EFFECTS OF D-DELTA TOCOTRIENOL ON HIGH-FAT DIET-INDUCED PERIPHERAL INFLAMMATION

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by

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Under the Direction of Desiree Wanders, PhD

ABSTRACT

Little is known about the ability of d-δ-tocotrienol to protect against obesity-induced inflammation. These studies were conducted to determine whether d-δ-tocotrienol inhibits high-fat diet (HFD)-induced peripheral inflammation, and to explore potential mechanisms by which d-δ-tocotrienol affects inflammation. In two animal experiments, mice were fed a low-fat control diet, a high-fat control diet, or a HFD supplemented with d-δ-tocotrienol: 400 mg/kg diet (experiment #1) or 60 mg/kg body weight (experiment #2) for 14 weeks. Expression of inflammatory and anti-inflammatory markers was measured in liver and white adipose tissue, and phosphorylation of STAT3 was measured using western blot. d-δ-tocotrienol mitigate HFD-induced hepatic inflammation, despite having no effect on body weight, suggesting direct anti-inflammatory effects of d-δ-tocotrienol independent of body weight loss. Mechanistic studies in 3T3-L1 adipocytes indicated that d-δ-tocotrienol suppresses LPS-induced inflammation through down-regulating STAT3 signaling. The potential for d-δ-tocotrienol as a treatment of obesity and related metabolic diseases requires further investigation.

INDEX WORDS: d-δ-tocotrienol, Anti-inflammatory, Signal Transducer and Activator of Transcription (STAT3)
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INFLAMMATION

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DEDICATION

I would like to dedicate my work to my Mother, Brother and Sister in law, who insisted

to in me the merits of perseverance and commitment and persistently encouraged me to thrive for

excellence.
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<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AT</td>
<td>Adipose Tissue</td>
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<tr>
<td>ATM</td>
<td>Adipose tissue macrophage</td>
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<tr>
<td>EWAT</td>
<td>Epididymal White Adipose Tissue</td>
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<tr>
<td>FGF21</td>
<td>Fibroblast Growth Factor-21</td>
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<td>HFD</td>
<td>High-Fat Diet</td>
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<tr>
<td>pSTAT3&lt;sup&gt;Tyr705&lt;/sup&gt;</td>
<td>Phosphorylated STAT3 at Tyr705</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
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<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signaling 3</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription</td>
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<tr>
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<td>Tumor necrosis factor-alpha</td>
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1 INTRODUCTION

1.1 Obesity-induced inflammation

1.1.1 Adipose tissue inflammation

Adipose tissue (AT) is now recognized as more than simply a storage depot for excess energy. The endocrine nature of AT has been well-established by the discovery of proteins, termed “adipokines”, that are produced and secreted by adipocytes. Adipokines can act locally in an autocrine/paracrine manner, or on distant tissues through their endocrine function. In addition to its endocrine nature, AT possesses various signaling receptors that establish bidirectional crosstalk with other parts of the body through the central nervous system (CNS) or traditional circulating hormones [1]. This crosstalk is carried out by a number of proteins produced by adipocytes, including leptin, TNFα, IL-6, MCP-1, adiponectin, plasminogen activator inhibitor 1 (PAI1), adipsin, acylation stimulating protein (ASP), and resistin, among others [1]. During high-fat diet feeding and obesity, adipocytes undergo hypertrophy, and adipocyte production of inflammatory cytokines becomes dysfunctional [2, 3]. During obesity, adipocytes display increased production of the pro-inflammatory cytokines and chemokines, IL-6, TNFα, and MCP1, and decreased secretion of the anti-inflammatory cytokine, adiponectin [3-5]. This leads to a low-grade inflammation within the adipose tissue, and increased infiltration of the fat depot by inflammatory immune cells [6, 7].

In normal, healthy adipose tissue there are several resident immune cells present, including macrophages. These native adipose tissue immune cells, including M2 macrophages, IL-4- and CD4+ Tregs among others, appear to exert protective effects against the development of metabolic dysfunction [8-11]. Functional diversity of adipose tissue macrophages (ATM)
depends upon stimulation of leukocytes by local stimuli [12]. When administered \textit{in vitro} or \textit{in vivo} the inflammatory stimulators lipopolysaccharide (LPS) or IFN-\(\gamma\) induce macrophages to attain the classical pro-inflammatory (M1) state, generating a Th1 response to exaggerate obesity [13, 14]. However, when polarized to the M2 state, macrophages stimulate Th2 cytokine response promoting IL-4 and IL-13, thus attenuating the classical inflammatory NF-\(\kappa\)B dependent pathways [13, 14]. Activation and maintenance of the M2, or “alternatively activated” state of adipose tissue macrophages is intrinsically regulated by production of IL-4, PPAR\(\gamma\), PPAR \(\delta\), and regulation of lipid metabolism and mitochondrial activity [15].

Obesity-associated inflammation, insulin resistance and pathogenesis is mediated by ATM. Phenotypic heterogeneity of these macrophages is characterized by their activation or polarization between M2 (alternative) and M1 (classical) state [6, 16, 17]. These macrophages are responsible for sensing, integrating and maintaining the microenvironment of fat depots [18]. Upon receiving specific stimulus, these macrophages polarize into M1 or M2 state. When stimulated with LPS or interferon gamma, M1 upregulates IRF5, priming Th1 innate immune response by producing IL-12, IL-23, TNF involved in activation of Th1 and Th17 response [19, 20]. Alternatively, M2 becomes activated by production of immunomodulatory Th2 cytokines (IL-4 and IL-13), CD4\(^+\) T cells and eosinophil derived Th2 cytokines [9, 21]. With high-fat diet feeding and obesity, the adipose tissue immune cell population shifts to a more pro-inflammatory state [7]. The population shift from anti-inflammatory (M2) macrophages to a more pro-inflammatory (M1) profile correlates with insulin resistance [22]. Variation in gene expression and ATM profile coordinates with
progression of IR mediated by accumulation of CD8$^+$ T cells, Th1 polarization CD4$^+$ T cells, natural killer, natural killer T cells, and mast cells [10, 23-25].

