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# The adverse effects of an in vivo inflammatory challenge on the vitamin E status of rats is accentuated by fish oil feeding[1–3]

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*To investigate the impact of dietary fish oil on tissue α-tocopherol concentration following an acute inflammatory challenge, weanling Sprague-Dawley female rats were fed diets containing 20% lard or menhaden fish oil. Dietary α-tocopherol was equalized across treatment groups (60 mg/kg). After 5 weeks, rats were injected intraperitoneally with one of two sterile inflammatory agents (i.e., glycogen or thioglycollate broth) or received no injection (control). Vitamin E (i.e., α-tocopherol) concentration was measured by reverse-phase HPLC for plasma, liver and immune cells isolated from the blood, lungs, spleen, and peritoneum. Aliquots of peripheral blood and peritoneal immune cells were analyzed for $H_2O_2$ production by flow cytometry. We found that fish oil-fed rats had lower baseline plasma ($P < 0.0001$) and liver ($P < 0.005$) α-tocopherol concentrations than lard-fed rats. The differences in plasma and hepatic α-tocopherol concentration between rats fed fish oil and those fed lard were increased following inflammatory challenge. In general, fish oil did not reduce immune cell α-tocopherol, with one notable exception. Peripheral blood immune cells from glycogen-challenged fish oil-fed rats had 45% less α-tocopherol than lard-fed counterparts ($P < 0.05$). The effect of diet on in vitro immune cell $H_2O_2$ production was modest, with the only significant difference occurring in peritoneal cells isolated from thioglycollate-challenged rats. In those animals, fish oil feeding led to an increased capacity to produce $H_2O_2$ in response to PMA stimulation. In summary, dietary fish oil adversely affects vitamin E status of rats and an in vivo inflammatory challenge accentuated this effect.* © Elsevier Science Inc. 1996 (J. Nutr. Biochem. 7:623–631, 1996.)

**Keywords:** fish oil; inflammation; immune cells; vitamin E; rats

## Introduction

Our laboratory has been interested in defining the relationship between dietary n-3 polyunsaturated fatty acids (n-3 PUFA)[4] and vitamin E[5] concentration of immune cells. We have reported that feeding rats diets high in n-3 PUFA can result in decreased immune cell α-tocopherol concentration.[2,3] Further, consumption of diets rich in n-3 PUFA is associated with increased susceptibility of membrane lipids to oxidative attack, as demonstrated in erythrocytes[4] and liver microsomes.[5] We designed this study to test two hypotheses: First, in vivo inflammatory challenges deplete tissue vitamin E; second, animals whose tissues were enriched with n-3 PUFA show a greater loss of vitamin E.

Inflammation is a complex process initiated by the host in response to tissue injury. Phagocytes (i.e., neutrophils and monocyte/macrophages) that are attracted to inflamma-

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Throughout this paper the term "vitamin E" is used as a generic descriptor for all tocol- and tocotrienol derivatives that exhibit vitamin E activity. This approach is in accordance with the nomenclature policy established by the American Institute of Nutrition.



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tory sites produce large quantities of highly reactive oxygen species (e.g., $H_2O_2$, superoxide anion, hydroxy radical), a process known as respiratory burst.[6,7] These compounds, often referred to as reactive oxygen species (ROS), help the phagocyte kill invading microorganisms and preventing infection. However, phagocytes must protect themselves from the damaging effects of ROS. Protection from autooxidation within the cytoplasm can be enzymatic (e.g., catalase) or chemical (e.g., reduced glutathione), while the primary protector of cellular membranes from oxidative attack is vitamin E (i.e., α-tocopherol). The concentration of α-tocopherol in macrophages[8] and neutrophils[9] has been reported to decline significantly (~40%) after in vitro stimulation of respiratory burst activity. These data suggest that α-tocopherol may play an important role in protecting immune cells from the toxic effects of ROS during inflammation and is perhaps consumed in the process. Little is known, however, about immune cell α-tocopherol metabolism during in vivo inflammatory responses.

In this study we measured the ability of n-3 PUFA-enriched and non-enriched immune cells to produce ROS (i.e., $H_2O_2$) for two important reasons. First, the existing data are in conflict, with two studies showing n-3 PUFA enhanced oxidative metabolism,[10,11] whereas two others report reductions.[12,13] Second, an alteration in ROS production, exposing immune cells from n-3 PUFA fed rats to different levels of in vivo oxidative stress than those from rats fed lard, could affect α-tocopherol utilization. Assessing diet-induced changes in respiratory burst will allow us to determine whether changes in ROS are responsible for the observed post-inflammatory challenge differences in tissue α-tocopherol concentration.

## Methods and material

### *Chemicals*

Minimal Essential Medium supplemented with 10 mmol/L HEPES and 2 mmol/L L-glutamine and Hank's balanced salts solution ($Ca^{++}$ & $Mg^{++}$ free) was provided by the MU Cell & Immunobiology Core facility (Columbia, MO, USA). Diet ingredients were obtained from U.S. Biochemicals (Cleveland, OH, USA). tertiary-butyl hydroquinone (TBHQ) was obtained from Eastman Kodak Company (Rochester, NY, USA). Histopaque 1077, glycogen (type II from oyster, No. G8751) and phorbol 12-myristate 13-acetate (PMA, No. P8139) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Brewer thioglycollate medium was purchased from Difco Laboratories (Detroit, MI, USA). 2,7-dichlorofluorescin diacetate was purchased from Molecular Probes, Inc. (Eugene, OR, USA). All other chemicals were purchased from Fisher Scientific (St. Louis, MO, USA).

