Prostaglandins, Leukotrienes and Essential Fatty Acids 111 (2016) 37–44

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Concentration and ratio of essential fatty acids influences the inflammatory response in lipopolysaccharide challenged mice ☆

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ARTICLE INFO

Article history:
Received 17 February 2016
Accepted 1 March 2016

Keywords:
Omega-3
Omega-6
Polyunsaturated fatty acids (PUFA)
Inflammation
Lipopolysaccharide (LPS)

ABSTRACT

The goal of this study was to evaluate the role of both the % of dietary, 18-carbon PUFA (2.5%, 5% and 10%) and the n-6:n-3 ratio (1:1, 10:1 and 20:1) on the acute inflammatory response. Mice were fed diets for 8 weeks and injected intraperitoneally with LPS to induce acute inflammation. After 24 h mice were sacrificed and plasma cytokines measured. Diets significantly affected the erythrocyte PUFA composition and the effect of PUFA ratio was more prominent than of PUFA concentration. The % dietary PUFA affected feed efficiency ($p < 0.05$) and there was a PUFA × ratio interaction with body fat ($p < 0.01$). In mice fed high %kcal from PUFA, those given a low n-6:n-3 ratio had more body fat than those fed a high ratio. Of the twelve cytokines measured, eleven were significantly affected by the % PUFA ($p < 0.05$), whereas five were affected by the ratio ($p < 0.05$). For seven cytokines, there was a significant PUFA × ratio interaction according to a two way ANOVA ($p < 0.05$). These data indicate that dietary polyunsaturated fatty acids can affect LPS induced-inflammation.

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1. Introduction

Essential fatty acids are required nutrients in animal diets because they cannot be synthesized *de novo*. The two 18 carbon essential fatty acids (EFA), linoleic acid (18:2n-6; LA) and linolenic acid (18:3n-3; ALA) can be further elongated and desaturated to create long chain unsaturated fatty acids such as arachidonate (20:4n-6; AA), eicosapentaenoate (20:4n-3; EPA) and docosahexenoate (22:6n-3; DHA) [1]. As the typical American diet contains a substantial excess of LA (n-6) over ALA (n-3), there is a greater proportion of AA (n-6) compared to EPA (n-3) in tissue long chain PUFA, which may influence inflammatory processes [2]. The 20 carbon PUFA species AA, EPA and dihomo-gamma-linoleate (20:3 n-6; DGLA) serve as signaling molecules (eicosanoids) when they are enzymatically released from membranes [3]. In response to stimuli, the fatty acid is cleaved from the cell membrane via a phospholipase (such as phospholipase A2) [4]. The process by which these 20 carbon fatty acids are recognized and cleaved from the membrane seems to be nonspecific for the particular class (n-6 vs. n-3) and thus the fatty acids released reflect their relative membrane proportions. The 20 carbon PUFA can then be acted upon by cyclooxygenases (COX) or lipoxygenases (LOX) which result in prostaglandins, prostacyclins and thromboxanes (COX metabolites) or leukotrienes (LOX metabolites). The net effect of eicosanoid release is complex, as it depends on the relative proportions of the different eicosanoids present, as well as the sensitivity of the tissues that sense them. In general, the eicosanoids generated from arachidonic acid (AA) are considered to be more potent mediators of inflammation than those generated from EPAs [5]. However, even COX metabolites of AA, such as PGE_2 can have both pro-inflammatory and anti-inflammatory activity [5]. Thus, dietary ratios of the EFAs directly impact tissue structural lipid composition and consequently systemic inflammatory and immune processes [2].

