</td>
<td>110.7 ± 2.6</td>
<td>85.9 ± 5.3</td>
<td>76.7 ± 2.6</td>
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<tr>
<td>γ-Toc</td>
<td>91.7 ± 3.3</td>
<td>74.4 ± 2.2</td>
<td>77.7 ± 1.7</td>
<td>114.3 ± 2.9</td>
<td>117.0 ± 2.6</td>
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<tr>
<td>δ-Toc</td>
<td>84.5 ± 4.4</td>
<td>77.7 ± 1.7</td>
<td>79.0 ± 1.8</td>
<td>85.5 ± 3.6</td>
<td>82.0 ± 4.0</td>
</tr>
</tbody>
</table>

* Toc content is expressed relative to initial Toc content of culture media containing respective serum ratios. Values represent mean ± standard deviation (n = 3)
Appendix 2. Fractionation Control Using Nuclear and Cytoplasmic Proteins

To determine Nrf-2 and NfκB presence in the nucleus, Caco-2 and FHs 74 Int cells were fractionated to obtain a nuclear extract that is subjected to further analysis. Satisfactory separation between the nuclear and cytoplasmic extract was confirmed by testing nuclear and cytoplasmic extracts for the specific protein markers, histone and GAPDH, respectively. Caco-2 cells were fractionated using the NE-PER kit as described in Chapter 3. Nuclear and cytoplasmic fractions were standardized for protein content, and the extracts were analyzed by Western blotting.

Proteins were resolved by 12.5% SDS-PAGE at 100 Volts for 1.5 hours. Separated proteins were transferred to nitrocellulose membranes followed by blocking of the membrane for one hour with 5% skim milk in tris-buffered saline-tween (TBS-T). Histone and GAPDH proteins were then probed using the respective primary antibodies diluted in 5% skim milk at 4°C overnight. Histone rabbit polyclonal (ab1791) and mouse monoclonal GAPDH primary (ab8245) antibodies were applied at dilutions of 1/2000 and 1/2500, respectively. Membranes were washed three times with TBST for five minutes each and then incubated with secondary antibody diluted in 5% skim milk for 1 hour at room temperature. Goat-anti rabbit secondary antibody and goat anti-mouse antibody were applied at 1/1000 dilution. The membranes were washed with TBS-T three times for five minutes each and then once with tris-buffered saline (TBS) for five minutes. Chemiluminescent signals were developed and band intensity was quantified using ImageQuant TL software (GE Healthcare).
Appendix Figure A1. Evidence of Separation between Nuclear and Cytoplasmic Fraction Probed for Histone (A) and GAPDH (B) Proteins.

Histone and GAPDH proteins were detected only in the nuclear and cytoplasmic fractions, respectively, indicating functional separation of a nuclear fraction that was free of cytoplasmic protein.
Appendix 3. List of Genes Included in Custom Real Time PCR Array