1.1.2 Hepatic inflammation

Similar to AT inflammation and obesity, non-alcoholic fatty liver disease (NAFLD) is also associated with elevated levels of M1/Th1 cytokines and infiltration of macrophages. Odegaard et al., and Kang et al., in 2008, suggested that modulation of PPARδ-dependent polarization of M2 NAFLD in mice [26, 27]. In obese and diabetic patients, the hepatic macrophages are largely accounted for by the presence of kupffer cells (KCs), with higher level of MCP-1 which serves recruitment of monocyte in liver. Monocytes mature and exaggerate obesity-induced hepatic inflammation and insulin resistance, thus stimulating hepatocyte lipogenesis, ceramide production and adipocyte lipolysis [28]. Obese individuals show higher proinflammatory cytokines (IL-6, IL-1β, TNF-α, MCP-1) in circulation, suggesting that obesity is the underlying cause of insulin resistance, where the paracrine function of MCP1 suppress PPARγ expression thus contributing toward insulin resistance [29-32]. Modulation of these proteins (IL-1β, TNF-α) potentially depends upon activation of JNK and IKKβ/NF-κB, protein kinase R and IKK signaling through classical receptor-mediated signaling mechanism [29, 33-36].

1.1.3 Link between obesity-induced inflammation and metabolic disease

There is now strong evidence supporting the idea that obesity-induced inflammation leads to cardiovascular and metabolic diseases [37]. Increased systemic inflammation and inflammatory proteins like CRP, IL-6, PAI-1, VCAM-1, α1-glycoprotein is directly correlated with increase in body mass index (BMI) [38]. Additionally, accumulation of monocytes, macrophages, T lymphocytes and activated endometrium results in
cardiovascular disease [39]. Accumulated monocyte and macrophages promote lipid peroxidation of LDL through the production of ROS, and inflammatory cytokines like IL-1β, TNFα and IL-6, resulting in endothelial dysfunction [40]. Defective endothelial membrane induce adhesion and transendothelial migration of monocytes [40]. Subsequently IL-1β, and TNF-α stimulate expression of VCAM-1 and ICAM-1, E selectin, and movement of monocytes into sub endothelial layer is regulated by MCP-1, and [41, 42]. Adhesion and accumulation of platelets leads to the formation of thrombus, contributing toward acute coronary infection and myocardial infraction [39]. According to Festa et al. (2000), chronic inflammation plays important role initiating insulin resistance to induce hyperinsulinemia [2]. Further insulin resistance leads to enhanced tumor growth (colorectal and endometrial), as reported by Women Health Initiative (WHI) and European prospective investigation into Cancer and Nutrition (EPIC) [43-46]. This increased risk of cancer is driven either by mitogen effect of hyperinsulinemia or by increased production of insulin like growth factor (IGF1) in circulation [47, 48], as concluded by Renehan et al (2004), Rinaldi et al (2010) and using meta-analysis[46, 48]. In 2011 Eltzschig & Carmeliet characterized T2DM and obesity by increased production of inflammatory cytokines (IL-6 and TNFα) from AT [49]. Increased level of IL-6 correlated in patients with breast cancer, prostate cancer, B-cell lymphoma and myeloma [49-51]. Subsequently, TNFα solicited proliferation and progression of tumors by activating NF-kB pathway [52]. Together, studies indicate that obesity-induced inflammation may be a factor contributing to the development of cardiovascular disease, insulin resistance, type 2 diabetes, and cancer.
1.2 Tocotrienols

Given the rising global obesity epidemic, the development of novel nutritional and pharmacological approaches to reduce the deleterious consequences of obesity has become a major focus of research initiatives. Despite these efforts, most treatments have proven ineffective, dangerous, or largely unsustainable. Tocotrienols have emerged as a natural treatment of several metabolic disorders, including inflammation [39]. Vitamin E is also referred to as tocochromanols because of the presence of chroman-6-ols in its two isoforms, the tocopherol and tocotrienol [53, 54]. Dietary tocotrienols can be obtained from seed endosperm of monocots, a limited number of dicots, palm oil, and cereal grains such as wheat, rice, rice bran, and barley. Highest concentration of tocotrienols can be obtained from Elaeis guineensis (800 mg/kg) through crude palm oil extract, primarily consisting of alpha (α), beta (β), delta(δ), and gamma (γ) tocotrienol [55]. Since the recognition of alpha tocopherol as isoform of vitamin E, eight chemically distinct isoforms have been classified into two categories, consisting of alpha (α), beta (β), delta(δ), and gamma (γ)-tocopherol (TP) and α, β, δ, and γ-tocotrienol. These isoforms of tocotrienol are classified based on number and location of methyl group on chromanol rings (Figure 1). At the molecular level, the effects of tocotrienols are mediated through transcriptional factors, translational and post-translational modifications [56]. Tocotrienols exhibit strong antioxidant, anti-proliferative, anti-survival, proapoptotic, antiangiogenic and anti-inflammatory activity [57].
Figure 1 Structural classification of alpha, beta, gamma and delta tocotrienols based on the number and location of methyl group. α- tocotrienol: 5,7,8, trimethyl; β-tocotrienol: 5,8 dimethyl; γ-tocotrienol: 7,8 dimethyl; d-δ-tocotrienol: 8, monomethyl.

The presence of the unsaturated side chain of the tocotrienols structure allows for effective penetration into tissues with saturated fatty layer such as brain and liver [57].

Mechanistically, absorption and distribution of tocotrienols isoforms depends upon the binding affinity with signaling molecules in brain, liver, intestine and AT [58]. Once reached to the bloodstream and exposed to chain of free radicals, tocotrienols neutralize free radicals before oxidative damage can occur to cellular membranes. Moreover, tocotrienols have been shown to decrease serum cholesterol and reduce atherogenic apolipoprotein B
concentrations [58, 59]. At the tissue level, absorption takes place with the help of enzyme lipoprotein lipase, or via receptor-mediated endocytosis of lipoproteins [58]. Lipoprotein lipase then degrades lipid into remnant for absorption by liver and other peripheral tissues. Interestingly bioavailability of tocotrienols is highest in adipose tissue followed by adrenal gland, suggesting potential involvement of d-tocotrienol in ameliorating low grade chronic inflammation [58].