### *Animals and diets*

Specific pathogen-free weanling female Sprague-Dawley rats (initial weight, 44.5 ± 2.3 g; Sasco, St. Louis, MO, USA) were housed individually in hanging wire stainless steel cages. Air temperature and relative humidity in the room were 21 to 24°C and 45 to 50%, respectively, with a diurnal 12 hr light cycle. Housing, handling, and sample collection procedures conformed to policies and recommendations of the University of Missouri's Animal Care and Use Committee.

Rats were allotted to one of two dietary treatment groups. These diets were isocaloric and formulated according to AIN guidelines[14] with minor modifications as noted above. Treatments were based solely on the type of fat added to a semi-purified diets: 20% by weight tocopherol-stripped lard or menhaden fish oil (a gift from Zapata Haynie Protein Corp., Reedville, VA, USA). Composition of these diets was as follows (g/100 g): casein, 20; DL-methionine, 0.3; corn starch, 20; dextrose, 29.3; α-cellulose, 5; mineral mix (AIN-76), 4; vitamin mix (AIN-76A), 1.2; choline bitartrate, 0.2; fat, 20. All dietary ingredients were purchased from U.S. Biochemical (Cleveland, OH, USA), unless otherwise noted.

Autooxidation of the diets was prevented by adding 1.2 μmol/L tertiary-butyl hydroquinone (Eastman Kodak Co.) to the oils upon receipt as described by Fritsche and Johnston.[15] Oils were mixed into the dry components of the diet in small batches, and stored at 4°C. Endogenous α-T concentration of each oil was determined in duplicate, as described by Slover and Thompson.[16] The vitamin E content of the lard was matched to that of the menhaden fish oil by adding α-T (a gift from Eastman Kodak Chemical Company, Rochester, NY, USA). After extraction and saponification of samples of the mixed diets, the final analyzed concentration of α-T in the experimental diets was 57 ± 3 mg/kg, which accounted for over 98% of the total vitamin E activity in these fats. Animals had ad libitum access to fresh water and fresh diet each day with remaining diet discarded.

### *Inflammatory challenge*

After 5 weeks on the experimental diets, rats were lightly anesthetized prior to receiving an i.p. injection of sterile saline (control) or one of two inflammatory challenges: 1% glycogen solution (40 mL, type II from oyster, Sigma Chemical Co., St. Louis, MO, USA) or Brewer's thioglycollate broth (10 mL, Difco Laboratories, Detroit, MI, USA). We selected two different inflammatory challenges in order to examine different inflammatory cell populations. There were two major phagocytic cell populations that we were interested in studying: neutrophils and macrophages. Harvesting cells from the peritoneum and blood just 7 hr after the glycogen challenge should provide us with a large quantity of relatively pure neutrophils. In contrast, collecting immune cells from the peritoneum 72 hr after a thioglycollate-challenge should provide a relatively pure sample of inflammatory macrophages.

### *Sample collection*

Feed was removed from rats 12 hr before sample collection. Rats were anesthetized by intramuscular injection of ketamine-HCl (50 μmol/100 g body weight; Aveco Co., Inc., Fort Dodge, IA, USA) and xylazine (4 μmol/100 g body weight; Mobay Co., Animal Health Division, Shawnee, KS, USA). Blood (5 to 10 mL) was collected by cardiac puncture into a syringe containing 300 U of heparin (Elkins-Sinn, Inc., Cherry Hill, NJ, USA). Livers were placed in re-sealable polyethylene bags and immediately frozen in liquid nitrogen. Plasma from a 1 mL aliquot of blood was separated by centrifugation and stored (−80°C) for later α-tocopherol and fatty acid determination. Peripheral blood leukocytes were isolated from the remaining blood by layering the heparinized blood over Lymphocyte Separation Medium, density 1.077 to 1.080 kg/L at 20°C, as described by the manufacturer (Organon Teknika Corp., Durham, NC, USA). The mononuclear leukocytes at the interface were collected, washed twice, and resuspended in 1 mL PBS (pH 7.4).

Alveolar macrophages were collected by flushing the lungs with five, 3 mL aliquots of ice-cold PBS w/o $Ca^{++}$ and $Mg^{++}$. Single cell suspensions of splenocytes were obtained by forcing tissue through a sieve (Sigma Chemical Co., St. Louis, MO, USA) equipped with a 80-mesh stainless steel screen into PBS. Using a 10 mL syringe without a needle, cell clumps were dispersed by

several gentle washings through the sieve. A single cell suspension was obtained by allowing cell clumps to sediment at room temperature for 10 min. Cells were then transferred into another tube and splenocytes were isolated from the crude cell suspension by density gradient centrifugation over Histopaque 1.077 (Sigma Chemical Co.). Erythrocytes were lysed by treatment with 0.19 mol/L of $NH_4Cl$. All immune cell samples were enumerated by a Coulter Counter, Model ZBI (Coulter Electronics, Hialeah, FL, USA) and a small aliquot of cells was subjected to cytospin for 5 min at 250 × g (Shandon, Inc., Pittsburgh, PA, USA) and stained (Wescor 7100 slide stainer, Wescor Inc., Logan, UT, USA). Differential counts on all immune cells preparation were conducted. All cells samples were pelleted and stored in 1 mL of 10 mmol/L EDTA at −80°C for α-tocopherol analyses.

