Enriching the Starter Diet in n–3 Polyunsaturated Fatty Acids Reduces Adipocyte Size in Broiler Chicks

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Abstract
Epidemiologic studies associate perinatal intake of eicosapentaenoic acid (EPA, 20:5n–3) and docosahexaenoic acid (DHA, 22:6n–3) with reduced adiposity in children, suggesting that these fatty acids may alter adipose tissue development. The objective of this study was to determine whether enriching the perinatal diet in EPA and DHA reduces fat deposition in young chicks. Cobb 500 broiler chicks were fed isocaloric diets containing fat (8% wt:wt) from fish oil (FO), lard, canola oil, or flaxseed oil from 7 to 30 d of age. Adiposity (abdominal fat pad weight/body weight) at 30 d was not significantly affected by diet, but FO significantly reduced adipocyte size, increasing the abundance of small adipocytes. Plasma nonesterified fatty acid concentrations suggest that reduced adipocyte size was due, in part, to enhanced mobilization of fatty acids from adipose tissue. Our work indicates that dietary EPA and DHA effectively reduce the size of developing adipocytes in juveniles, which may limit adipose deposition and provide metabolic benefits.

Introduction
Approximately 27% of children in the United States are classified as overweight or obese by age 5 y (1). Obese children are more likely to be obese adults, and both childhood and adult obesity increase the risk of cardiovascular disease, diabetes, and other comorbidities (2). Limiting excess fat accumulation in the first few years of life is therefore therapeutically important for children and for the prevention of adult obesity. A plethora of studies have identified factors that influence adiposity in mature animals and humans, in which changes in adipocyte size are the primary basis for differences in fatness. Much less is known about control of adipose mass in juveniles, when both adipocyte hypertrophy and hyperplasia actively contribute to fat deposition.

PUFAs of the n–3 and n–6 series differentially regulate preadipocyte proliferation, adipogenesis, and TG storage, all of which contribute to deposition of adipose tissue before adolescence. n–6 PUFAs tend to be pro-adipogenic, whereas long-chain n–3 PUFAs (particularly EPA and DHA) attenuate lipid accumulation and promote an oxidative adipocyte phenotype [reviewed in (3)]. Large-scale studies in mother-child pairs have associated n–6 PUFA intake with increased adiposity in children, whereas an inverse relation has been shown with dietary n–3 PUFAs (4, 5). These associations suggest that the types of FAs consumed early in life may influence the course of adipose development and subsequently affect the predisposition to obesity.

Avian models are useful for testing the effects of diet on early adipose development because chicks eat independently at hatch, allowing direct manipulation of the diet very early in life. Broiler chickens in particular are a valuable polygenic model of susceptibility to obesity due to inadvertent consequences of selection for rapid growth (6). Broiler chicks begin to deposit excess abdominal fat compared with other breeds within 2 wk of hatch. In contrast, most rodent models of obesity are monogenic or are induced by feeding a diet very high in fat.
high in fat. We used Cobb 500 broiler chicks, one of the most widely used commercial broiler lines, to determine whether providing EPA and DHA in the diet early in life attenuates adipose tissue deposition in juveniles. Diets enriched in fish oil (FO; as a source of EPA and DHA) were compared with diets containing equal amounts of lard (LA), canola oil (CA), or flaxseed oil (FL) to evaluate the effects relative to other types of FAs. Experimental diets were provided beginning at 7 d of age to coincide with initial deposition of the abdominal depot and to focus on a developmental window in which hypertrophy and hyperplasia contribute comparably to adipose growth.

Methods

Animals and diets

All animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee. Forty mixed-sex Cobb 500 broiler chicks were fed ad libitum a commercial starter diet from hatch until 7 d, then switched to 1 of 4 experimental diets. Experimental diets were produced by adding fat (8% wt:wt) from LA, CA, FL, or FO into a commercially formulated starter diet base (Supplemental Table 1). Final energy content of each diet was 3018 kcal/kg. Chicks were fed experimental diets from 7 to 30 d. At 30 d, chicks were weighed, then killed by carbon dioxide asphyxiation, and tissues and blood were collected for experimental procedures. Complete experimental details are provided in Supplemental Methods.

Statistical analysis

Data were analyzed for effects of diet by using ANOVA implemented in SAS (version 9.4; SAS Institute, Inc.), with P < 0.05 as the criterion for significance. A significant F-test was followed by post hoc comparisons with the use of Fisher’s least-significant-difference test to identify pairwise differences between diet groups.