According to Sylvester and Shah (2005), tocotrienols possess a challenge in terms of their bioavailability since it can be difficult to obtain therapeutic level of γ-tocotrienols in circulation [60]. The bioavailability of tocotrienols increases when it is administrated with food, owing to the necessity of micelle formation for optimal tocotrienols absorption [61]. Regardless of its relatively low bioavailability, accumulation of tocotrienols is high in AT, skin and peripheral tissue [62, 63]. Accumulated tocotrienols in AT remain metabolically active and effective against obesity due to its prolong stay in AT and slows rate of degradation [64]. This suggests that tocotrienols might have prominent effect against obesity and obesity-induced inflammation and metabolic disease.

1.3 Anti-inflammatory properties of tocotrienols

Anti-inflammatory activity of tocotrienols has been studied extensively in cancer cell models. Though these studies provide promising evidence for their additional research, and is required to determine optimal isoforms and doses for understanding their mechanism of action. Inflammation involves cascade of reactions, led by the activation of transcription factors such as NF-κB, STAT3, and Hypoxia induced factor -1 (HIF-1) [65-71]. Activated transcription factors stimulate production of inflammatory markers such as nitric oxide synthase (iNOS), cyclooxygenase (COX), nitric oxide (NO) and prostaglandin E2 (PGE2)
Activation of these factors are closely associated with enhance expression of IL-1, IL-6, and IL-8, to promulgate inflammation. According to Jiang et al. 2008, long-chain carboxychromanols of vitamin E mediated ω- and β-oxidation of hydrophobic side chain to inhibit COX [73]. This underscores the importance of carboxylic acid on C-13 and the length of side chain, among the isoforms of tocotrienol and tocopherol with chromanol analogs, in inhibiting COX [73]. Additionally, tocotrienols have been shown to suppress expression of NF-κB in diabetic rats and cancer cell lines [74, 75], IL-1 and [76], IL-6 in rats bones [77], IL-8 in angiogenesis and telomerase activity [78], iNO [79], and COX2 in cancer cells [73, 75]. Most studies utilized either γ-tocotrienol or tocotrienol-rich fractions of palm oil. Few studies examined the anti-inflammatory properties of tocotrienols in models of obesity. Our study is novel in that it evaluates the anti-inflammatory effects of δ-tocotrienol in two mouse models of obesity.

Inflammation of AT is associated with infiltration of macrophages; however, administration of gamma-tocotrienol has been shown to inhibit infiltration of macrophages, limits transformation of M2 to M1 macrophages in bone marrow derived macrophages, and suppress conversion of monocytes to macrophages via multiple signaling pathways [79-84]. α- Tocotrienol inhibits proteasome via activation of P27 and P57 in human monocyte cells, suppression of LPS induced NO and PGE2, NF-κB activation in human monocyte cells, and inhibition of VCAM-1 and ICAM-1 from vascular endothelium cells [79, 82-84]. Further, Wang et al. concluded that gamma TT suppresses activity of CEBP/alpha and NF-κB in bone marrow derived macrophages when stimulated by LPS [85]. In addition, Li et al. suggested that tocotrienol-rich fraction of palm oil potentiates activation of PPARγ and
PPARα in macrophages and human THP-1 cells [86]. Together, studies conducted *in vitro* and *in vivo* model systems suggest that TT exhibit strong anti-inflammatory effects.

1.4 **Signal transducer and activator of transcription 3 (STAT3) and inflammation.**

STAT3 acts as DNA binding transcription factor that plays a vital role in proliferation (c-fos, c-myc and cycin D1), invasion (e.g. matrix metalloproteins-2), apoptosis (e.g. bcl-xL and survivin), and angiogenesis (e.g. VEGF) [33, 87-90]. Similar to other STAT family proteins, STAT3 also possesses a DNA binding domain, amino-terminus for tetramerization, and SH2 domain for recruitment of receptors and carboxy-terminal transactivation domain.

Activation of STAT3 occurs via phosphorylation at tyrosine residue 705 (Tyr705 or Y705) or at Serine-727 (Ser727 or S727), and results in formation of STAT3 homodimer along with SH2 and SHP domain and that is translocated into the nucleus [73, 87, 89-91]. These molecules get recruited to the receptor and activate JAK1, which phosphorylates Tyr705 of STAT3. Phosphorylation of Tyr705 mediated by JAK1 drives STAT3 dimerization, and subsequent translocation into the nucleus, where they bind to gene promoters [92]. In contrast, phosphorylation at Ser727 is driven by various factors like tyrosine motifs, ERK1, 2, p38, JNK and H-7 sensitive kinase [93], or by recruitment of transcription cofactors as in STAT1 [94]. Activation of STAT3 by phosphorylation on either Tyr705 or Ser 727 results in activation of downstream gene or signaling molecules such as Ras/Raf/MAP kinase and the phosphatidylinositol 3-kinase (PI3-kinase) [95].

STAT3-mediated inflammation in cancer cells is well characterized, while its role in obesity is obscure. Obesity-promoted development of hepatocellular carcinoma depends on the production of IL-6 and TNF-α, and subsequent activation of STAT3 [96]. STAT3 plays
crucial role in propagating inflammation by activating downstream pathways like epidermal growth factor (EGFR), and platelet derived growth factor receptor (PDGFR) [97, 98]. Activated STAT3 can cause enhanced expression of NF-kB upon activation by IL-6 [99-101]. Activation of NF-kB depends upon RELA-p50 and REL, where RELA in nucleus cause acetylation of p300 (EP300) to activate STAT3 signaling [102, 103] in cancer cells. Additional activators of STAT3-induced inflammation includes IL-23, IL21, IL-6, COX2, IL-17 and IL-23 [100, 103-108].

