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Dietary α- and γ-tocopherol supplementation attenuates lipopolysaccharide-induced oxidative stress and inflammatory-related responses in an obese mouse model of nonalcoholic steatohepatitis☆

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Abstract

Oxidative stress contributes towards the development of nonalcoholic steatohepatitis (NASH). Thus, antioxidants may decrease oxidative stress and ameliorate the events contributing to NASH. We hypothesized that α- or γ-tocopherol would protect against lipopolysaccharide (LPS)-triggered NASH in an obese (*ob*/*ob*) mouse model. Five-week-old obese mice (n=18/dietary treatment) were provided 15 mg/kg each of α- and γ-tocopherol or 500 mg/kg of α- or γ-tocopherol for 5-weeks. Then, all mice were injected ip once with LPS (250 μg/kg) before being sacrificed at 0, 1.5 or 6 h. Body weight and hepatic steatosis were unaffected by tocopherols and LPS. Hepatic α- and γ-tocopherol increased ($P<.05$) ~9.8- and 10-fold in respective tocopherol supplemented mice and decreased in response to LPS. LPS increased serum alanine aminotransferase (ALT) by 86% at 6 h and each tocopherol decreased this response by 29–31%. By 6 h, LPS increased hepatic malondialdehyde (MDA) and tumor necrosis factor-α by 81% and 44%, respectively, which were decreased by α- or γ-tocopherol. Serum ALT was correlated ($P<.05$) to hepatic tumor necrosis factor-α ($r=0.585$) and MDA ($r=0.592$), suggesting that inflammation and lipid peroxidation contributed to LPS-triggered hepatic injury. α- and γ-Tocopherol similarly attenuated LPS-triggered increases in serum free fatty acid, and α-tocopherol only maintained the LPS-triggered serum triacylglycerol responses at 6 h. These findings indicate that increasing hepatic α- or γ-tocopherol protected against LPS-induced NASH by decreasing liver damage, lipid peroxidation, and inflammation without affecting body mass or hepatic steatosis. Further study is needed to define the mechanisms by which these tocopherols protected against LPS-triggered NASH.

Keywords: Vitamin E; Nonalcoholic steatohepatitis; Lipid peroxidation; Inflammation; Alanine aminotransferase

1. Introduction

The incidence of hepatic steatosis and NASH has increased dramatically in parallel with the underway obesity epidemic that currently afflicts two thirds of Americans [1]. Approximately 40 million American adults are estimated to have hepatic steatosis or NASH with as many as 58–74% of obese individuals being afflicted [2]. At present, there are no validated treatments for these diseases beyond co-morbidity management. Dietary modification and weight loss are first lines of treatment [2], but poor compliance limits their effectiveness [3]. Thus, in the absence of validated therapies, the identification of easily implemented and effective strategies to prevent the development of NASH is needed.

Early evidence suggested that hepatic steatosis was relatively benign, but continued study has led to the understanding that patients with steatotic livers are at increased risk for progressing towards NASH and liver-related morbidity and mortality [4]. The mechanisms leading to hepatic steatosis and subsequently NASH remain poorly understood, but are often characterized by a "two-hit" mechanism [5]. The "first-hit," triggered by obesity and insulin resistance, results in excess hepatic lipid accumulation causing hepatic steatosis and injury. Steatotic livers are vulnerable to "second-hits," mediated by inflammation and/or oxidative stress, resulting in lipid peroxidation, exacerbated hepatic injury, inflammatory infiltration, and NASH [5]. Importantly, the inflammatory cytokine tumor necrosis factor-α (TNF-α) contributes to the progression to NASH by stimulating lipogenesis and lipolysis as well as by provoking hepatic injury, likely mediated by inducing mitochondrial dysfunction and oxidative stress [6].

Although numerous models of NASH exist [7,8], obese (*ob*/*ob*) mice have been routinely used because they become obese, dyslipidemic, insulin resistant, and develop hepatic injury and steatosis. An advantage of *ob*/*ob* mice is that they do not spontaneously develop

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NASH. However, NASH can be reproducibly and rapidly induced by acute LPS administration [9–11], which triggers hepatic oxidative stress and inflammatory infiltration, increases inflammatory cytokines including TNF-α, and substantially increases circulating ALT, a commonly used surrogate marker of hepatic damage [12]. Thus, this model mimics a proposed etiology of NASH. Indeed, obese mice [13], like obese humans [14], have greater intestinal permeability resulting in translocation of gut-derived bacteria and LPS, which induce inflammatory- and oxidative stress-mediated responses contributing to NASH. Thus, LPS treatment to *ob*/*ob* mice conveniently facilitates the evaluation of targeted therapies against simple steatosis or NASH.