### α-Tocopherol determination by HPLC

α-Tocopherol concentration of plasma and isolated immune cells was determined by HPLC as described by Bieri et al.[17] Proteins were precipitated with ice-cold ethanol (100 μL or 1.5 mL, for plasma and immune cell preparations, respectively). Tocopherols were extracted by the addition of heptane (200 μL or 2 mL, for plasma and immune cell preparations, respectively). Plasma and immune cell samples were centrifuged to separate the phases and the organic (top) phase was transferred into a glass test tube. The extracts were evaporated to dryness under a stream of $N_2$ gas, then resuspended in methanol (100 μL). An internal standard (δ-tocopherol) was introduced into each sample to monitor extraction efficiency, which generally exceeded 90%. The amount of internal standard added to each sample was adjusted to approximately the molar amount of α-tocopherol previously reported,[3] which was ~2.5 μmoles for plasma and alveolar macrophages and 0.2 μmol for rest of the immune cell preparations.

Liver α-tocopherol determination was carried out as described by Zaspel and Csallany.[18] Frozen liver tissue samples (100 mg) were thawed and then homogenized in acetone (20 vol), which contained ~25 μmol of δ-tocopherol as an internal standard. The homogenate was centrifuged (1300 × g for 10 min at 10°C) and supernatants filtered through a 0.2 μm Nylon-66 filter (Rainin, Woburn, MA, USA). A 1 mL aliquot of sample extract was evaporated to dryness with $N_2$ gas. After solvent removal, samples were resuspended in methanol (100 μL).

These tissue extracts were then separated by a Perkin-Elmer Series 4 HPLC (Norwalk, CT, USA) equipped with a C-18 reverse-phase column (15 cm × 4.6 mm; 3 μm; Supelco Inc., Bellefonte, PA, USA). The mobile phase consisted of 98% methanol and 2% water (flow rate, 1.5 mL/min). Typical retention times were (min): 4.8, δ-tocopherol; 5.5, γ-tocopherol; 6.3, α-tocopherol. Plasma and immune cell tocopherols were monitored at 292 nM (Perkin-Elmer LC-75 UV detector). Sample α-tocopherol concentration was calculated from peak area responses using a standard curve that was established from known amounts of pure α-tocopherol. Values were corrected for losses during processing by following the recovery of the internal standards, which generally exceeded 90%.

### Total lipid determination

Plasma samples (1 mL) were diluted 1:1 with a Tris/EDTA/sucrose buffer (50 mmol/L Trizma-HCl; 1 mmol/L EDTA; 0.32 mol/L sucrose; pH 7.4). Liver samples were thawed quickly and homogenized in 10 volumes of the Tris/EDTA/sucrose buffer. Lipids were extracted with 4 volumes of chloroform and methanol (2:1, v/v). The organic phase containing the lipid extract was removed and the aqueous phase reextracted with two volumes of chloroform/methanol/12 mol/L HCl (4:1:.013, v/v/v). After neutralization of the second extract with $NH_4OH$, the organic layers were pooled, filtered, then reduced in volume under $N_2$. The weight of total lipid extracts was determined gravimetrically, in duplicate.

### Oxidative burst determination by flow cytometry

Cells were analyzed for the $H_2O_2$ production by flow cytometry according to the method of Bass et al.[19] Peripheral blood and peritoneal immune cell preparations (5 × $10^6$ cells) were resuspended in PBS w/o $Ca^{++}$ and $Mg^{++}$, which contained 2% BSA and 0.2% sodium azide. To this cell suspension, 5 μmol/L of 2,7-dichlorofluorescin diacetate was added (1 μL/mL of cells, for a final ethanol concentration of 0.1%). After 15 min at 4°C, an aliquot of dye-loaded cells was transferred into a new tube that contained phorbol myristate acetate (10 ng/mL; PMA). Control and PMA-stimulated cell suspensions were incubated 60 min at 37°C. Following this period, green fluorescence was measured using a Model Epics 753 flow cytometer (Coulter Electrics Inc., Hialeah, FL, USA) equipped with a 4 W argon laser (488 nm emission, 100 mW output) and a 530 nm log band-pass filter. Fluorescence data were collected on 1 × $10^4$ cells as determined by forward light scatter intensity using logarithmic amplification and by gating on viable leukocytes to exclude cell debris and erythrocytes. Data are expressed as mean fluorescence intensity (arbitrary units).

### Statistical analyses

Data were analyzed as a completely randomized design using the general linear model (GLM) procedures of SAS.[20] The model included effects due to diet, inflammatory challenge, and diet X inflammatory challenge. Each rat served as an experimental unit. Flow cytometric data were subjected to square root transformation in order to obtain homogeneous variance. Mean differences were ascertained using a protected (i.e., $P < 0.05$) least significant difference method. All values represent least square means and are accompanied by a pooled SEM.