Results

Effects on body composition and growth rate

The FA composition of the diet significantly affected final body weight (P = 0.045), with FO birds weighing less than those in the LA group (Table 1; P = 0.02). Neither absolute nor relative (adjusted for body weight) weights of the abdominal adipose depot or of breast muscle differed significantly between diet groups (P > 0.05). Plasma concentrations of NEFAs (P = 0.002), but not glucose (P = 0.130), were affected by diet, with increased NEFAs in FO chicks compared with each of the other diet groups. As expected, the FA profile of the abdominal fat reflected the dietary FA composition (Supplemental Table 2). Feed intake did not differ across diets (data not shown).

Despite similarities in fat pad weight, diet significantly affected abdominal adipocyte volume (P = 0.020). Average adipocyte size was smallest in FO chicks, differing significantly from chicks fed LA or CA diets (Table 1). Adipocyte number varied with diet (Table 1), but differences were not significant (P = 0.093). FO promoted a shift in adipocyte size, favoring the abundance of relatively small adipocytes compared with diets enriched in LA or CA (Figure 1A). The frequency of very small (<2000 μm³) adipocytes was significantly increased in FO compared with either LA or CA (P < 0.05). Conversely, frequencies of cells in each size bin >4000 μm³ were lower (P < 0.05) in FO than in CA chicks and in FO than in LA chicks for most bins. Adipocyte volumes tended to be smaller in chicks fed the FL diet, with frequencies intermediate between those of FO and LA or CA in most size bins.

Effects on relative mRNA expression in visceral white adipose tissue and liver

Dietary fat source significantly influenced expression of PPAR-γ (PPARG), early growth response transcription factor 1 (EGR1), patatin-like phospholipase domain–containing protein 8 (PNPLA8), and pyruvate dehydrogenase kinase 4 (PDK4) in abdominal adipose tissue (Figure 1B). Both PPARG and EGR1 were expressed at significantly lower levels in FO and CA chicks relative to LA. The expression of PNPLA8 was significantly reduced in FO, CA, and FL chicks compared with LA chicks. Genes associated with FA oxidation [acyl-CoA oxidase 1 (ACOX1) and carnitine palmitoyl acyltransferase 1 (CPT1)], lipogenesis [FA synthase (FASN)], lipid storage [lipoprotein lipase (LPL)], gluconeogenesis [phosphoenolpyruvate carboxykinase 1 (PCK1)], and

### Table 1: Effects of dietary LA, CA, FL, and FO on body, adipose, and muscle weights and on serum metabolites in broiler chicks

<table>
<thead>
<tr>
<th></th>
<th>LA</th>
<th>CA</th>
<th>FL</th>
<th>FO</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>1752 ± 46.7ab</td>
<td>1650 ± 54.6ab</td>
<td>1694 ± 45.1ab</td>
<td>1562 ± 45.0a</td>
<td>0.045</td>
</tr>
<tr>
<td>Breast weight, g</td>
<td>337.9 ± 13.8</td>
<td>360.2 ± 16.5</td>
<td>354.6 ± 13.6</td>
<td>346.1 ± 7.9</td>
<td>0.573</td>
</tr>
<tr>
<td>Relative breast weight, %</td>
<td>19.4 ± 1.0</td>
<td>21.9 ± 0.6</td>
<td>21.0 ± 1.0</td>
<td>22.1 ± 1.7</td>
<td>0.400</td>
</tr>
<tr>
<td>Adipose tissue weight, g</td>
<td>26.0 ± 1.9</td>
<td>24.5 ± 1.2</td>
<td>22.3 ± 1.2</td>
<td>24.3 ± 1.8</td>
<td>0.455</td>
</tr>
<tr>
<td>Relative adipose weight, %</td>
<td>1.48 ± 0.09</td>
<td>1.51 ± 0.07</td>
<td>1.33 ± 0.07</td>
<td>1.56 ± 0.12</td>
<td>0.499</td>
</tr>
<tr>
<td>NEFAs, mM</td>
<td>6.25 ± 1.98ab</td>
<td>6.49 ± 0.79ab</td>
<td>6.55 ± 1.41ab</td>
<td>10.04 ± 0.96a</td>
<td>0.002</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>175.7 ± 12.8</td>
<td>184.5 ± 10.8</td>
<td>189.4 ± 7.0</td>
<td>168.1 ± 5.7</td>
<td>0.130</td>
</tr>
<tr>
<td>Adipocyte volume, μm³</td>
<td>3651 ± 420.7a</td>
<td>3706 ± 184.1a</td>
<td>3098 ± 95.2ab</td>
<td>2546 ± 153.1b</td>
<td>0.020</td>
</tr>
<tr>
<td>Adipocyte number</td>
<td>75.4 ± 13.3</td>
<td>70.8 ± 9.5</td>
<td>93.7 ± 9.8</td>
<td>90.3 ± 8.1</td>
<td>0.093</td>
</tr>
</tbody>
</table>

