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The delta 6 desaturase knock out mouse reveals that immunomodulatory effects of essential n-6 and n-3 polyunsaturated fatty acids are both independent of and dependent upon conversion☆,☆☆

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Abstract

Typically fatty acids (FA) exert differential immunomodulatory effects with n-3 [α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] and n-6 [linoleic acid (LA) and arachidonic acid (AA)] exerting anti- and pro-inflammatory effects, respectively. This over-simplified interpretation is confounded by a failure to account for conversion of the parent FA (LA and ALA) to longer-chain bioactive products (AA and EPA/DHA, respectively), thereby precluding discernment of the immunomodulatory potential of specific FA. Therefore, we utilized the Δ6-desaturase model, wherein knockout mice (D6KO) lack the *Fads2* gene encoding for the rate-limiting enzyme that initiates FA metabolism, thereby providing a model to determine specific FA immunomodulatory effects. Wild-type (WT) and D6KO mice were fed one of four isocaloric diets differing in FA source (9 weeks): corn oil (LA-enriched), arachidonic acid single cell oil (AA-enriched), flaxseed oil (ALA-enriched) or menhaden fish oil (EPA/DHA-enriched). Splenic mononuclear cell cytokine production in response to lipopolysaccharide (LPS), T-cell receptor (TCR) and anti-CD40 stimulation was determined. Following LPS stimulation, AA was more bioactive compared to LA, by increasing inflammatory cytokine production of IL-6 (1.2-fold) and TNFα (1.3-fold). Further, LPS-stimulated IFNγ production in LA-fed D6KO mice was reduced 5-fold compared to LA-fed WT mice, indicating that conversion of LA to AA was necessary for cytokine production. Conversely, ALA exerted an independent immunomodulatory effect from EPA/DHA and all n-3 FA increased LPS-stimulated IL-10 production *versus* LA and AA. These data definitively identify specific immunomodulatory effects of individual FA and challenge the simplified view of the immunomodulatory effects of n-3 and n-6 FA.

Keywords: Polyunsaturated fatty acid; Cytokines; Inflammation; Fatty acid desaturase 2; Fatty acid metabolism

1. Introduction

Δ6-desaturase (D6D), encoded by the *Fads2* gene, is the rate-limiting enzyme that initiates the metabolism of the dietary essential plant-derived n-6 and n-3 polyunsaturated fatty acids (PUFA), linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), respectively, into their downstream long chain (LC) FA conversion products [1]. The principal FA produced from the n-6 PUFA LA is arachidonic acid (AA, 20:4n-6, direct conversion product), whereas the n-3 PUFA ALA is converted to eicosapentaenoic acid (EPA, 20:5n-3) and subsequently to docosahexaenoic acid (DHA, 22:6n-3), with limited conversion efficiency (in both rodents and humans) [2]. Specifically, in humans, approximately up to 8% of ALA is converted to EPA and<0.1% is converted to DHA [2–5]. In humans, essential PUFA intakes are disproportionate; n-3 PUFA intake in the form of ALA is low, whereas n-6 PUFA intake as LA is typically 5- to 20-fold greater [6–8] and this surplus LA is either utilized for energy production/storage or converted to AA. Differential effects of n-3 and n-6 PUFA are reported following FA immune cell membrane incorporation, impacting

Abbreviations: AA, arachidonic acid; ALA, alpha-linolenic acid; APC, antigen presenting cell; ARASCO, arachidonic acid single cell oil; D6D, Δ6-desaturase; D6KO, D6D knock out; DHA, docosahexaenoic acid; DHASCO, docosahexaenoic acid single cell oil; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; FO, fish oil; LA, linoleic acid; LC, long chain; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PE, phycoerythrin; TCR, T-cell receptor; TLR, toll-like receptor.

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membrane structure and function including lipid mediator production, lipid raft formation and signaling, leading to altered gene expression and pathophysiological outcomes [9–15]. However, dietary interventions attempting to discern specific protective and/or therapeutic immunomodulatory effects of individual FA are confounded due to an inability to determine if the outcome is attributable to the parent compound (LA or ALA), the downstream FA conversion product (AA or EPA/DHA), or both.