## Results

The effects of diet and inflammatory challenge on final body, liver and spleen weight are shown in *Table 1*. Body weight was not affected by diet or by inflammatory challenge (*Table 1*). Data for liver and spleen weight were presented on a body weight basis as well as on a per rat basis, because lard-fed control rats weighed significantly less than other groups of rats. Fish oil-fed rats had significantly heavier livers than lard-fed rats ($P < 0.005$). These diet-induced differences remained when liver weight was expressed as a percentage of body weight. Inflammatory challenge modestly affected liver weight. Significant interactions between main effects (i.e., dietary and inflammatory challenge) were noted for liver weight, both on an organ basis and as a percentage of body weight ($P < 0.05$). Independent of inflammatory treatment, spleens from fish oil-fed rats were ~25% heavier than those from lard-fed rats ($P < 0.01$). Inflammatory challenge significantly affected spleen weight on a whole organ basis and as a percentage of body weight.

The effects of diet and inflammatory challenge on plasma vitamin E (i.e., α-tocopherol) concentration are shown in *Table 2*. Plasma α-tocopherol concentration was significantly lower in fish oil-fed rats than those fed lard. Inflammatory challenge appeared to accentuate the adverse

**Table 1** Body, liver and immune organ weights of female rats fed diets containing lard or fish oil for 5 weeks and then subjected to an inflammatory challenge[1,2,3]

| | Control | | Glycogen | | Thioglycollate | | | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Dietary fat source: | Lard | Fish oil | Lard | Fish oil | Lard | Fish oil | SEM[4] | Diet | Challenge | D × C |
| Body weight, g[5] | 146.3 | 173.5 | 170.5 | 173.0 | 167.5 | 167.8 | 7.2 | NS | NS | NS |
| Liver, g | 3.94$^{c}$ | 6.13$^{a}$ | 5.82$^{ab}$ | 5.96$^{ab}$ | 5.03$^{b}$ | 6.49$^{a}$ | 0.35 | 0.0005 | 0.05 | 0.05 |
| Liver, g/100g bwt | 2.70$^{c}$ | 3.48$^{ab}$ | 3.48$^{ab}$ | 3.43$^{ab}$ | 3.03$^{bc}$ | 3.85$^{a}$ | 0.17 | 0.005 | 0.07 | 0.05 |
| Spleen, g | 0.380$^{b}$ | 0.544$^{a}$ | 0.408$^{b}$ | 0.429$^{b}$ | 0.483$^{ab}$ | 0.590$^{a}$ | 0.040 | 0.01 | 0.05 | NS |
| Spleen, mg/100g bwt | 2.75$^{ab}$ | 3.25$^{a}$ | 2.25$^{b}$ | 2.50$^{b}$ | 3.25$^{a}$ | 3.50$^{a}$ | 0.27 | NS | 0.005 | NS |

[1]Weanling female Sprague-Dawley rats were fed semipurified diets that contained either 20% lard or menhaden fish oil. Diets were equalized in vitamin E content. After 5 weeks, rats received a sterile inflammatory challenge, then 7 hr (glycogen-injected) or 72 hr (thioglycollate-injected) later rats were sacrificed and tissues harvested.
[2]Values represent the least square means ($n$ = 4).
[3]Values with different letters within a row are significantly different at $P < 0.05$
[4]Pooled standard error (SEM).
[5]Weight immediately before sacrifice, *after* inflammatory challenge.

effect of fish oil feeding on plasma α-tocopherol, whereby the difference was 30% in control rats and 55% and 45% for glycogen and thioglycollate-injected rats, respectively. However, the overall effect of inflammatory challenge on plasma α-tocopherol concentration was not statistically significant.

Both diet and inflammatory challenge significantly altered liver lipid concentration (*Table 2*). Total liver lipids in fish oil-fed rats were ~50% higher than those in lard-fed rats ($P < 0.0005$). The effect of diet on liver lipids was accentuated by inflammatory challenge, particularly for thioglycollate-injected rats, where fish oil-fed rats had liver lipids that were twice as high as those fed lard. Glycogen-injected rats had significantly lower liver lipids compared to thioglycollate-injected rats, whereas livers from control rats had an intermediate amount of lipids.

The effects of diet and inflammatory challenge on liver α-tocopherol concentration are complex. Both diet and inflammatory challenge significantly altered liver α-tocopherol when it was expressed on the basis of total lipids (*Table 2*). As in plasma, the effect of diet on liver α-tocopherol was accentuated by inflammatory challenge, with liver α-tocopherol being 27%, 35%, and 45% lower in fish oil-fed rats compared with those fed lard for control, glycogen, and thioglycollate-injected rats, respectively. Glycogen-injected rats had significantly higher liver α-tocopherol concentration compared with control and thioglycollate-injected rats. In contrast, when liver α-tocopherol concentration was expressed on a wet weight basis neither diet nor inflammation had a significant impact (data not shown).

