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Key words
- docosahexaenoic acid
- leptin
- adiponectin
- insulin
- fetal programming

Abstract

Maternal factors can have major imprinting effects on homeostatic mechanisms in the developing fetus and newborn. Here we studied whether supplemented perinatal polyunsaturated fatty acids (PUFAs) influence energy balance and fuel homeostasis later in life. Between day 10 after conception and day 10 after delivery, female rats were subjected to chow enriched with 10% fish-oil (FO-rich). Fish oil contains high concentrations of n-3 biosynthesis endpoint products, which may have caused the increased membrane phospholipid incorporation (particularly derived from the long-chain 20+:n-3 PUFAs) in 10-day old pup brains. Adult male offspring of FO-rich fed rats had reduced body weight (−20%) at 3 months, and had lower levels of plasma leptin (−54%), insulin (−41%), triglycerides (−65%), and lactate (−46%) than controls. All differences between groups were lost 48 h after streptozotocin (STZ) treatment. At 4.5 months of age, body weights of FO-rich were still lower (−6%) than controls, but were associated with increased food intake, and increased insulin sensitivity (following intraperitoneal injection) to lower blood glucose levels relative to controls. We concluded that perinatal FO supplementation has lasting effects on body weight homeostasis and fuel metabolism in male offspring, but does not offer resistance against STZ-induced diabetes.

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Introduction

There is an increasing amount of evidence that maternal factors related to the nutritional status can have an effect on fetal and post-natal development in human as well as rodent offspring. These imprinting effects can be long-lasting and may affect metabolic, physiological and structural parameters at adulthood. In his fetal origins hypothesis, Barker proposed that gestational undernourishment programmes later coronary heart disease, non-insulin dependent diabetes [1], and several other derangements, together often termed as the "metabolic syndrome" [2]. Although the precise mechanisms that program susceptibility to chronic metabolic and cardiovascular diseases still have to be defined (see [3,4]), there are strong indications that they are the result of fetal adaptations invoked when the nutrient supply fails to match the fetal demand [5]. Over the last few years, however, data are emerging that increased birth weight and maternal obesity in humans are associated with increased adult obesity and chronic diseases in the offspring as well [6,7]. While this apparent inconsistency is a continuing matter for debate (see [8]), there might be a common basis underlying its etiology. One of the common mechanism might involve the maternal availability of long-chain polyunsaturated fatty acids (lc PUFAs), and in particular the n-3 type, for fetal development [9].

It is well known that lc PUFAs have profound influences on the cellular regulation of fuel metabolism (e.g., [10]). For example, they stimulate transcription factors that augment fat oxidation and reduce those involved in lipogenesis [10]. In particular, the n-3 PUFAs might be considered protective against obesity [11]. In addition, dietary n-3 PUFAs appear to offer resistance against spontaneous [12] or streptopzotocin (STZ)-induced [13] type 1 diabetes mellitus. Moreover, cellular reduction of n-6 to n-3 increases pancreatic B cell survival against cytokine-induced [14] and streptozotocin-induced [15] cell death.

What is less-well appreciated is that docosahexaenoic acid (DHA; 22:6 n-3); that is, one of the endpoint biosynthesis products of n-3 PUFAs, is essential for brain growth and maturation during fetal and post-natal development and thus for

normal functioning of the CNS at adulthood [16,17]. The biosynthesis of end-point PUFAs is limited [18], and a relative deficiency of these PUFAs could easily occur under conditions of famine [19,20], which than could be transferred into diminished DHA availability for fetal and early post-natal development. It might be hypothesized that this reduced availability of DHA during the perinatal stage is a key factor in "programming" the developing fetus towards a thriftier phenotype at adulthood. As in the condition of famine, feeding of a western-type high fat diet can also cause a relative reduction of DHA in pregnant rats, as well as in their offspring [21]. Again, this might then underlie obesity and related derangements later in life [22], and the same could be anticipated in humans as well [23,24]. Siemelink et al. previously found that providing diets with a low ratio of unsaturated vs. saturated fatty acids during gestation causes more disturbances in fuel homeostasis than diets with a relatively high ratio of unsaturated vs. saturated fatty acids [25]. The present study was designed to confirm this earlier work of Siemelink [25], and to investigate – in light of the acute protective effects of n-3 PUFAs against experimental or spontaneous type 1 diabetes – whether also perinatal PUFA supplementation offers resistance to streptozotocin-induced diabetes.