1 Values are means ± SEMs for all chicks in each diet group, n = 10/group. Means with shared superscript letters do not differ significantly, P < 0.05. CA, canola oil; FL, flaxseed oil; FO, fish oil; LA, lard.
2 Derived by using single-factor ANOVA for effect of diet.
3 Abdominal adipose depot weight (g)/body weight (g) × 100.
4 Calculated from adipocyte volume and adipocyte depot weight.
5 Adipocyte number × body weight (g).

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inflammation [chemokine C-C ligand 20 (CCL20), colony stimulating factor 1 receptor (CSF1R)] were not significantly affected by dietary fat type. In liver, expression of ACOX1, but not CPTI or FASN, was significantly affected by diet (Figure 1C). Expression of ACOX1 was higher in FO than in all other diet groups.

FIGURE 1  Effects of dietary FO, FL, CA, and LA on abdominal adipocyte size distribution and on gene expression in liver and adipose tissue. (A) Adipocyte area was measured from images of hematoxylin-and-eosin–stained sections of abdominal adipose tissue (Supplemental Figure 1) by using ImageJ (version 1.48; NIH); areas <500 μm² were removed from analyses. Adipocyte volume (μm³) was calculated from area (μm²). Frequency distributions were produced by grouping adipocytes into bins on the basis of volume and counting the frequency of cells within each bin. ANOVA was used to evaluate the effect of diet within each bin. (B and C) Relative mRNA expression of genes involved in lipid metabolism, adipogenesis, and inflammation in abdominal adipose tissue (B) and in lipid metabolism in liver (C); n = 6 birds/diet. Values are group means ± group SDs. Means without a common lowercase letter differ significantly (P, 0.05) based on post hoc testing when ANOVA indicated a significant effect of diet (P, 0.05). ACOX1, acyl-CoA oxidase 1; CA, canola oil; CCL20, chemokine C-C ligand 20; CPT1α, carnitine palmitoyl acyltransferase 1α; CSF1R, colony stimulating factor 1 receptor; EGR1, early growth response transcription factor 1; FASN, FA synthase; FL, flaxseed oil; FO, fish oil; LA, lard; LPIN1, lipin-1; LPL, lipoprotein lipase; PCK1, phosphoenolpyruvate carboxykinase 1; PDK4, pyruvate dehydrogenase kinase 4; PNPLA8, patatin-like phospholipase domain containing protein 8; PPARG, PPARGC1B, PPARG coactivator 1 beta.
Discussion

A number of studies in rodents have shown that dietary FO can attenuate the obesogenic effects of a high-fat diet. These studies have largely used mature animals, in which growth-related adipose expansion has ceased and changes in adipose mass result from effects on adipocyte size. In contrast, we focused on the first few weeks after hatch to capture the period when the abdominal depot develops and rapidly expands through both adipocyte hyperplasia and hypertrophy (7). Evaluating the effects of FO on these pathways is important because both contribute to childhood obesity (8, 9). A specific benefit of using an avian model, compared with a rodent model, is that they lack uncoupling protein 1 (UCP-1) and thus the capacity for adipose browning. This is valuable because recent studies indicate that at least part of the antiobesity effects of FO in rodents are due to induction of beige adipocytes in white adipose depots (10). Therefore, our model enables us to evaluate the specific effects of dietary FO on white adipocytes.