Immune cell α-tocopherol concentration was affected by both diet and inflammatory challenge (*Table 3*). These effects were highly dependent on which location or tissue the immune cells were collected from. For example, the α-tocopherol concentration of immune cells isolated from the lungs, spleen, peritoneum and blood were consistently lower in fish oil-fed rats as compared to those fed lard, but these differences were only significant for peripheral blood immune cells (averaging 36% lower across all inflammatory treatments; $P < 0.05$). Inflammatory challenge significantly altered peritoneal and peripheral blood immune cell α-tocopherol content, but in opposite directions. In the peritoneum, glycogen-injection significantly decreased and thioglycolate-injection increased immune cell α-tocopherol concentration as compared to control rats. In contrast, glycogen-injected significantly increased peripheral blood immune cell α-tocopherol concentration, whereas thioglycollate had no effect.

Immune cell yield from the peritoneum and spleen were affected by inflammatory challenge, while diet only affected the latter (*Table 4*). Glycogen injection caused a dramatic (~20 fold) increase in the number of cells in the

**Table 2** Plasma and liver α-tocopherol total lipids of female rats fed diets containing lard or fish oil for 5 weeks and then subjected to an inflammatory challenge[1-3]

| | Control | | Glycogen | | Thioglycollate | | | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Dietary fat source: | Lard | Fish oil | Lard | Fish oil | Lard | Fish oil | SEM[4] | Diet | Challenge | D × C |
| Plasma, μmol/L | 14.8$^{a}$ | 10.3$^{b}$ | 16.8$^{a}$ | 7.6$^{b}$ | 15.0$^{a}$ | 8.5$^{b}$ | 1.2 | 0.0001 | NS | NS |
| Liver lipids, mg/g | 65.0$^{bc}$ | 75.0$^{b}$ | 48.0$^{c}$ | 66.0$^{c}$ | 56.3$^{bc}$ | 108.0$^{a}$ | 7.42 | 0.0005 | 0.005 | 0.05 |
| Liver, μmol/g lipid | 1.93$^{b}$ | 1.40$^{bc}$ | 2.98$^{a}$ | 1.93$^{b}$ | 1.82$^{a}$ | 1.00$^{c}$ | 0.27 | 0.005 | 0.01 | NS |

[1]Weanling female Sprague-Dawley rats were fed semipurified diets that contained either 20% lard or menhaden fish oil. Diets were equalized in vitamin E content. After 5 weeks, rats received a sterile inflammatory challenge, then seven hr (glycogen-injected) or 72 hr (thioglycollate-injected) later rats were sacrificed and tissues harvested. Vitamin E (α-tocopherol) was measured by reverse-phase HPLC after heptane (plasma) or acetone (liver) extraction as described in detail in the methods and materials section.
[2-4](See Table 1).

**Table 3** Immune cell α-tocopherol of female rats fed diets containing lard or fish oil for 5 weeks and then subjected to an inflammatory challenge[1–3]

| | Control | | Glycogen | | Thioglycollate | | | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Dietary fat source: | Lard | Fish oil | Lard | Fish oil | Lard | Fish oil | SEM[4] | Diet | Challenge | D × C |
| | | | | | nmoles α-tocopherol/$10^9$ cells | | | | | |
| Peripheral blood cells | 37.9[b] | 22.9[b] | 73.3[a] | 41.4[b] | 38.4[b] | 31.7[b] | 10.1 | 0.05 | 0.05 | NS |
| Lung macrophages | 525 | 332 | 379 | 318 | 487 | 510 | 103 | NS | 0.10 | NS |
| Peritoneal cells | 29.8[ab] | 22.5[bc] | 16.2[bc] | 9.9[c] | 46.5[a] | 39.7[a] | 5.30 | NS | 0.005 | NS |
| Splenocytes | 11.3 | 11.9 | 10.2 | 7.50 | 12.2 | 13.1 | 2.08 | NS | 0.08 | NS |

[1]Weanling female Sprague-Dawley rats were fed semipurified diets that contained either 20% lard or menhaden fish oil. Diets were equalized in vitamin E content. After 5 weeks, rats received a sterile inflammatory challenge, then 7 hr (glycogen-injected) or 72 hr (thioglycollate-injected) later rats were sacrificed and tissues harvested. Vitamin E (α-tocopherol) was measured by reverse-phase HPLC following heptane extraction as described in detail in the methods and materials section.
[2–4](See Table 1).

peritoneum after 7 hr, whereas the increase with thioglycollate treatment after 72 hr was not significant. In the spleen, thioglycollate challenge led to a significant increase in immune cell yield as compared to control rats. An intermediate number of splenocytes were collected from glycogen-injected rats, which were not different from the controls. Additionally, ~33% more splenocytes were recovered from fish oil-fed rats than from lard-fed rats ($P < 0.05$). The number of immune cells collected from the blood and lungs were not significantly affected by diet or inflammatory challenge.

Immune cell type in the blood was substantially altered by inflammatory challenge. Specifically, glycogen-injection significantly increased the percentage of neutrophils found in the peripheral blood immune cell population to over 80% compared with approximately 15% in control and thioglycollate-injected rats. There was a corresponding alteration in the percentage of lymphocytes found in the peripheral blood. Diet had a modest, but significant, impact on the immune cell population found in the blood. Specifically, there were twice as many neutrophils and three times as many monocytes in the peripheral blood of non-challenged fish oil-fed rats than those fed lard. Significant interactions between main effects (i.e., diet and inflammatory challenge) were noted for peripheral blood immune cells.