One reason intakes of EFA are so high in the US that replacing saturated fatty acids with EFA in the diet leads to lower total and LDL cholesterol levels, with LA being the most effective fatty acid in achieving this effect [6]. Thus, dietary advice given to Americans has been to replace saturated fats with foods containing more EFA. Over the last 100 years, an increase of vegetable oils in Western diets has affected both total PUFA intake and the n-6:n-3 ratio. Blasbalg and colleagues [7] estimated that LA consumption increased from 2.23% to 7.21% of daily calories from 1909 to 1999 and in this same time frame, the ratio of dietary n-6 to n-3 fatty acids changed from 5.4 to 9.6. It has been suggested that the increased LA intake and high n-6:n-3 ratio may negatively affect health through excessive eicosanoid signaling [8].

☆Funding for this project was provided by grants from the Utah State University Office of Research and Graduate Studies and from the United States Department of Agriculture NIFA-AFRI-UTA1065

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http://dx.doi.org/10.1016/j.plefa.2016.03.003
0952-3278/Published by Elsevier Ltd.

Adding n-3 PUFA to the diet is generally considered as an effective strategy for reducing inflammation, and these molecules may affect inflammation in several ways (reviewed by Calder [4]). For example, high dietary intake of EPA results in the displacement of AA in membrane phospholipids which reduces the pool of available AA for eicosanoid synthesis. In addition, EPA inhibits the hydrolysis of AA from immune cell membranes. Furthermore, when fatty acids are cleaved from membranes enriched in EPA by PLA_2, the freed EPA competes with free AA for conversion by COX and LOX enzymes. Lastly, EPA-derived eicosanoids compete with those derived from AA at target cell binding sites, and the individual eicosanoids have different effects. While the rationale behind replacing AA with EPA is based on the weaker activity of the eicosanoids derived from EPA, there are also metabolites from EPA as well as DHA that actively promote inflammation resolution. Specialized pro-resolving mediators (SPM) is a term for lipid mediators derived from EPA and DHA that promote the active resolution of inflammation and SPM includes resolvins, protectins, and maresins [9]. Omega-3 fatty acids may also induce anti-inflammatory effects in macrophages and mature adipocytes via GPR120 signaling [10]. [11] In addition, long chain n-3 PUFA enhance B cell-mediated immunity in mice fed both control and high fat diets [11].

Most studies that have examined the inflammatory potential of dietary PUFA have focused on long chain PUFA (EPA and DHA) interventions that may not be relevant to modern, western populations. For example, EPA and DHA intake is estimated to account for less than 0.05% of daily caloric intake compared to ~8% of daily caloric intake from plant derived LA and ALA [7]. While there is a general consensus in the literature that high intakes of n-6 fatty acids can result in a pro-inflammatory state, there are few studies in which both n-6:n-3 ratios and total PUFA concentrations have been investigated together. Moreover, most studies investigating inflammatory potential of dietary PUFA have focused on long chain n-3 interventions.

The goals of this study were to determine if the total PUFA content of the diet and/or the n-6:3 ratio has an effect on the acute inflammatory response in C57BL6/J mice. Consequently, we formulated nine diets in which the fat source of the AIN-93G diet was modified to produce 3 different PUFA levels (2.5%, 5% and 10% of total kcal) at three ratios of n-6 to n-3 (1:1, 10:1 and 20:1) using fatty acids exclusively from plant sources. These PUFA concentrations, n-6 to n-3 ratios and dietary sources are physiologically relevant in the context of modern western diets, however long chain PUFA and animal fats were purposefully avoided as each may have complex actions on the inflammatory response. Mice were then challenged with LPS, a stressor that is well-known to induce an acute inflammatory response.

2. Materials and methods

2.1. Diet formulation

Nine diets differing in total PUFA content and n-6 to n-3 ratios were formulated using the AIN-93G purified rodent diet. This diet has a caloric density of 3.8 kcal/g and derives 18.8% kcal from protein, and 63.9% kcal from carbohydrate and 17.2% kcal from fat. Nine diets were formulated with three different PUFA levels (2.5%, 5% and 10%) at three different ratios (1:1, 10:1 and 20:1). To produce the different fat compositions, seven different vegetable oils were blended at various proportions (Table 1). Diets were produced by Harlan Laboratories (Madison, WI), and individual catalog numbers are provided in Table 1. To protect against lipid oxidation, all diets contained 14 mg/kg TBHQ and were stored at 4 °C.