To date, a gap exists with regard to our basic understanding of how individual FA influence the diverse spectrum of immune cell effector functions, in particular inflammatory cytokine production, wherein n-3 PUFA are generally regarded to exert anti-inflammatory biological effects and high intakes of n-6 PUFA typically promote a pro-inflammatory phenotype [9,16]. Complicating this interpretation, pro- and anti-inflammatory effects have been attributed to some n-6 PUFA-derived lipid mediators [17–19], and pro-resolving effects of some AA-derived lipid mediators that down-regulate inflammatory responses are also reported [20–24]. Therefore, this paradigm in FA immunobiology remains controversial due to the growing evidence that individual FA within these families can exert unique biological effects. Using cytokine production as one aspect of immune competence that is influenced by dietary FA (assessed in the absence of a confounding pathology), the majority of studies utilize human mononuclear cells. In this context, EPA and DHA have been shown to reduce peripheral blood mononuclear cell synthesis and/or secretion of the inflammatory cytokines tumor necrosis factor (TNF) α, interleukin (IL)-1β, IL-6 and/or interferon (IFN) γ [25–31], while other studies have shown stimulatory [32] or no effect on these parameters [33–35]. Conversely, AA has been shown to increase immune cell gene expression of the inflammatory cytokines TNFα and IL-1β [36], increase IL-6 secretion [37], and decrease anti-inflammatory IL-10 secretion [38]. Conversely, human supplementation with 1.5 g/day of AA showed no change in mononuclear cell inflammatory cytokine secretion, although the sample size was limited [39]. Data pertaining to the biological effects of the FA parent compounds, ALA and LA, on cytokine production are less clear, and tend to be confounded by downstream conversion to the more biologically active FA products, as discussed above. Despite this limitation, anti-inflammatory effects of ALA have been demonstrated based on decreased serum levels or mononuclear cell secretion of TNFα, IL-1β and/or IL-6 [40–42], whereas a stimulatory effect on macrophage TNFα production has been reported [43]. Although these outcomes cannot preclude the involvement of LC n-3 FA conversion products, they demonstrate the potential for an independent biological effect of ALA. Finally, despite a lack of independent LA-centered investigations, many studies commonly use high LA diet formulations as the control diet for comparison purposes to discern the biological effects of n-3 PUFA [27,31,34,40–42], but fail to determine the independent effects of LA compared to other FA. Few studies have attempted to address this question and have shown LA to increase IL-6 bioactivity [31] or have no effect on inflammatory cytokine secretion levels [35,41,44]. LA is generally regarded as a pro-inflammatory n-6 PUFA, however, no human clinical evidence supports this dogmatic view [45] and protective effects of n-6 PUFA in inflammatory diseases have been reported (reviewed elsewhere [46]). Collectively, these studies confound our understanding of the effects of specific FA and highlight that this component of FA immunobiology (in both the healthy unchallenged and disease states) requires revisitation, particularly since any beneficial or deleterious pro- or anti-inflammatory effects of individual FA will be context-dependent. For example the anti-inflammatory effects of n-3 PUFA are broadly interpreted to be beneficial, which is true with respect to inflammatory pathologies and associated inflammation-driven tissue damage, however, a robust inflammatory response represents a normal physiological function and is necessary in situations such as opportunistic infections.

The Δ6-desaturase knock-out (D6KO) mouse model provides a means to determine the specific effects of individual FA and avoids the confounding effect of FA conversion to LC products [47,48,49,50]. The D6KO mouse exhibits similar FA conversion efficiency as humans [7], thereby providing a mouse model with translational utility. The D6KO mouse lacks a functional copy of the *Fads2* gene, rendering it unable to produce the D6D protein [49,50], thereby blocking the rate-limiting step in FA metabolism [1]. Therefore, D6KO mice represent a tool to determine the effects of specific FA when provided in the diet and can be used to delineate differences between parent chain FA and their respective LC FA conversion products. In this model, WT mice provide insight into the influence of FA conversion on study endpoints, whereas comparison to D6KO mice demonstrates the effects of individual FA (when conversion is inhibited). The objective of this study was to feed wild-type (WT) and D6KO mice specific FA-enriched diets to assess the role of LA, AA, ALA and EPA/DHA on splenic mononuclear cell cytokine production in response to an inflammatory stimulus (lipopolysaccharide, LPS) and within specifically activated immune cell compartments, namely T cells [*via* activation of the T cell receptor (TCR)] and antigen presenting cells (APC, *via* anti-CD40 ligation).