Immune cell type in the peritoneum was substantially altered by inflammatory challenge. Similar to the blood, the immune cell population in the peritoneum was greatly enriched with neutrophils 7 hr after the glycogen injection such that ~90% of the cells collected were neutrophils, whereas 72 hr after the thioglycollate injection only 15% of the cells were neutrophils. In control rats (i.e., those not receiving any inflammatory challenge) the peritoneal cell population was generally less than 5% neutrophils and 45% macrophages. We observed a significant increase in the percentage of eosinophils in the peritoneum in response to the thioglycollate injection. Dietary fat significantly affected the migration of eosinophils into the peritoneum, in that fish oil-fed rats challenged with thioglycollate had more eosinophils than those fed lard (55% vs. 42%, respectively; $P < 0.005$). There were no other diet effects observed on the type of cells collected from the peritoneum. Furthermore, the type of immune cell isolated from the lungs and spleen were not affected by diet or inflammatory challenge. Greater than 95% of lung cells showed morphological and staining properties characteristic of macrophages. The density gradient-purified splenocyte population was approximately 95% lymphocytes, 4% monocytes and 1% granulocytes across all treatment groups.

The effects of diet and prior in vivo inflammatory challenge on the in vitro $H_2O_2$ production by peripheral and peritoneal immune cells are shown in *Table 5*. Peritoneal cells collected from thioglycollate-challenged rats that had been fed fish oil produced significantly more $H_2O_2$ in response to PMA than those from lard-fed rats. A significant interaction between diet and challenge was observed with PMA-stimulation of peritoneal cells. Basal $H_2O_2$ production by peritoneal immune cells from glycogen-challenged rats

**Table 4** Yield of immune cells from female rats fed diets containing lard or fish oil for 5 weeks and then subjected to an inflammatory challenge[1,2,3]

| | Control | | Glycogen | | Thioglycollate | | | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Dietary fat source: | Lard | Fish oil | Lard | Fish oil | Lard | Fish oil | SEM[4] | Diet | Challenge | D × C |
| | | | | | cell number ($\times 10^{-6}$) | | | | | |
| Peripheral blood cells | 18.5 | 30.5 | 28.5 | 31.6 | 23.9 | 19.4 | 5.85 | NS | NS | NS |
| Lung macrophages | 0.303 | 0.333 | 0.330 | 0.405 | 0.308 | 0.310 | 0.072 | NS | NS | NS |
| Peritoneal cells | 8.33[b] | 12.27[b] | 209.5[a] | 170.5[a] | 20.8[b] | 11.0[b] | 51.8 | NS | 0.005 | NS |
| Splenocytes | 30.9[a] | 39.6[ab] | 32.4[a] | 55.5[b] | 45.1[ab] | 48.2[b] | 5.41 | 0.02 | 0.05 | NS |

[1–4](See Table 1).

**Table 5** Relative $H_2O_2$ production of peripheral blood and peritoneal immune cells from female rats fed diets containing lard or fish oil for 5 weeks and then subjected to an inflammatory challenge[1,2,3]

| | Control | | Glycogen | | Thioglycollate | | | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Dietary fat source: | Lard | Fish oil | Lard | Fish oil | Lard | Fish oil | SEM[4] | Diet | Challenge | D × C |
| | | | | Mean fluorescence intensity (arbitrary units) | | | | | | |
| *Peripheral blood cells* | | | | | | | | | | |
| Basal | 3.01 | 2.75 | 4.05 | 2.78 | 2.79 | 3.35 | 0.05 | NS | NS | NS |
| PMA-stimulated | $18.2^{b}$ | $22.7^{b}$ | $61.9^{a}$ | $62.2^{a}$ | $16.5^{b}$ | $20.2^{b}$ | 3.65 | NS | 0.0001 | NS |
| *Peritoneal exudate cells* | | | | | | | | | | |
| Basal | $17.7^{bc}$ | $10.7^{cd}$ | $32.6^{a}$ | $24.1^{ab}$ | $5.63^{d}$ | $15.2^{bcd}$ | 4.23 | NS | 0.005 | 0.10 |
| PMA-stimulated | $74.6^{a}$ | $65.2^{ab}$ | $82.6^{a}$ | $79.7^{a}$ | $45.5^{b}$ | $79.0^{a}$ | 7.5 | NS | 0.07 | 0.05 |

[1]Weanling female Sprague-Dawley rats were fed semipurified diets that contained either 20% lard or menhaden fish oil. Diets were equalized in vitamin E content. After 5 weeks, rats received a sterile inflammatory challenge, then 7 hr (glycogen-injected) or 72 hr (thioglycollate-injected) later rats were sacrificed and tissues harvested. Aliquots of immune cells from each rats were labeled with 2′,7′-dichlorofluorescin and analyzed for $H_2O_2$ production by flow cytometry before and after in vitro stimulation by phorbol myristate acetate (PMA).
[2–4](See Table 1).

was two fold higher than from control and thioglycollate-challenged rats, but was not affected by diet. Neither diet nor inflammatory challenge affected basal $H_2O_2$ production by peripheral blood immune cells. However, reflecting the effects of the large influx of neutrophils into the blood compartment, PMA-stimulated production was significantly (3 fold) higher in peripheral blood immune cells from glycogen-challenged rats than from control and thioglycollate-challenged rats. Dietary fat source did not affect peripheral blood immune cell $H_2O_2$ production stimulated by PMA.