1.5 Tocotrienols and STAT3

Tocotrienols have been shown to suppress cancer cell proliferation through downregulating STAT3 signaling. γ-TT effectively suppressed proliferation of multiple myeloma cells by blocking activation of Src kinase and Janus kinase 1 (JAK1), regulators of STAT3 [109]. Accordingly, Liu et al., (2012) found that expression of STAT3 is proportional to S1P receptor expression (S1PR1) in tumor cells, upregulating IL-6 expression [110]. Inhibition of STAT3 by γ-tocotrienol in mesothelioma cells occur via redox-silent analog of α-tocotrienol, 6-O-carboxypropyl-α-tocotrienol [32, 33]. γ-tocotrienol has been shown to suppress STAT3 activity in human hepatocellular carcinoma (Hep3b) cells [111]. Likewise, the literature support a possible anti-inflammatory effect of tocotrienols mediated through STAT3 signaling, but these studies were conducted in cancer cells. Inhibition of these activating factors may contribute toward prevention or restriction of obesity induced inflammation of peripheral tissue. According to Kannappan 2012, tocotrienol was able to suppress activation of Src kinase, Janus kinase 1 (JAK-1) and JAK2 the regulators of STAT3 by inducing expression of tyrosine phosphatase SHP-1 [109].
There have been a limited number of studies to examine the effects of tocotrienols in obesity-induced inflammation in adipocytes [81, 112-114]. Further, there have been no studies examining the effects of d-δ-tocotrienol on HFD-induced inflammation in the liver. The purpose of this study was to determine if d-δ-tocotrienol inhibits HFD-induced inflammation in liver and adipose tissue, and to identify if STAT3 mediates any anti-inflammatory effects of d-δ-tocotrienol. Therefore, the following aims were pursued.

Specific Aim #1. Test the hypothesis that d-δ-tocotrienol inhibits HFD-induced inflammation in liver and adipose tissue. Gene expression of inflammatory cytokines and immune cell markers will be measured in livers and adipose tissue from mice fed HFD with or without tocotrienol. Cell culture studies in 3T3-L1 adipocytes will identify whether d-δ-tocotrienol can inhibit LPS-induced inflammation.

Specific Aim #2. Test the hypothesis that d-δ-tocotrienol inhibits STAT3 signaling in liver and adipose tissue of HFD-fed mice. pSTAT3<sup>tyr705</sup> and pSTAT3<sup>Ser727</sup> will be measured by immunoblot analysis from livers and adipose tissue of mice fed HFD with or without TT. Cell culture studies in 3T3-L1 adipocytes will identify whether d-δ-tocotrienol can inhibit IL-6-stimulated STAT3 activation (phosphorylation).

2 MATERIAL AND METHODS

2.1 Animal studies

Animal studies were conducted at the Department of Pathology, Texas Tech University Health Sciences Center (TTUHSC), Lubbock, TX, USA and tissues were provided by Dr.
Chwan-Li Shen. Experimental protocols were reviewed and approved by Institutional Animal Care and Use Committee at TTUHSC.

2.1.1 Experiment #1

Male C57BL/6 mice (Charles River Laboratories), aged 5-6 weeks, were fed a low-fat control diet (n=9), a high-fat control diet (n=12), or a high-fat diet supplemented with d-δ-tocotrienol (400 mg/kg diet; n=9) for 14 weeks. All of the diets are tocopherol-free basal diet with TT added to tocopherol-free soybean oil for the groups receiving d-δ-tocotrienol. Liver samples were harvested and flash frozen in liquid nitrogen.

2.1.2 Experiment #2

Male CD-1 mice (Charles River Laboratories), aged 5-6 weeks, were randomly assigned to one of three treatment groups: 1) low-fat diet (n=10), 2) high-fat diet vehicle (n=10); 3) high-fat diet TT (n=10). The mice in the HFD vehicle group received distilled water by oral administration 5 times per week. The mice in the HFD TT group received d-δ-tocotrienol (60 mg/kg body weight) 5 times per week for 14 weeks. The TT was DeltaGold product at 70% purity containing ~ 90% delta and 10% gamma-tocotrienol, and was generously provided by American River Nutrition (Hadley, MA). Liver and inguinal white adipose tissue (IWAT) were harvested and flash frozen in liquid nitrogen.

2.2 Cell culture studies

3T3-L1 cells (ATCC) were cultured in high-glucose DMEM (Dulbecco’s modified Eagle medium) fortified with 10% FBS (fetal bovine serum albumin), 1% penicillin and streptomycin in a humidifier of 10% CO₂ at 37°C. After 3T3-L1 preadipocytes reached confluence, cells were differentiated using medium with, 1 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM IBMX. After 2-3 days of treatment with differentiation media,
media was changed to DMEM with 10% FBS and 1% penicillin and streptomycin, there after changed after 2 days until fully confluent (~10 days after induction of differentiation).

Fully differentiated 3T3-L1 adipocytes were treated with variable concentrations of d-δ-tocotrienol (0, 2.5, 5, 10, 25 and 50 μM) over-night in serum free media (SFM). Cells were treated with 20 ng/ml of IL-6 (Cell signaling technology) for 20 minutes, and protein was extracted for western blot analysis. In other 3T3-L1 studies, fully differentiated 3T3-L1 adipocytes were treated with variable concentrations of d-δ-tocotrienol over-night in serum free media (SFM). Cells were then incubated for 24 hours with lipopolysaccharides (LPS).

2.3 RNA extraction

Liver tissue- RNA was extracted from liver samples using Qiagen RNeasy tissue kit 50, DNA digestion was performed to improve the purity of RNA and to avoid DNA contamination. Concentration of RNA was measured using Nanodrop (Core facility, Department of Biology). Isolated RNA was used to prepare cDNA using the protocol of Promega, containing initial concentration of 2 μg RNA. Prepared cDNA was diluted up to 3.33 ng/μl for Real-time PCR.