The involvement of oxidative stress suggests that antioxidants could potentially mitigate the "second-hit," by the enhanced degradation, or prevention, of the formation of reactive oxygen species (ROS), provided that they could be targeted to hepatic tissue. Vitamin E is the term given to eight closely related lipophilic compounds including four tocopherols and four tocotrienols that function as chain breaking antioxidants to cease the propagation of lipid peroxidation [15]. α- and γ-Tocopherol are most important to humans due to their dietary and biological abundance [15]. Preliminary humans trials with α-tocopherol supplementation have been equivocal with some demonstrating decreases in serum ALT in children with NASH [16] and improvements in serum ALT and histologic evidence of hepatic inflammation and fibrosis in NASH patients [17]. In contrast, 6-week [18] or 12–24-month [19,20] α-tocopherol supplementation had no benefit over lifestyle modification on serum markers or hepatic histopathology of NASH in children or adults. However, no studies have examined γ-tocopherol in mitigating hepatic injury or NASH. γ-Tocopherol, in addition to its antioxidant function, has unique anti-inflammatory activities not shared with α-tocopherol [21–23] suggesting that γ-tocopherol may attenuate underlying pathogenic events leading to NASH. Thus, given that vitamin E therapy does not routinely improve markers or pathology of NASH and lifestyle modifications have poor long-term success rates, a need exists for studying α- and γ-tocopherol as potential preventative strategies against inflammatory and oxidative stress-mediated second-hits leading to NASH.

The role of unique vitamin E forms in mitigating oxidative stress and inflammatory responses during the development of NASH in *ob*/*ob* mice has not been studied. Thus, we hypothesized that α- or γ-tocopherol accumulation in steatotic livers would decrease hepatic oxidative stress, inflammation, and injury responses implicated in the progression of NASH in *ob*/*ob* mice. To this end, we fed *ob*/*ob* mice diets containing α- or γ-tocopherol and then injected LPS to induce hepatic injury and NASH. We then assessed the tocopherol-mediated effects on hepatic lipid, oxidative stress and inflammation, and serum ALT and dyslipidemia during LPS-induced NASH.

2. Materials and methods

2.1. Materials

High-performance liquid chromatography (HPLC)-grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA) as were the following chemicals: ascorbic acid, butanol, butylated hydroxytoluene (BHT), phosphate-buffered saline (PBS), chloroform, ethanol, methanol, hexane, potassium chloride (KCl), potassium hydroxide (KOH), lithium perchlorate, sodium chloride (NaCl), sodium phosphate, sulfuric acid. Vitamin E standards (*RRR*-α- and *RRR*-γ-tocopherol), LPS (*E. coli* 0111:B4), protease inhibitor cocktail (P8340), 1,1,3,3-tetramethoxypropane, sodium deoxycholate, sodium dodecyl sulfate (SDS), Trizma hydrochloride (Tris-HCl), and 2-thiobarbituric acid (TBA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Nonidet-P40 (NP-40) was purchased from US Biological (Swampscott, MA, USA).

2.2. Animals and study design

The protocol for the care and use of animals was approved by the Institutional Care and Use Committee at the University of Connecticut. Male leptin-deficient obese (*ob*/*ob*) mice (n=54; 4-5 week of age) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and individually housed in a temperature (20–22°C) and humidity (50–60%) controlled room with a 12-h light-dark cycle. After 1-week acclimation, mice (n=18 mice/dietary treatment) weighing 27.3±0.5 g (mean±S.E.) were assigned randomly to one of three modified AIN-93G diets [24] for 5 weeks: (A) a vitamin E adequate control diet containing 15 mg/kg each *RRR*-α-tocopherol and *RRR*-γ-tocopherol, (B) a diet containing 500 mg/kg of *RRR*-α-tocopherol, or (C) a diet containing 500 mg/kg *RRR*-γ-tocopherol (Table 1). *Ob*/*ob* mice at 4–5-week of age were chosen to allow the 5-week dietary intervention to occur during the time period when these mice are known to become obese and develop severe hepatic steatosis, hepatic injury, and dyslipidemia [25–27]. The tocopherol doses were selected based on prior studies indicating that similar dietary levels of α- and γ-tocopherol significantly increased plasma and liver tocopherol concentrations [24,28]. The basal as well as the α-tocopherol- and γ-tocopherol-supplemented diets were prepared by Harlan Teklad (Madison, WI, USA) using tocopherol-stripped soybean oil to control the dietary vitamin E content. *RRR*-α-tocopherol (>99% α-tocopherol) and *RRR*-γ-tocopherol (97%-γ-tocopherol) were a kind gift from Dr. Brent Flickinger (Archer Daniels Midland; Decatur, IL, USA).

Mice had free access to the diet and water throughout the study and food intake and body weight were recorded weekly. After the 5-week feeding, all mice were injected ip with LPS (250 μg/kg body weight) to rapidly induce hepatic injury and NASH as described previously [11]. Mice were then sacrificed at 0, 1.5 or 6 h post injection. Regardless of the sacrifice time point, all mice were fasted for 5–6 h. Blood was collected from the retro-orbital sinus, serum was obtained by centrifugation (1500×g, 4°C, 10 min) and subsequently snap-frozen in liquid nitrogen and stored at −80°C. Liver was harvested, rinsed in PBS, blotted, snap frozen, and stored at −80°C until analysis.