## Discussion

Consistent with our previous findings[2,3] and many other reports, we find that plasma vitamin E (i.e., α-tocopherol) concentration is reduced by fish oil feeding. Although, inflammatory challenge itself does not significantly affect plasma α-tocopherol concentration, differences in plasma α-tocopherol concentration between lard- and fish oil-fed rats are accentuated by an in vivo inflammatory challenge. In contrast, Demling and coworkers[21] reported a 20% decline in plasma α-tocopherol 24 hr after intraperitoneal administration of a zymosan challenge. Additionally, Sakaguchi et al.[22] reported that after an intraperitoneal endotoxin challenge, plasma α-tocopherol concentration was significantly reduced during the first 6 hr, rebounding to levels that were 2 fold higher at 18 to 24 hr post-challenge than in non-challenged mice. The rebound of plasma α-tocopherol appeared to be a result of mobilization from liver stores.

Understanding the effect of diet and inflammation on hepatic α-tocopherol concentration is complicated by the significant impact these two variables have on liver lipid levels, which, in turn, appeared to influence the α-tocopherol concentration of this tissue in a manner similar to that reported for plasma.[23] When diet-induced differences in hepatic α-tocopherol concentration are expressed on the basis of total lipids, the negative impact of fish oil feeding on vitamin E status becomes more apparent. In the study of Sakaguchi et al.[22] hepatic α-tocopherol concentration showed a continuous decline through 18 hours, followed by a gradual return to near control levels after 48 hr post-challenge. Since we only measured α-tocopherol concentration at a single time point for each type of inflammatory challenge, possible time-dependent effects on vitamin E metabolism could not be assessed in our study. Furthermore, as with plasma α-tocopherol, it was quite clear that inflammatory challenge accentuates the adverse effect of dietary fish oil on hepatic vitamin E status.

A novel aspect of this study was our determination of immune cell vitamin E content after an in vivo inflammatory challenge. Alpha-tocopherol has been shown to protect cell membranes from oxidative damage and may be destroyed in the process.[4,24,25] We hypothesized that immune cells enriched with (n-3) PUFA would show a greater net loss of α-tocopherol post-challenge than those not enriched. In this study, fish oil feeding had a significant negative effect on α-tocopherol concentration of peripheral blood immune cells isolated from glycogen-challenged rats. Similarly the tendency of fish oil feeding to accentuate decreases in α-tocopherol concentration was observed in immune cells isolated from the peritoneum and spleen of glycogen-challenged rats. In contrast, we found that by itself fish oil feeding did not lead to a generalized reduction in immune cell α-tocopherol, which is in agreement with some of our other recent findings.[3] Unfortunately, we were unable to measure PUFA content in our immune cell samples, because the entire sample was needed for vitamin E analysis. However, the changes in immune cell fatty acid composition upon fish oil consumption in rodents has been well documented by us and others.[26–32]

The inflammatory challenges used in this study induced significant alterations in the number (4 to 20 fold increase) and type of immune cells in the blood and peritoneum. For example, the peritoneal cavity of glycogen-injected rats contained over 150 million cells of which more than 90% were neutrophils. In contrast, the number of resident immune cells in the peritoneum of a control rat were usually less than 15 million and typically were characterized as 45% macrophages, 25% eosinophils, 15% lymphocytes and few neutrophils. Such alterations in immune cell number and type provided strong evidence that our inflammatory challenges were effective.

We were not surprised to find that the effect of inflammation, per se, on immune cell α-tocopherol was not the

same for each of the two types of inflammatory challenge. For example, glycogen-challenge significantly reduced the α-tocopherol concentration of peritoneal immune cells, while thioglycollate increased it. Since glycogen led to an influx of neutrophils, which reportedly have a much lower α-tocopherol content than macrophages,[33] and macrophages are the predominate immune cell in the peritoneum of control rats, it is logical that the average immune cell α-tocopherol content would go down following this challenge. In fact, changes in the predominate immune cell population found in the peritoneum following the in vivo inflammatory challenge may account for most of the observed alterations in α-tocopherol concentration in this tissue compartment as well as in the blood.

That α-tocopherol concentration of peripheral blood immune cells doubled after the glycogen challenge was unexpected. The predominate immune cell in the blood shifted from over 80% lymphocytes in control rats to over 80% neutrophils in glycogen-injected rats. These data suggest that neutrophils have twice as much vitamin E as lymphocytes. In contrast, Hatam and Kayden[33] reported that human peripheral blood neutrophils and lymphocytes contain similar amounts of α-tocopherol. This contradiction may reflect a species difference (i.e., rats versus humans) or the difference in the way these cells were obtained (i.e., inflammatory vs. resting). Most of the neutrophils in the peripheral blood of the glycogen-injected rats had just recently emigrated from the bone marrow, a potential site of vitamin E loading. Once these cells are released into the circulation their ability to replenish membrane α-tocopherol may be limited. Under such a scenario, we would have anticipated a time-dependent decline in neutrophil α-tocopherol concentration until levels reached those in lymphocytes. However, little is known about how and where immune cells acquire α-tocopherol. It would be of great interest to determine if immune cells possess the recently discovered intracellular 15 kDa tocopherol-binding protein, which is thought to be responsible for intracellular transport and retention of α-tocopherol in many tissues.[34] Furthermore, confirmation that α-tocopherol content varies across immune cell subpopulations will require additional studies utilizing modern cell separation techniques (e.g., flow cytometry, panning).