Inguinal white adipose tissue (IWAT) tissue – Approximately 80-100 mg of IWAT tissue was homogenized in cold Trizol reagent, and was centrifuge at 12000RPM for 30 minutes. Infranatant was transferred to spin columns provided in the Qiagen RNeasy Lipid Tissue kit. DNA digestion was performed to improve the purity of RNA and to avoid DNA contamination. Concentration of RNA was measured using Nanodrop (Core facility, Department of Biology). Isolated RNA was used to prepare cDNA using the protocol of Promega, containing initial concentration of 2 μg RNA.
3T3-L1 cells – LPS-treated cells were harvested for extraction of RNA using Trizol reagent. Cells were washed with PBS once, followed by addition of 300 µl of Trizol into each well of 6 well plates. Plates were incubated on ice for 20 minutes. Cells were transferred into a new 1.7ml tubes, 250 µL of chloroform were added to the tubes, and tubes were shaken vigorously for 10 sec. Tubes were incubated at room temperature for 15 minutes. Tubes were centrifuged at 12,000 RPM for 15 min at 4°C, and supernatant was transferred to a new tube. 550 µL of isopropanol was added to each tube, and tubes were mixed by inversion. Tubes were incubated at room temperature for 15 minutes. Tubes were centrifuged at 12,000 RPM for 30 min at 4°C, and supernatant was removed without disturbing the pellet. 800 µL of cold 70% ethanol (prepared in nuclease free water) was added to each tube, and the solution was centrifuged at 12,000 RPM for 15 min at 4°C. The supernatant was removed, followed by quick spin for 2 min at 12,000 RPM, remove leftover ethanol. The tubes were incubated on ice for 10 min, followed by addition of 20 µL of nuclease-free water. RNA concentration and quality was measured using Nanodrop (Core facility, Department of Biology). Isolated RNA was used to prepare cDNA using the protocol of Promega, containing initial concentration of 2ug RNA.

2.4 Real-time PCR

Real-time PCR was performed on liver cDNA (3.33 ng/µl) using SYBR-green reagent. Expression of macrophage-associated markers [Emr1(F4/80), Itgam (CD11b), Itgax (CD11c)], inflammatory/anti-inflammatory markers (Ifng, Il2, Ccl2 (MCP-1), Il6, Tnf, Il1b, Il17, Il23, Il10), and the STAT3-specific markers [Stat3, Ccnd1 (Cyclin D1), Socs3, and Kfl5] was measured and normalized with cyclophilin (Ppia).
Real time PCR was performed on IWAT cDNA (3.33ng/ul) using SYBR-green reagent, expression of macrophage associated markers (Ccl2 (MCP-1), Emr1(F4/80), Itgam (CD11b), Itgax (CD11c), inflammatory markers Il6 was measured and normalized with cyclophilin.

Real time PCR was performed on 3T3-L1 cell treated with variable concentration of tocotrienol and LPS. Isolated RNA was used to prepare cDNA (3.33 ng/µl). mRNA expression of the inflammatory markers Tnf, Il6, Ccl2 and the anti-inflammatory cytokine, Il10 and was detected using SYBR green reagent and was normalized to cyclophilin. Primer sequences are found in table 1.

Table 1 Primers used for gene expression studies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2</td>
<td>5-GGAGAGACACCGGTCGTA AG</td>
<td>5-CCAGCCGGCAACTGTGA</td>
</tr>
<tr>
<td>Ccl5</td>
<td>5-GCTCCAATCTTGAGTCGTG</td>
<td>5-GAGCGCTAGATGCCCATT</td>
</tr>
<tr>
<td>Ccnd1</td>
<td>5-CAAGTGTGACCCCGACTGC</td>
<td>5-GCTCCCTACTTCAAGGTTGA</td>
</tr>
<tr>
<td>Emr1</td>
<td>5-GTGCCATCATTCAGGGAT TC</td>
<td>5-GACCGGTGAGCAGACGAGTGA</td>
</tr>
<tr>
<td>Fgf21</td>
<td>5-AGCATAACCCCATCCCTGACT</td>
<td>5-TCTCCCTGATCTCCAGGTTGA</td>
</tr>
<tr>
<td>Il1b</td>
<td>5-TGCCAATTTGAGGCTATG</td>
<td>5-TTCTTGAGGCCCTGAGAGC</td>
</tr>
<tr>
<td>Il2</td>
<td>5-CATGCAGCCTGCATCTGT</td>
<td>5- AAGTGGGCTTGAAGTGGG</td>
</tr>
<tr>
<td>Il6</td>
<td>5-ATGGATGCTCATAACTGTGATG</td>
<td>5-TGAAGGACTTGCTTTTGCTCT</td>
</tr>
<tr>
<td>Il10</td>
<td>5-TAATCGACCCACTTCCCAG</td>
<td>5-AGGCTTGGAACCACAGTAA</td>
</tr>
<tr>
<td>Il17</td>
<td>5-TTCTGGGGTGAGGTACAGA</td>
<td>5-GACATTGTCACCACAGGCAG</td>
</tr>
<tr>
<td>Ifng</td>
<td>5-GAGGTCAACACCCACAGGT</td>
<td>5-GGGACAATCTCTCCACACC</td>
</tr>
<tr>
<td>Itgax</td>
<td>5-GGGACGCTTTACCTGGGTTAC</td>
<td>5-CCTGGAAATCTCTGAGGTTG</td>
</tr>
<tr>
<td>Itgam</td>
<td>5-CCACACTAGCATCAAGGG CA</td>
<td>5-CCCTGTACCGTGAGAGA AG</td>
</tr>
</tbody>
</table>
In order to determine the effect of d-δ-tocotrienol on STAT3 signaling in 3T3-L1 adipocytes, western blot analysis was performed for STAT3. Phosphorylation of STAT3 at Tyr705 and Ser 727 was measured using the protein extracted from 3T3-L1 cells. Fully differentiated 3T3-L1 cells treated with d-δ-tocotrienol were treated with 300 µl of radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. Plates were incubated in ice for 20 minutes. Cell lysates were scraped and collected into microcentrifuge tubes, followed by centrifugation at 13,200 rpm for 30 minutes at 4°C. The semitransparent solution between the pellet and the supernatant was pipetted into a new tube for protein estimation using Lowry Protein Assay. Approximately 40 µg of concentrated protein was diluted using 4X laemmli buffer and was boiled for 5 minutes at 100°C.