2.3. Hepatic vitamin E

Hepatic vitamin E, as α- and γ-tocopherol, was measured as described [25]. In brief, liver was saponified in alcoholic KOH in the presence of ascorbic acid for 30 min at 70°C. Subsequently, the sample was extracted with hexane, and then the extract was evaporated under nitrogen gas and dissolved in ethanol:methanol (1:1) prior to injection on an HPLC-Coularray system (ESA Inc; Chelmsford, MA, USA) consisting of 2 solvent delivery modules (Model 582), a refrigerated autosampler (Model 542) maintained at 4°C and a 4-channel coulometric cell (Model 6210) set to 150, 250, 350, and 450 mV. Tocopherols were separated isocratically (0.6 ml/min) at room temperature on a Phenomenex Luna $C_{18}(2)$ column (150 mm×3.0 mm id; 3 μ; Torrance, CA, USA) using 10 mM lithium perchlorate in methanol:water (98:2) as the mobile phase. Tocopherol standards were prepared in ethanol and verified spectrophotometrically using molar absorption coefficients for α-tocopherol ($\varepsilon^{292\ nm}$=3270 $M^{-1} \cdot cm^{-1}$) and γ-tocopherol ($\varepsilon^{298\ nm}$=3810 $M^{-1} \cdot cm^{-1}$) [29].

2.4. Hepatic and serum lipid

Total hepatic lipid was determined gravimetrically as described previously [30]. Briefly, liver was minced and incubated overnight in 2:1 chloroform:methanol containing 151 μmol/L BHT. The sample was filtered and the lipid extract was separated with 0.05% (vol/vol) sulfuric acid. The lower phase was collected, evaporated under nitrogen, and the lipid content was weighed. Serum triacylglycerol (ThermoElectron; Louisville, CO, USA) and free fatty acid (Wako Diagnostics; Richmond, VA, USA) were analyzed using separate clinical assays and performed in accordance with the manufacturer's instructions using a microplate reader (Molecular Devices SpectraMax M_2; Sunnyvale, CA, USA).

2.5. Serum alanine aminotransferase activity

Serum alanine aminotransferase (ALT) activity was measured spectrophotometrically using a commercially available clinical assay and was performed in accordance with the manufacturer's instructions (ThermoElectron; Louisville, CO, USA). In brief, sample was mixed with kit reagent and the reaction was monitored at 340 nm for 3 min at 37°C on a peltier, temperature-controlled spectrophotometer (Beckman Coulter DU 800; Fullerton, CA, USA).

2.6. Hepatic malondialdehyde

Hepatic malondialdehyde (MDA) was extracted [31] and measured [32] by HPLC-FL. In brief, liver was homogenized in KCL and a portion of the homogenate was mixed with water, BHT prepared in ethanol, SDS and TBA reagent. The sample was incubated (60 min, 100°C), rapidly chilled, and mixed with butanol. Following centrifugation

Table 1
Dietary vitamin E concentrations

Diet	α-Tocopherol (mg/kg)	γ-Tocopherol (mg/kg)
Vitamin E adequate diet	15	15
α-Tocopherol diet	500	15
γ-Tocopherol diet	15	500

(16,000×g, 4°C, 10 min), the butanol layer was injected on the HPLC system (Beckman Coulter; Fullerton, CA, USA) consisting of a Gold 126 solvent delivery module, a Gold 508 refrigerated autosampler maintained at 4°C, and a Jasco FP-2020 plus (Easton, MD, USA) florescence detector. The sample was separated isocratically (0.9 ml/min) on a Phenomenex Luna C18 (2) column (250×4.6 mm id; 5 μ; Torrance, CA, USA) using methanol and 50 mM phosphate buffer (60:40, pH 5.5). MDA was detected at excitation and emission settings of 532 and 553 nm, respectively, and was quantified against standards prepared in parallel from 1,1,3,3-tetramethoxypropane. MDA was normalized to hepatic protein, which was measured using a commercially available Bradford assay (Bio-Rad Laboratories; Hercules, CA, USA).

2.7. Hepatic TNF-α

Livers were prepared as described previously [33], with minor modifications. In brief, liver was homogenized in ice-cold RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors. The homogenate was incubated 30 min on ice, centrifuged (17,000×g, 30 min, 4°C), and the supernatant diluted 1:350 for ELISA analysis (eBioscience; San Diego, CA, USA), which was performed in accordance with the manufacturer's instructions. Hepatic TNF-α was normalized to hepatic protein as described above.

2.8. Statistical analysis

Data were analyzed using GraphPad Prism (Version 4.03; GraphPad Software, La Jolla, CA, USA). Initial and final body weights of mice were evaluated using two-way analysis of variance (ANOVA) with repeated measures. Two-way ANOVA with Bonferroni's posttest was used to evaluate the effects attributed to dietary treatment, LPS administration, and their interaction. Specific dietary treatment effects within an LPS time point are illustrated in the figures using lettered superscripts. Correlations were performed by linear regression analysis. All data are expressed as means±S.E. The α-level for statistical significance was set at $P<.05$.