The ability of n-3 PUFA to affect immune cell oxidative burst (e.g., chemiluminescence, $H_2O_2$ production) has been reported previously. However, the findings from those studies were contradictory in that (n-3) PUFA-enriched immune cells were shown to have higher[10,11] and lower[12,13] oxidative burst activity than cells not enriched with (n-3) PUFA. In this study, we found no evidence of a uniform alteration of immune cell $H_2O_2$ production by dietary fish oil feeding. However, we did find significantly greater PMA-stimulated $H_2O_2$ production by peritoneal immune cells isolated from fish oil versus lard-fed rats after the thioglycollate challenge. In agreement with our findings, Berger et al.[10] reported that superoxide anion release from PMA-stimulated peritoneal macrophages was twice as high for rats fed fish oil or linseed oil than those fed safflower or olive oil. Similarly, Yaqoob and Calder[11] found that thioglycollate-elicited peritoneal macrophages from fish oil-fed mice produced more $H_2O_2$ than those from mice fed any other fat source (e.g., olive oil, safflower oil, hydrogenated coconut oil). Our current hypothesis is that fish oil-fed rats maintain higher $H_2O_2$ production due to lower production of prostaglandin $E_2$, which has been shown to inhibit phagocyte oxidative burst.[35] However, it is uncertain why similar fish oil-induced increases in $H_2O_2$ production were not observed under any other circumstance within our study, because a reduced capacity to produce prostaglandin $E_2$ would have been evident in all fish oil-fed rats. However, the selectivity of this effect may be related to the nature of the inflammatory challenge and/or the time required for prostaglandin $E_2$ to down-regulate the response. Thus, only rats challenged with thioglycollate for 72 hr and consuming fish oil may have had sufficient exposure, in vivo, to the lower endogenous prostaglandin $E_2$ levels to be affected.

It has been proposed that high cellular oxidative metabolism might lead to greater destruction of vitamin E, such that there might be an inverse relationship between production of ROS and immune cell vitamin E.[8,36,37] Direct evidence for such an effect was provided by Coquette et al.[8] who observed a 40% reduction in α-tocopherol concentration in resident rat peritoneal macrophages 1 hr after an in vitro challenge with phorbol myristate acetate (PMA), a potent stimulant of oxidative burst activity. Our data do not support this hypothesis. We found that immune cells from the peritoneum generally had higher basal $H_2O_2$ production than those from the peripheral blood, yet, the α-tocopherol concentration of peritoneal immune cells was generally greater. Such differences, as discussed previously, may have more to do with the substantial differences in the immune cell populations fluxing into the peritoneum and blood from the bone marrow. Paradoxically, in glycogen-challenged animals, we found that basal $H_2O_2$ production by peritoneal cells tended to be lower in those fed fish oil, yet α-tocopherol concentration was 45% lower than lard-fed rats. It is possible that immune cells from fish oil-fed rats could have shown greater depletion of endogenous α-tocopherol upon in vitro stimulation of oxidative burst activity, but we did not measure α-tocopherol content after PMA treatment. Of particular relevance are the findings of Ho and Chan,[38] which raise doubts about the relevance of the acute depletion of immune cell α-tocopherol observed in vitro. They demonstrated that ascorbic acid or reduced glutathione were capable of quickly and non-enzymatically regenerating α-tocopherol in rat neutrophil homogenates following an acute oxidative stress. The importance of ascorbic acid in protecting phagocytes from self-produced oxidants has been reviewed elsewhere.[39]

Finally, we cannot explain why control rats fed lard seemed to weigh less than all other groups of rats. Previous experience with a much greater number of rats (same strain, sex, and age) as well as the same diet formulation never revealed an adverse effect of lard on weight gain.[2,3] An assessment of the individual response of rats in this lard-fed control group revealed that two gained at a normal rate, whereas the other two grew at much slower rate throughout the 5-week feeding trial. Such a failure to thrive has occasionally been observed in our laboratory, but rarely have two such animals been in the same treatment group. That this group only contained a total of four animals was disquieting. However, based on other data collected from these slower growing rats (i.e., organ weights as a percentage of

body weight, immune cell yields, and populations in the blood and peritoneum) these animals were normal and healthy. For this reason, we chose not to exclude the data from these animals.

In summary, our data support the following conclusions. First, fish oil feeding accentuates the adverse effects of inflammation on the vitamin E status of the host, as evidenced by decreased blood and liver α-tocopherol. Second, in general, immune cell α-tocopherol content is not compromised by fish oil feeding. By carefully defining isolated immune cell populations, we were able to exclude the possibility that dietary treatment-associated differences in immune cell α-tocopherol concentration were a result of differential immune cell migration and infiltration. Third, dietary-induced alterations in basal or PMA-stimulated immune cell oxidative burst activity do not seem to explain any subsequent destruction of cellular α-tocopherol.

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