Prepared protein sample was loaded in precast gel (Criterion Precast) purchased from Bio-Rad Laboratory, and electrophoresis was performed at 90-110V for 2.5-3-hours. Electrophorotic gel was transferred to PVDF membrane overnight at 10V. Next day, PVDF membrane was washed with deionized water and was incubated in blocking buffer (5% BSA in TBS) for 1hr followed by addition of primary antibody prepared in 5% BSA.
(pSTAT3\textsuperscript{Tyr705} and pSTAT3\textsuperscript{Ser727} in 5% BSA and total STAT3 in 1% BSA). Incubated for 1 hr at 4°C, followed by 3 washes for 5 minutes each. Secondary antibody was added to the membrane (secondary Ab is anti-rabbit in 1% or 5% BSA or NFDM) and incubate for one hour, followed by 3 wash (5min each) by TBSt. Membrane was loaded with ECL for 5minutes and was rushed to ImageQuant LAS4000 equipment and software (GE Healthcare). pSTAT3\textsuperscript{Tyr705} and pSTAT3\textsuperscript{Ser727} was purchased from Cell Signaling Technology (Danvers, MA), STAT3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Band intensity and density was quantified using ImageJ (software) and were normalized with total STAT3.

2.6 Enzyme-linked Immunosorbent Assay (ELISA)-

Serum was obtained from mice in experiment # 2 at the end of the study. Serum adiponectin concentrations were analyzed using an ELISA kit from EMD Millipore Corporation (Billerica, MA, USA) per manufacturer’s instructions.

2.7 Statistical analysis

RT-PCR data was analyzed using one-way ANOVA. Turkey’s multiple comparison was used to identify significant differences between the groups. Western blot was analyzed by two-way ANOVA. Data were analyzed using Prism software (version 7.0, GraphPad, La Jolla, California).
3 RESULTS

3.1 Background Information

3.1.1 Body weight –

In experiment #1, the C57BL/6 mice fed the HFD gained significantly more weight than the LFD-fed controls, and d-δ-tocotrienol (400 mg/kg diet) had no effect on body weight (Fig 2A). In experiment #2, the CD-1 mice fed with HFD for 14 weeks gained weight significantly compared to the LFD-fed controls, and in this study, d-δ-tocotrienol (60 mg/kg body weight) slightly, but significantly attenuated the HFD-induced increase in body weight (Fig 2B). EWAT was weighed in experiment #2, and HFD increased EWAT weight, and d-δ-tocotrienol had no effect on fat pad weight (Fig 3).

Figure 2  A) Body weight of C57BL/6 mice fed with normal chow diet (LFD), high fat diet (HFD) or HFD supplemented with d-δ-tocotrienol (400 mg/kg diet) for 14 weeks.  B) Body weight of CD-1 mice LFD, HFD or HFD and d-δ-tocotrienol (60 mg/kg body weight) for 14 weeks. Values are means ± SEM, n=9-12.
3.2 Anti-inflammatory effect of d-δ-tocotrienol in liver

HFD-fed mice showed increased expression of some inflammatory markers when compared with LFD-fed control mice. Monocyte chemoattractant protein-1 (MCP-1 or Ccl2) expression was significantly increased by HFD, but this increase was suppressed by d-δ-tocotrienol (400 mg/kg diet) in liver tissue (Fig 4). Hepatic expression of Emr1 was reduced when TT was administered orally at 60 mg/kg body weight (Fig. 5A), but not when given at 400 mg/kg diet (Fig 5B). Likewise, expression of the inflammatory macrophage marker, Itgax, was increased in the liver by HFD (Fig. 6A & B). While tocotrienol administered at a dose of 400 mg/kg diet had no significant effect on Itgax expression (Fig. 6A), 60mg/kg body weight TT administered orally prevented the HFD-induced increase in Itgax (Fig. 6B). Tocotrienol tended to reduce hepatic expression of Itgam (CD11b), but this did not reach statistical significance (Fig 6C).
Figure 4 Hepatic Ccl2 (MCP-1) mRNA expression in 14 weeks old C57BL/6 mice fed with normal chow diet (LFD), high fat diet (HFD) and d-δ-tocotrienol (400 mg/kg diet). Values are means ± SEM, n=7-9.

Figure 5 A) Hepatic Emr1 (F4/80) mRNA expression CD1 mice fed LFD, HFD, and HFD with d-δ-tocotrienol (60 mg/kg body weight). B) Hepatic Emr1 (F4/80) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (400 mg/kg diet), n=7-9 Values are means ± SEM, n=8-10.
Figure 6  
A) Hepatic Cd11c (ITGAX) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (400 mg/kg diet), n=7-9.  
B). Hepatic Cd11c (ITGAX) mRNA expression CD1 mice fed LFD, HFD, and HFD with d-δ-tocotrienol (60 mg/kg body weight). Values are means ± SEM, n=8-10.  
C) Hepatic Cd11b (ITGAM) mRNA expression in 14 weeks CD1 mice fed with LFD, HFD and d-δ-tocotrienol (60 mg/kg body weight), n=8-10. Values are means ± SEM

400 mg/kg diet d-δ-tocotrienol supplementation suppressed the HFD-induced increase in hepatic Tnf-α (Fig 7) and Il2 (Fig 8) mRNA expression. d-δ-tocotrienol supplementation had no
significant effect on expression of \textit{Il6} (Fig 9), \textit{Il23} (Fig 10) \textit{Interferon-gamma} (Fig 11), \textit{Il10} (Fig.12) or \textit{Il-1beta} (Fig 13).