3. Results

3.1. Body weight and food intake were unaffected by tocopherol supplementation

Initial body weights were not significantly different between control mice (26.7±0.9 g) and those provided α-tocopherol (27.7±0.9 g) or γ-tocopherol (27.2±1.0 g). Following the 5-week intervention, all mice gained ~50% greater ($P<.05$) body mass and no differences ($P>.05$) occurred between controls (42.9±1.0 g) and those fed diets containing α-tocopherol (41.6±1.0 g) and γ-tocopherol (40.7±1.2 g). Daily food intake (g/d) was not different ($P>.05$) between controls (6.1±0.1) and mice fed α-tocopherol (5.8±0.1) or γ-tocopherol (5.8±0.2).

3.2. Tocopherol supplementation did not affect hepatic steatosis

The severity of hepatic steatosis was unaffected ($P>.05$) by tocopherol supplementation or LPS. Hepatic total lipid (mg/g liver) of control mice at 0, 1.5, and 6 h post LPS injection was 297.9±50.1, 283.3±31.6, and 315.4±20.0, respectively. Mice provided diets supplemented with α-tocopherol had hepatic total lipid (mg/g liver) of 292.3±22.3, 311.2±31.0, and 324.8±12.8 at 0, 1.5, and 6 h, respectively, in comparison to those from γ-tocopherol supplemented mice (320.9±16.7, 295.6±22.3, 276.8±34.5) at the respective time points. Hepatic lipid was correlated ($r=0.585$, $P<.0001$) with body mass consistent with obesity as a risk factor for hepatic steatosis.

3.3. Dietary α- or γ-tocopherol supplementation increased hepatic tocopherols whereas LPS administration decreased hepatic tocopherols

Supplementation with α- or γ-tocopherol increased hepatic α- and γ-tocopherol concentrations by ~9.8- and 10-fold ($P<.05$), respectively, compared to obese controls (Fig. 1). Hepatic α-tocopherol concentration was 64-times greater ($P<.05$) than the γ-tocopherol concentration from mice supplemented with the respective tocopherol. Hepatic α-tocopherol was unaffected by LPS in obese controls (Fig. 1A and B). In α-tocopherol supplemented mice, LPS decreased hepatic α-tocopherol by 18-20% at 6 h ($P<.05$) compared to 0 and 1.5 h. Hepatic γ-tocopherol was unaffected by LPS in obese controls and α-tocopherol supplemented mice (Fig. 1B). However, in γ-tocopherol-supplemented mice, LPS time-dependently decreased ($P<.05$) hepatic γ-tocopherol by 14% and 33%. Collectively, α- or γ-tocopherol supplementation substantially increased hepatic tocopherol accumulation and LPS decreased tocopherols in mice provided tocopherol supplemented diets.

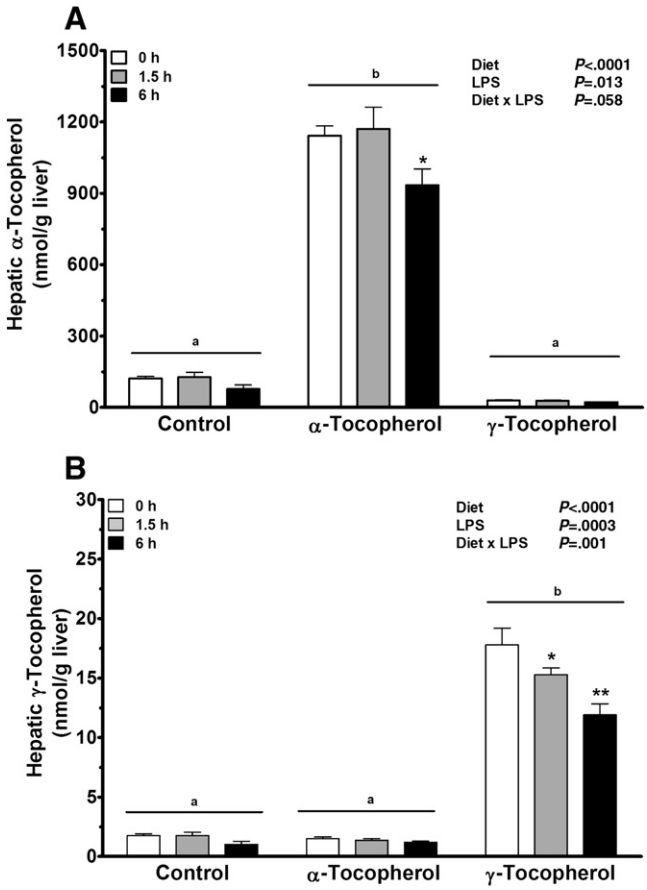

Fig. 1. Hepatic (A) α- and (B) γ-tocopherol were measured at 0, 1.5, and 6 h post-LPS injection from obese mice fed a vitamin E adequate control diet, 500 mg/kg of α-tocopherol, or 500 mg/kg of γ-tocopherol. Values are means±S.E., $n=5$–6 mice/dietary treatment/LPS time point. Between dietary treatments, labeled means without a common letter differ, $P<.05$. Within a dietary treatment, labeled means not sharing a common symbol differ, $P<.05$.