Appendix Table A2 Genes Tested in Custom PCR Array.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Function of Encoded Protein</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td><strong>Nrf-2 Target Genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPX1</td>
<td>Glutathione peroxidase 1</td>
<td>Classic GPX; detoxification of H$_2$O$_2$;</td>
<td>(259, 260)</td>
</tr>
<tr>
<td>GPX2</td>
<td>Glutathione peroxidase 2</td>
<td>Gastrointestinal GPX; detoxification of H$_2$O$_2$;</td>
<td>(222, 261)</td>
</tr>
<tr>
<td></td>
<td>(gastrointestinal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPX4</td>
<td>Glutathione peroxidase 4</td>
<td>Reduces H$_2$O$_2$, lipid hydroperoxides;</td>
<td>(260)</td>
</tr>
<tr>
<td></td>
<td>(phospholipid hydroperoxidase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTA1</td>
<td>Glutathione-S-transferase alpha 1</td>
<td>Detoxification mechanism of reactive xenobiotic</td>
<td>(229)</td>
</tr>
<tr>
<td>GCLC</td>
<td>Glutamate-cysteine ligase, catalytic subunit</td>
<td>Glutathione biosynthesis; rate limiting enzyme</td>
<td>(230)</td>
</tr>
<tr>
<td>GSS</td>
<td>Glutathione synthetase</td>
<td>Glutathione biosynthesis</td>
<td>(262)</td>
</tr>
<tr>
<td>GSR</td>
<td>Glutathione reductase</td>
<td>Reduction of oxidized GSSG to reduced GSH</td>
<td>(263)</td>
</tr>
</tbody>
</table>
Appendix Table A2 Genes Tested in Custom PCR Array Continued.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Function of Encoded Protein</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes Involved in NfκB Signaling</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFKB1</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells1</td>
<td>Precursor to p50 subunit of NfκB complex</td>
<td>(264, 265)</td>
</tr>
<tr>
<td>RELA</td>
<td>V-rel reticuloendotheliosis viral</td>
<td>Inactive p105 subunit acts as NfκB inhibitor</td>
<td>(120)</td>
</tr>
<tr>
<td><strong>NfκB Target Genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL8</td>
<td>Interleukin 8</td>
<td>Pro-inflammatory chemokine; recruitment of neutrophil</td>
<td>(266)</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
<td>Synthesis of prostaglandin (PGE2)</td>
<td>(227, 268)</td>
</tr>
<tr>
<td>IRAK2</td>
<td>Interleukin1 receptor-associated kinase 2</td>
<td>Component of Toll-like/IL-1 receptor signaling; positive regulator of NfκB</td>
<td>(223)</td>
</tr>
<tr>
<td>BID</td>
<td>BH3 interacting domain death agonist</td>
<td>Pro-apoptotic protein</td>
<td>(269)</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
<td>Pro-apoptotic protein</td>
<td>(269, 270)</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
<td>transcription factor mediating cell death</td>
<td>(271, 272)</td>
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<tr>
<td>Gene</td>
<td>Full Name</td>
<td>Function of Encoded Protein</td>
<td>Ref</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------</td>
<td>------</td>
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<tr>
<td><strong>Genes in Other Signaling Pathways</strong></td>
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<tr>
<td>ALOX15B</td>
<td>Arachidonate 15-lipoxygenase, type B</td>
<td>Lipoxygenase that converts arachidonic acid to 15S-hydroperoxyeicosatetraenoic acid</td>
<td>(273)</td>
</tr>
<tr>
<td>PKCA</td>
<td>Protein kinase C α</td>
<td>Serine/Threonine Kinase; proliferation; apoptosis; differentiation</td>
<td>(274)</td>
</tr>
<tr>
<td>MAPK1</td>
<td>Mitogen-activated protein kinase 1</td>
<td>ERK; p38; proliferation, differentiation, transcription regulation</td>
<td>(275)</td>
</tr>
<tr>
<td>MAPK8</td>
<td>Mitogen-activated protein kinase 8</td>
<td>JNK; proliferation, differentiation, transcription regulation</td>
<td>(275)</td>
</tr>
<tr>
<td><strong>Housekeeping Genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL13A</td>
<td>Ribosomal protein L13a</td>
<td>Housekeeping gene</td>
<td>(276)</td>
</tr>
<tr>
<td>ACTB</td>
<td>β-Actin</td>
<td>Housekeeping gene</td>
<td>(276)</td>
</tr>
</tbody>
</table>
Appendix 4. Time Course of Activation of Nrf-2 and NfκB Signaling by IFNγ/PMA in Caco-2 and FHs 74 Int Cells

Appendix Figure A2. Time Course Activation of Nrf-2 and NfκB Signaling by IFNγ/PMA in Caco-2 cells. Caco-2 cells (4.5 × 10^5 cells/mL) were seeded in six well plates and differentiated for 21 days. Cells were stimulated with IFNγ/PMA in media containing serum (1.5 mL) for various incubation times and then nuclear protein was extracted as described in Chapter 3. Nrf-2 protein in nuclear extract was determined using Western blotting, and results are normalized to actin as a housekeeping protein. NfκB activation was determined using Trans-Am ELISA kits as described in Chapter 3. * denotes significant difference at P < 0.05 vs. time zero.
Appendix Figure A3. Time Course Activation of Nrf-2 and NfkB Signaling by IFNγ/PMA in FHs 74 Int cells. FHs 74 Int cells were processed as described in Chapter 3. After two days of incubation at 37°C, cells were placed in serum-free media for 18 hours, followed by incubation in 1% serum for 24 hours. Media were removed and cells were stimulated with IFNγ/PMA in 1.5 mL of media containing 1% serum for various incubation times. Nrf-2 and NfkB activation was determined in nuclear protein using Trans-Am ELISA kit as described in Chapter 3. * denotes significant difference at P < 0.05 vs. time zero. Results are expressed as mean ± standard deviation (n = 3)