Unlike comparable diet studies in mature chickens [e.g., (11)], we did not find an effect of dietary n-3 PUFAs on adiposity. This may be due to the relatively brief period of feeding (23 d) or because FA type was not sufficient to influence the inherent stimulus for rapid adipose deposition during this age window. However, both of the diets that were enriched in n-3 PUFAs (particularly FO) favored the abundance of small adipocytes relative to diets enriched in LA or CA. Although adipocyte hypertrophy is a normal component of adipose development, excessive hypertrophy presents very early in obese children and promotes insulin resistance and adipose inflammation (9). Therefore, the ability of dietary n-3 PUFAs to reduce adipocyte size in juveniles, even in the absence of decreased fat mass, may provide metabolic benefits for children prone to obesity.

Adipocyte size results from a balance between FA uptake and mobilization, particularly in species (e.g., avians and humans) in which de novo lipogenesis in adipose tissue is relatively modest. Increased plasma NEFA concentrations suggest that enhanced FA mobilization may have contributed to reduced adipocyte size in FO chicks. Interestingly, elevated NEFAs, reduced adipocyte size, and enrichment of adipose tissue in EPA and DHA are also found in genetically lean lines of chickens relative to obesity-prone broilers (12). Both DHA and EPA have been shown to stimulate lipolysis and reduce adipocyte lipid accumulation in vitro (13, 14). The consumption of FO may therefore have reduced adipocyte size by promoting mobilization of FAs that are then oxidized by other tissues. Hepatic expression of ACOX1, the rate-limiting enzyme for oxidation of very-long-chain FAs in peroxisomes, was increased by dietary FO. However, expression of CPT1, which regulates mitochondrial FA oxidation and is often coregulated with ACOX1 (15), was not significantly affected by diet in liver. Therefore, increased expression of ACOX1 in the FO group may reflect a specific response to the abundance of very-long-chain PUFAs (EPA and DHA) rather than a net increase in hepatic FA catabolism. Dietary EPA has also been shown to enhance FA oxidation within white adipocytes (16). However, our data do not indicate that this pathway contributed to reduced adipocyte size in FO and FL chicks on the basis of expression of CPT1 and ACOX1. It is also possible that FO, and to some extent FL, increased the abundance of smaller adipocytes by suppressing their progression through differentiation, rather than altering the balance between lipid storage and mobilization. This possibility is supported by a study in which dietary perilla oil (∼52% Ω-linolenic acid, 18:3n–3) downregulated the later stages of adipocyte differentiation in rats (17). Additional characterization with stage-specific markers of adipocyte differentiation is necessary to investigate this possibility in our study.

None of the genes that we profiled were specifically affected by the diets (FO and FL) that reduced adipocyte size. However, all 3 diets enriched in unsaturated FAs reduced expression of PPARG, EGRI, and PNPLA8 compared with LA. PPARG is a well-characterized transcriptional regulator of both adipocyte differentiation and maintenance of the mature adipocyte phenotype (18). Increased PPARG expression with dietary SFA—compared with PUFA-enriched diets is consistent with comparable studies in mature broilers (19). EGRI is a pleiotropic transcription factor that has been linked to multiple aspects of adipocyte function (20). Elevated EGRI expression in adipose tissue is associated with obesity in humans and mice, whereas loss of EGRI enhances adipocyte metabolism and confers protection from obesity (21). Decreased expression of this gene in the FL, FO, and CA groups relative to LA may therefore reflect beneficial effects of dietary fat quality on adipocyte metabolism. Calcium-independent phospholipase A2γ (iPLA2γ, encoded by PNPLA8), is a phospholipase that catalyzes the release of FA side chains from mitochondrial phospholipids to generate production of eicosanoids and other lipid second messengers that regulate cellular energetics. The specific roles of iPLA2γ in adipose tissue are not known, but PNPLA8−/− mice are resistant to diet-induced obesity, with reduced adipocyte size relative to wild-type controls (22). Although expression was not consistently associated with adipocyte size in our study, reduced levels across all PUFA-enriched diets suggest that dietary FAs may regulate mitochondrial lipid mediators and subsequently adipocyte metabolism through PNPLA8.

In conclusion, we showed that dietary FO attenuates adipocyte hypertrophy in juvenile chicks that are prone to rapid fat accumulation. The mechanisms underlying this effect remain to be determined but may include increased mobilization of stored FAs for oxidation by other tissues or disruption of adipocyte maturation. Although the effect of diet on adipocyte size did not manifest as significantly less adipose mass during the relatively brief period of feeding used herein, it may nonetheless be sufficient to elicit favorable metabolic effects in children who are prone to obesity.

Acknowledgments

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References