3.4. Tocopherol supplementation protected against LPS-induced liver damage

Serum ALT activity is frequently increased in NASH patients [2]. Following the intervention and immediately after LPS administration (0 h), serum ALT was markedly elevated in all groups [normal reference interval of male C57BL6 mice = 14–38 U/L [34]] (Fig. 2). Serum ALT at 0 h was unaffected ($P>.05$) by vitamin E despite 15–22% lower ALT activities among mice fed α- and γ-tocopherol. Following LPS, ALT increased ($P<.05$) in obese controls by 57–86% at 1.5 and 6 h (Fig. 2). α- or γ-Tocopherol supplementation decreased LPS-triggered responses in serum ALT at 1.5 and 6 h. Serum ALT of α- and γ-tocopherol supplemented mice were 29–36% and 31–34% lower ($P<.05$), respectively, at 1.5 and 6 h compared to time-matched controls. Serum ALT was not different between mice fed α- and γ-tocopherol at any of the times studied. Thus, LPS exacerbated ALT

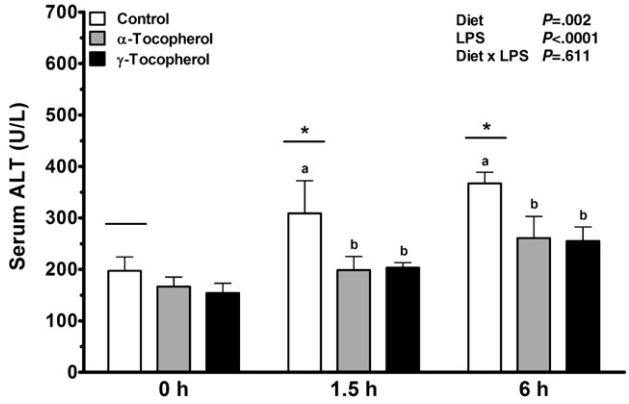

Fig. 2. Serum ALT activity was measured at 0, 1.5, and 6 h post-LPS injection from obese mice fed a vitamin E adequate control diet, 500 mg/kg of α-tocopherol, or 500 mg/kg of γ-tocopherol. Values are means±S.E., n=5-6 mice/dietary treatment/LPS time point. *Means were significantly different from 0 h in control mice only, P<.05. Within an LPS time point, labeled means without a common letter differ, P<.05.

levels and tocopherol supplementation decreased these responses in obese mice having steatotic livers.

3.5. Vitamin E supplementation blocked LPS-triggered hepatic lipid peroxidation

At 0 h, hepatic MDA was lower (P<.05; Fig. 3) only in α-tocopherol supplemented mice compared to obese controls. LPS increased hepatic MDA (P<.05) in control mice by 81% at 6 h. Hepatic MDA was unaffected (P>.05) by LPS in mice provided either tocopherol. Mice fed α-tocopherol had 58% and 70% lower (P<.05) hepatic MDA at 1.5 and 6 h, respectively, compared to time-matched controls. Hepatic MDA from γ-tocopherol supplemented mice was 44% lower, but without statistical significance, at 1.5 h compared to time-matched controls. However, hepatic MDA from these mice at 6 h was 61% lower compared to the controls. No differences in hepatic MDA were observed between α- and γ-tocopherol supplemented mice at any times studied (Fig. 3). Serum ALT and hepatic MDA were correlated (r=0.592, P<.0001) supporting that hepatic lipid peroxidation contributes to hepatic damage. Collectively, LPS increased hepatic lipid peroxidation in obese mice and α- or γ-tocopherol decreased lipid peroxidation without affecting hepatic steatosis.

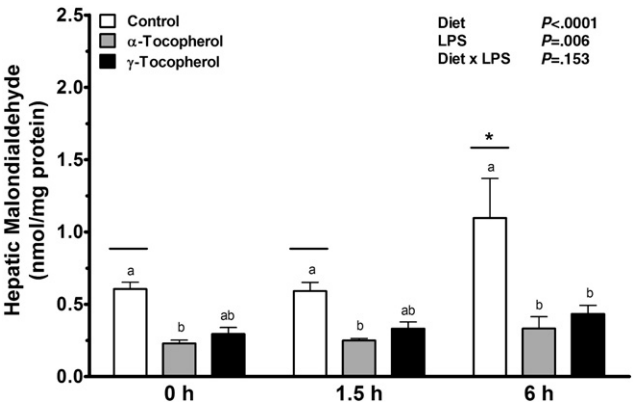

Fig. 3. Hepatic MDA was measured at 0, 1.5, and 6 h post-LPS injection from obese mice fed a vitamin E adequate control diet, 500 mg/kg of α-tocopherol or 500 mg/kg of γ-tocopherol. Values are means±S.E., n=5–6 mice/dietary treatment/LPS time point. *Means were significantly different from 0 and 1.5 h in control mice only, P<.05. Within an LPS time point, labeled means without a common letter differ, P<.05.