\textit{Figure 7} Hepatic Tumor necrosis factor- alpha (TNF-\(\alpha\)) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-\(\delta\)-tocotrienol (400 mg/kg diet). Values are means \(\pm\) SEM, \(n=7-9\)

\textit{Figure 8} Hepatic Interleukin-2 (IL-2) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-\(\delta\)-tocotrienol (400 mg/kg diet). Values are means \(\pm\) SEM, \(n=7-9\)

\textit{Figure 9} Hepatic Interleukin-6 (IL-6) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-\(\delta\)-tocotrienol (400 mg/kg diet). Values are means \(\pm\) SEM, \(n=7-9\)
Figure 10 Hepatic Interleukin-23 (IL-23) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (400 mg/kg diet). Values are means ± SEM, n=7-9

Figure 11 Hepatic Interferon-gamma (Ifn-γ) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (400 mg/kg diet). Values are means ± SEM, n=7-9

Figure 12 Hepatic Interleukin-10 (IL-10) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (400 mg/kg diet). Values are means ± SEM, n=7-9
STAT3 associated inflammatory genes – To understand the role of STAT3 in mediating systemic inflammation during obesity, we analyzed expression of Stat3 target genes. There was no effect of d-δ-tocotrienol on STAT3 or its target genes like IL-17 (Fig 15), Cyclin D1 (Fig 16) and Krüppel-like factors 5 (Fig 17) in the liver, except for Socs3. Expression of SOCS3 was significantly suppressed by d-δ-tocotrienol (Fig. 18).
Figure 15 Hepatic Interleukin-17 (IL-17) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (400 mg/kg diet). Values are means ± SEM, n=7-9.

Figure 16 Hepatic Cyclin D1 mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (400 mg/kg diet). Values are means ± SEM, n=7-9.

Figure 17 Hepatic Krüppel-like factors (KLF5) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (400 mg/kg diet). Values are means ± SEM, n=7-9.
3.3 Anti-inflammatory effect of d-δ-tocotrienol in inguinal white adipose tissue (IWAT)

Administration of d-δ-tocotrienol (60 mg/kg body weight) had no effect on the HFD-induced increase in MCP-1 expression (Fig 19) in IWAT tissue. Similar effects were observed with Emr1 (F4/80) (Fig 20), Cd11b (ITGAM) (Fig 21) and Cd11c (ITGAX) (Fig 22), and Il6 (Fig 23), though strong trends towards reductions in expression of Itgam (CD11b), and Itgax were observed in IWAT of TT-supplemented mice.

Figure 18 Hepatic Suppressor of cytokine signaling 3 (SOCS3) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (400 mg/kg diet). Values are means ± SEM, n=7-9.

Figure 19 IWAT Ccl-2 (MCP-1) mRNA expression in C57BL/6 mice fed with LFD, HFD, and d-δ-tocotrienol (60 mg/kg body weight). Values are means ± SEM, n=7-9.
Figure 20 IWAT Emr1 (F4/80) mRNA expression in 14 weeks old CD-1 mice fed with normal chow diet (LFD), high fat diet (HFD) and d-δ-tocotrienol (60mg/kg body weight), Values are means ± SEM, n=7-9.

Figure 21 IWAT Cd11c (Itgax) mRNA expression in 14 weeks old CD-1 mice fed with normal chow diet (LFD), high fat diet (HFD) and d-δ-tocotrienol (60mg/kg body weight), Values are means ± SEM, n=7-9.

Figure 22 IWAT Cd11b (Itgam) mRNA expression in 14 weeks old CD-1 mice fed with normal chow diet (LFD), high fat diet (HFD) and d-δ-tocotrienol (60mg/kg body weight), Values are means ± SEM, n=7-9.
3.4 STAT3 Phosphorylation in 3T3-L1 Adipocytes –

To test whether d-δ-tocotrienol can suppress LPS-induced inflammation in cultured adipocytes, 3T3-L1 adipocytes were cultured overnight in variable concentrations of d-δ-tocotrienol followed by 24 hour incubation with LPS (10 µM). As expected, LPS increased expression of Il6, Tnf, and Ccl2 (Fig 24 A-C). Treatment of 3T3-L1s with d-δ-tocotrienol decreased expression of Tnf and Ccl2 (Fig 24 B & C). Surprisingly, d-δ-tocotrienol had no effect on LPS-induced Il6 expression (Fig 24A). Interestingly, 50 µM d-δ-tocotrienol increased expression of the anti-inflammatory cytokine, IL-10 in LPS-treated adipocytes (Fig 25).
Figure 24. mRNA expression of A) Il-6 B) Tnf-α C) Ccl2 (Mcp-1) in 3T3-L1 cells treated with different concentration of δ-δ-tocotrienol overnight followed by addition of LPS (10 μg/ml) for 24 hr to induce inflammation. * represents significant difference compared to LPS-treated cells receiving vehicle.
Figure 25 mRNA expression of Interleukin-10 (IL-10) in 3T3-L1 cells treated with different concentration of d-δ-tocotrienol overnight followed by addition of LPS (10 μg/ml) for 24 hr to induced anti-inflammation response. * represents significant difference compared to LPS-treated cells receiving vehicle.

To determine signaling mechanisms mediating the anti-inflammatory effects of d-δ-tocotrienol on Tnf expression in 3T3-L1 adipocytes, phosphorylation of the inflammatory transcription factor, STAT3, was measured. d-δ-tocotrienol had variable effects on basal and IL-6-induced STAT3 signaling in 3T3-L1 adipocytes depending on the concentration used. Higher concentrations of TT reduced basal and IL-6-induced STAT3 phosphorylation at Tyr\(^{705}\) and Ser\(^{727}\) (Fig 26).
3.5 Serum adiponectin concentrations

To test whether anti-inflammatory effects of δ-tocotrienol in vivo were mediated through increases in concentrations of the anti-inflammatory adipokine, adiponectin, adiponectin concentrations in the serum of mice from experiment #2 were measured. There was no effect of HFD or TT on circulating adiponectin concentrations (Fig 27).
Figure 27 Effect of d-δ-tocotrienol on circulating adiponectin in CD-1 mice fed a LFD, HFD, or HFD with d-δ-tocotrienol (60 mg/kg body weight). Values are means ± SEM, n=7-9.
4 DISCUSSION

Obesity, inflammation and metabolic syndromes are closely associated, each being characterized by increased production of inflammatory cytokines, interacting with various inflammatory signaling pathways [115]. Obesity-induced low grade systemic inflammation contributes toward the development of NAFLD, characterized by accumulation of fat in liver with increased free fatty acids, reactive oxygen species, TNF-α, and enhanced activity of signaling molecules like IL-6, resulting in insulin resistance [116]. Development and progression of NAFLD primarily depends on a complex crosstalk between different organs and cells. This crosstalk is established by some major signaling pathways, including NFkB, AMPK, JAK/STAT, PPARs, PI3K, PKB/Akt, and Toll-like receptor (TLR) resulting in IR, oxidative stress and inflammation [117]. The ability to inhibit or suppress pace of any of these pathways will help us to ameliorate the effect of obesity-induced hepatic inflammation and steatosis.