Table 1
Fat formulas for experimental diets. Values are expressed as a percentage of total dietary fat.

Diet	Target % PUFA	n-6:n-3 Ratio	CB	CO	CSO	FSO	OO	SFO	SO	Catalog #
1	2.5	1:1	45.5	–	–	12.5	42.0	–	–	TD.10148
2	2.5	10:1	29.5	11.0	–	15.5	58.0	–	–	TD.10149
3	2.5	20:1	23.0	9.5	–	–	65.0	–	2.5	TD.10150
4	5	1:1	45.0	–	–	23.0	15.5	–	16.5	TD.10151
5	5	10:1	26.2	39.0	–	3.8	31.0	–	–	TD.10152
6	5	20:1	15	–	30.0	–	42.0	–	13.0	TD.10153
7	10	1:1	8.0	–	40.0	52.0	–	–	–	TD.10154
8	10	10:1	–	–	79.4	9.1	–	11.5	–	TD.10155
9	10	20:1	–	–	65.5	4.5	5.0	25.0	–	TD.10156

Abbreviations are: Cocoa butter (CB), corn oil (CO), cottonseed oil (CSO), flaxseed oil (FSO), olive oil (OO), sunflower oil (SFO) and soybean oil (SO). The diets were prepared by Harlan Laboratories (Madison, WI) and the catalog numbers are given in the table.

2.2. LPS challenge

All animal experimental protocols were approved by the Utah State University Institutional Animal Care and Use Committee. Male 4-5 week old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) (n = 12) were fed the experimental diets ad-libitum for 8 weeks. Food intake and animal weights were determined weekly. Three days prior to sacrifice, body composition was determined by NMR (EcoMRI LLC, Houston, TX). After 8 weeks, mice were injected intraperitoneally with 5mg/kg lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, MO) 24-h prior to sacrifice and six mice were injected with saline. Mice were euthanized by CO_2 asphyxiation, blood was collected via cardiac puncture, and plasma was isolated by centrifugation.

2.3. Plasma cytokine analysis

Plasma cytokine analysis was performed using the Q-Plex™ Mouse Cytokine - Screen (16-plex) array (Quansys Biosciences, Logan, UT, USA). Cytokines analyzed include: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1, IFNγ, TNFα, MIP-1α, GMCSF, and RANTES.

2.4. Fatty acid analysis

Lipids were quantified in diets and red blood cells using gas chromatographic analysis of fatty acid methyl ester derivatives (FAMEs). The direct derivitization method of O'Fallon [12] was used after adaption to smaller sample sizes. In short, diet or erythrocytes samples (~100 mg) were mixed with 234 μl of 10N KOH in water and 1.77 ml of methanol. Samples were vortexed for 30 s and then incubated at 55 °C for 1.5 h in a shaking water bath. Samples were removed every 20 m and vortexed for 30 s. Next, samples were cooled, and 193 μl of 24N H_2SO_4 was added. Samples were vortexed for 30 s and then incubated at 55 °C for 1.5 h in a shaking water bath with vortexing every 20 m for 30 s. Samples were cooled and 300 μl of hexane with 0.05% BHT was added. Samples were vortexed for 30 s and then centrifuged at 1000*g* for 5 min. The upper hexane layer was removed to a GC vial and analyzed according to the method of Zhou et al. [13] using a Shimadzu GC2010 gas chromatograph with flame ionization detection (Shimadzu Corporation, Columbia, MD). Fatty acids were separated using an HP-88 column (Agilent Corporation, Santa Clara, CA). The retention time for fatty acids and response factors were determined using GLC-463, a pure standard of fatty acid methyl esters (Nu-Chek Prep, Elysian, MN).