3.6. Vitamin E supplementation inhibited LPS-triggered increases in hepatic TNF-α

Inflammation, along with oxidative stress, have been strongly implicated as "second-hits" that contribute to the development of NASH [5]. At 0 h, hepatic TNF-α was not different (P>.05) between the groups (Fig. 4). In contrast, hepatic TNF-α increased 44–45% at 1.5 and 6 h (P<.05) following LPS administration in obese controls. Both tocopherols reduced (P<.05) LPS-triggered increases in hepatic TNF-α. Specifically, mice fed α-tocopherol had 30–32% lower (P<.05) TNF-α at 1.5 and 6 h compared to time-matched controls. γ-Tocopherol supplemented mice had 27% lower (P<.05) hepatic TNF-α at 6 h compared to controls. No difference was observed between mice fed α- and γ-tocopherol at 1.5 or 6 h. Hepatic TNF-α correlated with serum ALT (r=0.585, P<.0001) and hepatic MDA (r=0.584, P<.0001) supporting the relation between hepatic damage, lipid peroxidation and inflammation during the development of NASH. Collectively, α- or γ-tocopherol supplementation decreased the acute inflammatory response induced by LPS that otherwise leads to liver damage.

3.7. Serum triacylglycerol and free fatty acids were exacerbated by LPS and partially mitigated by tocopherol supplementation

Serum triacylglycerol increased in response to LPS (P<.05) and these increases were, in part, reduced by γ-tocopherol supplementation only (P<.05; Fig. 5A). Serum triacylglycerol increased in all mice at 6 h post-LPS compared to earlier times regardless of supplementation. Obese mice had serum triacylglycerol at 6 h that was 3.3–3.8 times higher (P<.05; Fig. 5A) than that from 0 and 1.5 h. Neither tocopherol affected serum triacylglycerol at 0 or 1.5 h. However, an interaction (P<.05) between LPS and tocopherol supplementation indicated serum triacylglycerol increased more substantially in LPS-treated obese controls and α-tocopherol supplemented mice compared to those fed γ-tocopherol (Fig. 5A). Indeed, serum triacylglycerol at 6 h from mice fed γ-tocopherol was 38-43% lower (P<.05) compared to time-matched controls and α-tocopherol-supplemented mice.

Serum free fatty acid increased (P<.05) by 6 h post-LPS, compared to 0 and 1.5 h, regardless of tocopherol supplementation (Fig. 5B). Supplementation or LPS did not affect free fatty acids at 0 or 1.5 h. However, free fatty acids increased (P<.05) by 119% at 6 h post-LPS in obese controls and by 93% and 38% (P<.05; Fig. 5B) in mice provided α- and γ-tocopherol, respectively. An interaction between tocoph-

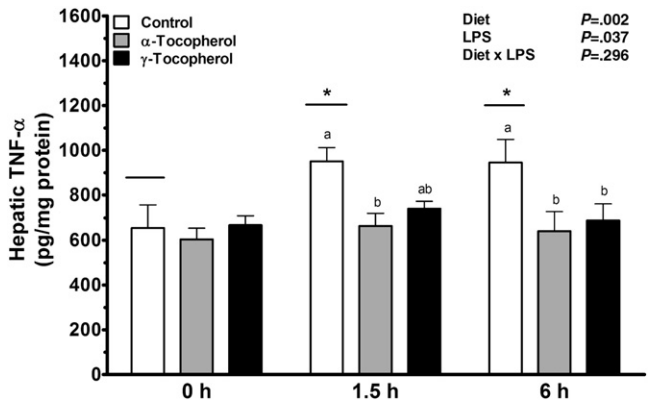

Fig. 4. Hepatic TNF-α concentrations were measured at 0, 1.5, and 6 hour post-LPS injection from obese mice fed a vitamin E adequate control diet, 500 mg/kg of α-tocopherol, or 500 mg/kg of γ-tocopherol. Values are means±S.E., n=5–6 mice/dietary treatment/LPS time point. *Means were significantly different from 0 h in control mice only, P<.05. Within an LPS time point, labeled means without a common letter differ, P<.05.