In our experiment, d-δ-tocotrienol suppressed the high-fat diet-induced increase in expression of Ccl2, Emr1, Cd11c, and CD11b in liver. However, not all inflammatory markers we measured followed this pattern. To our knowledge, there have been no studies examining anti-inflammatory properties of d-δ-tocotrienol in liver of high-fat diet-fed animals. Though further investigation is required, these data provide evidence that d-δ-tocotrienol holds promise as a natural therapeutic for obesity-related inflammation.

Extensive studies on the endocrine nature of WAT has revealed that WAT is a complex organ that has the ability to integrate with other systems. While the nature of obesity-induced inflammation is complex, it is known that obesity-associated inflammation comes about, in part,
due to the secretion of inflammatory cytokines (IL-6, TNF-alpha, MCP-1) either from adipocytes or immune cells whose presence in WAT and liver increases during obesity. These WAT-derived inflammatory cytokines simulate production of inflammatory markers from immune cells located in liver and other organs [118, 119]. These cytokines include MCP-1, TNF-α, IL-1, IL-6 and IL-8. These inflammatory molecules have been shown to activate c-Jun NH2-terminal kinase (JNK), phosphorylating IRS-1 at Ser 307, which has been linked to obesity-induced insulin resistance, metabolic disorders, CVD, and cancer [80, 81, 114, 120-123]. Downregulation of obesity-induced adipose tissue inflammation has the potential to ameliorate a number of obesity-associated metabolic diseases.

Though strong trends were seen regarding d-δ-tocotrienol-induced suppression of expression of the inflammatory immune cell markers \textit{Itgam} and \textit{Itgax}, d-δ-tocotrienol had no significant effects on inflammatory markers in IWAT of mice. These results are not in accordance with Zhao \textit{et al}., (2015), who reported that γ-tocotrienol administered by oral gavage to mice for 4 weeks decreased inflammatory gene expression in mice fed a HFD [81]. There are some differences between the study designs. For instance, Zhao \textit{et al}. used γ-tocotrienol, while our study used δ-tocotrienol. Further, Zhao \textit{et al}. studied the epididymal WAT, while we used inguinal WAT. There is evidence that visceral adipose tissue contains more inflammatory and immune cells than subcutaneous adipose tissue [124]. Therefore, the use of a subcutaneous depot may have masked any anti-inflammatory effects of d-δ-tocotrienol. Despite not seeing effects on adipose tissue inflammation \textit{in vivo}, we did see an effect of d-δ-tocotrienol to suppress LPS-induced inflammation in cultured adipocytes. Similar findings were seen in Matsunaga \textit{et al} (2012) when 3T3-L1 adipocytes were cultured in the presence of γ-tocotrienol with or without the
inflammatory stimulus, TNF-α [114]. Our data are novel in that d-δ-tocotrienol has not been tested for its anti-inflammatory properties in cultured adipocytes.

γ-tocotrienol has been shown to suppress STAT3 signaling in cancer cell lines [109, 111, 125-127]. Only one study, to our knowledge, has shown that d-δ-tocotrienol inhibits STAT3 signaling, and this was conducted in a model of human bladder cancer. We therefore wanted to test whether the anti-inflammatory effects of d-δ-tocotrienol in 3T3-L1 adipocytes was mediated through downregulation of STAT3. Interestingly, we did see that some concentrations of d-δ-tocotrienol suppressed basal and IL-6-induced STAT3 phosphorylation, and hence activation, in 3T3-L1 adipocytes. These data suggest that STAT3 may be the mechanism behind the anti-inflammatory effects of d-δ-tocotrienol in cultured adipocytes. Future studies are needed to see if d-δ-tocotrienol inhibits STAT3 \textit{in vivo}.

NF-κB, a multiprotein transcription factor regulating secretion of TNFα and MCP-1, which phosphorylate and degrade inhibitory IKB1 or IKB2, activating NF-κB to travel into the nucleus and activate NF-κB-dependent transcription factors modulating activity of target genes [128-133]. Tocotrienol has been shown to suppress constitutively active NF-κB in mammalian epithelial cell line, prostate cancer, multiple myeloma, and colon cancer [74, 134-136]. While we did not explore NF-κB, it is possible that this inflammatory transcription factor could be an additional mediator of the anti-inflammatory effects of d-δ-tocotrienol seen in the liver and adipocytes.

We postulated that d-δ-tocotrienol may have a global impact by enhancing secretion of the anti-inflammatory adipokine, adiponectin. We found no effect of HFD or d-δ-tocotrienol on
serum adiponectin concentrations. Our data are in accordance with the literature. It has been shown before that chronic (4 months) HFD feeding had no effect on serum adiponectin concentrations in mice [137]. Further, Zhao et al 2015) found no effect of γ-tocotrienol on serum adiponectin concentrations in mice fed a HFD [81, 138].

The majority of studies on STAT3 and its signaling pathways have been studied in cancer cells, where its activation is in response to IL-6, IL-10, IL-17/23 family of cytokine [139]. Suppressor of cytokine signaling 3 (SOCS3) is a downstream target of STAT3. SOCS3 is key to suppress or promote inflammation depending upon the method of STAT3 activation (either via gp130 family cytokine or by IL-10 cytokine family) [140]. d-δ-tocotrienol suppressed expression of SOCS in liver, indicating potential downregulation of STAT3 signaling. However, not all STAT3 target genes were downregulated, suggesting potential alternative signaling pathways downstream of STAT3.
5 CONCLUSION

Our data suggest that d-δ-tocotrienol decreases expression of some inflammatory markers in liver, but not IWAT of mice fed a HFD. d-δ-tocotrienol also has direct effects on adipocytes to suppress LPS-induced inflammation and IL-6-induced STAT3 signaling. Further studies are needed to determine the optimal isoform, dose, and treatment time of tocotrienol as a potential treatment for obesity-induced inflammation.
REFERENCES


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