2. Materials and methods

2.1. Animals, housing and diets

The creation of the D6KO mouse was described previously [50]. Breeders were transferred from University of Illinois at Urbana-Champaign to the University of Guelph to establish a breeding colony. Heterozygous D6KO male and female mice were bred to generate WT and KO offspring and harems were fed a basal diet, which is a modified AIN-93G diet with corn oil as the principal dietary fat source (D03090904P, Research Diets, New Brunswick, NJ, USA). The pups were fed the same diet until they were weaned and genotyped at 21 days of age, at which point male and female homozygous D6KO and WT mice were placed on one of four isocaloric experimental diets, differing only in their principal FA source: corn oil (LA-enriched), arachidonic acid single cell oil (ARASCO) (AA-enriched), flaxseed oil (ALA-enriched), and menhaden fish oil (EPA/DHA-enriched). Diet formulations and diet FA compositions are shown in Tables 1 and 2, respectively. No diet was deficient in LC PUFA (>20 carbon length), and therefore, the LA and ALA-enriched diets were supplemented with a minimal amount (0.2% w/w) of either AA or DHA from ARASCO and docosahexaenoic acid single cell oil (DHASCO), respectively (DSM Nutritional Products Canada Inc., Ayr, ON, Canada), to prevent LC PUFA deficiency in D6KO mice [47]. Specifically, the LA-enriched diet was devoid of AA but was supplemented with DHASCO, whereas the ALA-enriched diet was devoid of EPA/DHA but supplemented with ARASCO. All diet compositions were formulated to contain AA, which was not supplemented in the EPA/DHA-enriched diet because menhaden fish oil contains low levels of endogenous AA. The combination of these dietary formulations and the use of WT and D6KO mice allows for the discernment of the individual effects of specific FA. For example, in the LA-enriched diet, the specific effects attributed to AA can be determined in WT mice, wherein the downstream conversion of LA to AA is intact, *versus* D6KO mice wherein the downstream conversion of LA to AA is inhibited). Mice were fed experimental diets for a total of 9 weeks. At 12 weeks of age, final body weights were recorded and mice were euthanized using CO_2. During the experimental period, mice were housed as described [47], monitored daily, and food intake and changes in body weight were recorded. Water and diets were provided *ad libitum* and refreshed every 2–3 days. This investigation was approved by the University of Guelph Animal Care Committee in accordance with the requirements of the Canadian Council on Animal Care.

2.2. Genotyping

At 21 days of age, mice were weaned and tail snips were obtained for DNA extraction and PCR analysis to determine genotype as described [50]. The PCR primers utilized were D6D WT forward (CGGTGGGAGGAGGAGTAGAAGAC); D6D WT reverse (CCTCTCCCTGGTTACCTCCCTTC); D6D KO forward (GCTATGACTGGGCACAACAG); and D6D KO reverse (TTCGTCCAGATCATCCTGATC) [47].

2.3. Fatty acid analysis by gas chromatography

Lipids were extracted from whole spleens ($n=3$–4/diet/genotype) and from two individual pellets from each experimental diet using the Folch method [51]. FA methyl esters were prepared as described previously [52] and separated on Agilent Technologies 7890 A GC System with DB-FFAP fused-silica capillary column (15 m, 0.1 μm film thickness, 0.1 mm i.d.; Agilent Technologies, Palo Alto, CA, USA). FA were identified by comparing peak retention times with those of known standards (GLC463; Nu-Chek Prep,