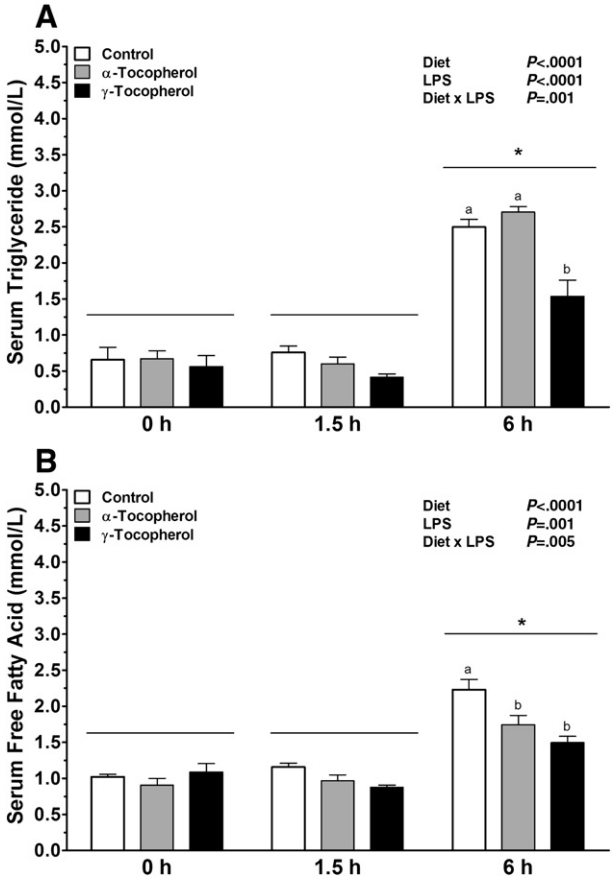

Fig. 5. Serum triacylglycerol (A) and free fatty acid (B) were measured at 0, 1.5, and 6 h post-LPS injection from obese mice fed a vitamin E adequate control diet, 500 mg/kg of α-tocopherol, or 500 mg/kg of γ-tocopherol. Values are means±S.E., n=5–6 mice/dietary treatment/LPS time point. *Means were significantly different from 0 and 1.5 h for each dietary treatment, P<.05. Within an LPS time point, labeled means without a common letter differ, P<.05.

erol supplementation and LPS (P<.05) indicated that both tocopherols, in part, reduced LPS-triggered increases in free fatty acids. Indeed, no differences were observed in free fatty acid concentrations at 6 h from α- and γ-tocopherol supplemented mice, but were 22-33% lower (P<.05) than those from obese controls. Serum ALT activity correlated with serum triacylglycerol (r=0.426, P<.005) and free fatty acid (r=0.525, P<.005) suggesting that liver damage coincided with inflammatory-mediated dyslipidemia. Collectively, LPS-triggered oxidative stress and inflammatory responses induced serum dyslipidemia and tocopherol supplementation decreased or maintained these responses.

4. Discussion

This study provides evidence that 5-week dietary supplementation of either α- or γ-tocopherol decreases LPS-triggered lipid peroxidation, inflammation, and hepatic damage without affecting hepatic steatosis or body mass in an obese (*ob*/*ob*) mouse model of NASH. Supplementation of α- or γ-tocopherol increased the hepatic accumulation of the respective tocopherol with α-tocopherol accumulating more substantially than γ-tocopherol. The levels of both tocopherols decreased from steatotic livers in response to LPS and reduced LPS-induced hepatic damage as suggested by decreases in serum ALT activity. These tocopherols also mitigated LPS-triggered increases in hepatic MDA, hepatic TNF-α, and serum free fatty acids and each of these responses correlated to serum ALT. Thus, the findings from this study suggest that α- and γ-tocopherol protected against several pathogenic events leading to liver damage and NASH, which were likely attributed to their known antioxidant and anti-inflammatory activities, but not to any anti-obesity effects.

To our knowledge, this was the first study examining the hepatoprotective activities of unique vitamin E forms in an obese (*ob*/*ob*) mouse model of NASH. The "first-hit" of NASH is characterized by obesity and excess hepatic lipid accumulation [5], which were observed in our mice and unaffected by tocopherols. The "second-hit" of NASH is characterized by exacerbated hepatic injury, inflammation, and lipid peroxidation [5] and our findings suggested that α- or γ-tocopherol decreased these events. These findings are important because hepatic steatosis is relatively asymptomatic and individuals with this disorder are at greater risk for developing NASH [2]. To date, human clinical trials have only examined α-tocopherol therapy in patients with existing NASH and the findings have been inconsistent [16–20]. Given these conflicting results, this emphasizes the need for preventative strategies such as improving hepatic α- and/or γ-tocopherol levels to attenuate oxidative stress and inflammatory-mediated responses that otherwise cause NASH. Such approaches in high-risk individuals could decrease the risk for developing NASH while enabling individuals to manage their comorbidities through currently recommended lifestyle changes and pharmacological approaches.

Oxidative stress has been identified as a central mechanism contributing to hepatic damage in NASH [2,5]. Multiple sources of ROS formation have been identified including electron leakage from mitochondria, up-regulation of cytochrome P450 metabolism of fatty acids, peroxisomal β-oxidation, and from activated inflammatory cells. Consistent with oxidative stress, our obese (*ob*/*ob*) mice treated with LPS had increased hepatic lipid peroxidation, TNF-α, and serum ALT and dietary supplementation with either tocopherol attenuated these responses. Also, α- or γ-tocopherol decreased the LPS-mediated response leading to greater serum free fatty acids. Collectively, the LPS-mediated increases in TNF-α [35] likely resulted in free fatty acid release from adipocytes by stimulating lipolysis [36]. In turn, serum free fatty acids are readily taken up by the liver where they are esterified to triacylglycerol and exacerbate liver steatosis. Likewise, hepatic free fatty acid accumulation induces oxidative stress through several of the aforementioned intracellular pathways. The selected LPS time points of this study were likely too early to observe alterations in liver lipid accumulation. Indeed, C57BL6 mice treated with LPS had increased serum free fatty acids by 6 h, but increased hepatic triglyceride at 24 h, an effect that was ameliorated using anti-TNF-α antibodies [35]. Future studies may consider examining later time points following LPS, but caution is needed since *ob*/*ob* mice are hypersensitive to LPS resulting in premature mortality by 12-24 h [11].

Our data indicated that LPS-mediated oxidative stress occurs rapidly in steatotic livers leading to greater serum ALT levels. Serum ALT correlated with serum free fatty acids and hepatic TNF-α supporting that inflammatory-mediated dyslipidemia contributed to liver damage. Although no studies have examined γ-tocopherol on NASH, our findings are in agreement with those demonstrating a protective role of α-tocopherol against NASH. In rats fed a methionine-choline deficient diet (MCD) to induce NASH, α-tocopherol supplementation decreased histologic evidence of liver steatosis, inflammation, and fibrosis, decreased hepatic lipid peroxidation, and restored hepatic glutathione [37]. Likewise, mice fed a MCD and α-tocopherol had lower serum aminotransferase activities and hepatic MDA concurrently with improved histologic scores of hepatic steatosis and necroinflammation [38]. These were also accompanied by a suppression in hepatic nuclear factor kappa B activity, cyclooxygenase (COX)-2 protein levels, and increases in

hepatic superoxide dismutase activity. Thus, a common feature of *ob*/*ob* mice and rodents fed a MCD is that α-tocopherol protects against NASH by improving inflammatory and oxidative stress events that otherwise lead to lipid peroxidation and liver injury. Existing evidence demonstrates that subcutaneous administration of α-tocopherol to Zucker rats enriches mitochondrial α-tocopherol and decreases mitochondrial lipid peroxidation and hepatic injury [39]. Thus, more work is warranted to determine intracellular tocopherol localization (e.g., mitochondria, peroxisome) in *ob*/*ob* mice following dietary supplementation to better define their site(s) of hepatoprotection during NASH.

The protective effects of α- and γ-tocopherol against the pathogenic events involved in NASH are explained by their hepatic accumulation. As expected [24,40], supplementation substantially increased hepatic tocopherol accumulation. Likewise, LPS-triggered oxidative stress likely decreased these tocopherols consistent with their antioxidant function. Obese mice having steatotic livers accumulated 2-6-times greater α- and γ-tocopherol compared to those from lean mice [40] without affecting the hepatic status of the alternative tocopherol. The lower hepatic accumulation of γ-tocopherol compared to α-tocopherol is consistent with its active metabolism to γ-CEHC (carboxyethyl-hydroxychromanol) whereas α-tocopherol is metabolized limitedly to α-CEHC [24,40,41]. Regardless, γ-tocopherol or γ-CEHC inhibited COX activity [42] and prostaglandin E_2 production [41] as well as inhibited LPS-induced increases in nuclear factor κB activation, TNF-α and ROS generation [43]. Thus, α- and γ-tocopherol have direct hepatoprotective effects in addition to the indirect effects of γ-tocopherol via γ-CEHC that may mitigate inflammatory responses contributing to hepatic injury and NASH.

Numerous factors are implicated to contribute towards the development of NASH [5]. Unfortunately, there are no validated biomarkers for NASH, but serum ALT is commonly used as a surrogate for assessing liver injury and defining the response to therapy. In NASH patients, the rate of serum ALT change was correlated with changes in histology-derived semi-quantitative scores of inflammation, but not to liver steatosis [44]. Thus, our observed relation between hepatic TNF-α and serum ALT links a specific inflammatory response to NASH that can be pursued in future human trials. We also observed a significant correlation between hepatic MDA and serum ALT supporting the involvement of lipid peroxidation on hepatic damage. Given the known relation between inflammation and oxidative stress, human studies are needed to define the specificity and sensitivity of these responses on serum ALT. Additional work is also warranted to determine if ALT or other serum markers can predict the future incidence of NASH.

No animal model fully recapitulates the pathology or developmental events of NASH [8]. We used *ob*/*ob* mice because their metabolic abnormalities and histopathology resemble that observed in humans. However, they are with limitations because (1) most humans with NASH express leptin and (2) these mice do not spontaneously develop NASH in the absence of an inflammatory challenge. Although the distinct "stages" of NASH do not mimic the human condition, this was a strength of our study because we were able to control the induction of NASH by LPS to better define the hepatoprotective activities of dietary tocopherols.

In summary, this study provides novel evidence that α- or γ-tocopherol similarly decreased LPS-triggered pathogenic responses by mitigating liver damage, lipid peroxidation, and inflammation in a genetic obese mouse model of NASH. Studies examining vitamin E on NASH are of importance because >92% of Americans fail to ingest the estimated average requirement of vitamin E [45] suggesting that they may be vulnerable to oxidative insults that contribute to hepatic disorders. This is particularly problematic since two-thirds of Americans are overweight or obese [1], which is a significant risk factor for hepatic steatosis [2]. Thus, future studies are warranted to more fully examine the specific mechanisms underlying the hepatoprotective effects of these tocopherols against NASH.

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