Material and Methods

Cycling female Wistar rats (n=16) were housed individually in clear plastic cages, in a temperature (20±2°C) and humidity controlled environment with a 12:12-h light:dark schedule. Food (pelleted laboratory chow, Charles River diet number #5075) and water were available ad libitum. The energy content of the diet was 14.4 kJ/g, the macronutrient ratio of carbohydrate/protein/fat was 57.3/18.1/4.5, and the remaining 20.1% was composed of vitamins, minerals and fiber. After establishing estrus, all females were made pregnant by 8 males (each cage contained 2 females and 1 male), and successful copulations and pregnancies were evidenced by observation of the vaginal plug and absence of di-estrus, respectively. Between day 10 of their pregnancy until day 10 after delivery of pups, 8 mother rats were fed the same diet as above, but enriched with fish-oil (FO-rich, enrichment 10%), and the remaining rats were left on the normal chow (control). Fish oil (a generous gift of BLT Berg Lipidtech; Aale, Norway) was used as a dietary supplement to increase the availability of certain PUFAs for the fetal and newborn rats. The fish oil was inserted chemically into the chow by organic solvent, which was fully evaporated afterwards (for details, see [26]), and the dietary content of the different fatty acids are presented in **Table 1**. There were some fatty acids which appeared in negligible amounts (under 0.5% in each diet) and not listed in the table. In case of FO-rich food there were another 15 fatty acids which could be detected (under 0.5%).

At day 10 of age, all litters were reduced to 7 (male:female ratio, 4:3). Superfluous pups were killed, and 1 male from each nest was used to investigate membrane phospholipid incorporation of fatty acids in brain tissue of to assess efficacy of maternal FO supplementation [26]. Body weights of remaining offspring were assessed at 3 months of age. 1 male rat from each of 4 FO-rich litters and 1 from each 4 control litters was sacrificed for assessment of circulating hormone (all with radioimmuno assays from Linco Research) and fuel levels (all with enzymatic kits from Sigma). Another male from each of the 4 remaining FO-rich litters and of each of the 4 control litters were injected with streptozotocin through the tail vein (STZ; Sigma, 55 mg/kg b.w., i.v.), dosed to cause destruction of pancreatic B-cells. 48 h after STZ treatment, these rats were sacrificed and the same parameters were assessed as above. The remaining males were regrouped to 5–6 cages (2–3 rats in each cage) per treatment. At

Table 1 Fatty acid composition of diets

FA	FO-rich	Control
14:0	1.9	0.6
16:0	18.4	13.7
16:1	3.2	0.8
18:0	7.1	3.2
18:1	23.0	17.1
18:2n6 (LA)	21.3	51.2
18:3n3 (LNA)	2.0	6.8
20:4n6 (AA)	0.9	0.1
20:5n3 (EPA)	2.8	0.7
22:4n6	0.8	ND
22:5n3	0.5	ND
22:6n3 (DHA)	11.3	1.2
Total %	93.2	95.4

Levels are expressed as percentage of total fatty acid content
ND: Nondetectable

Table 2 Phospholipid content in total forebrain homogenates of control or FO-rich offspring

		PE	PS	PC
14:0	FO-rich	0.82±0.10	1.24±0.07	3.35±0.17
	control	ND	1.45±0.42	2.87±0.12
16:0DMA	FO-rich	4.67±0.07**	ND	ND
	control	5.88±0.15	ND	ND
16:0	FO-rich	11.69±0.64*	9.85±2.16	48.42±1.35
	control	9.54±0.22	7.01±0.61	51.97±0.87
16:1	FO-rich	1.12±0.14	2.06±0.34	5.54±0.17
	control	0.82±0.08	1.80±0.29	4.70±1.05
16:3	FO-rich	0.98±0.07	ND	ND
	control	0.86±0.11	ND	ND
18:0DMA	FO-rich	4.6±0.07*	ND	ND
	control	5.35±0.17	ND	ND
18:1DMA	FO-rich	0.57±0.03***	ND	ND
	control	0.86±0.03	ND	ND
18:0	FO-rich	20.43±0.58	37.20±1.82	8.29±0.33
	control	20.01±0.42	37.24±0.80	7.24±0.40
18:1n9	FO-rich	4.74±0.07	4.81±0.31	15.40±0.49
	control	4.74 0.06	4.94±0.27	13.81±0.18
18:1n7	FO-rich	0.93±0.04	ND	3.11±0.11
	control	1.04±0.04	ND	3.33±0.04
18:2n6	FO-rich	1.12±0.09	1.65±0.18*	2.23±0.21*
	control	0.78±0.15	1.00±0.11	1.42±0.09
20:4n6	FO-rich	11.19±0.15***	2.84±0.29***	4.61±0.22***
	control	19.26±0.37	7.24±0.41	7.36±0.18
20:5n3	FO-rich	0.55±0.12	ND	0.53±0.15
	control	ND	ND	ND
22:4n6	FO-rich	1.73±0.06***	1.86±0.19***	ND
	control	5.72±0.19	6.12±0.52	0.68±0.04
22:5n6	FO-rich	0.85±0.05***	1.14±0.03***	ND
	control	4.65±0.29	6.66±0.28	0.54±0.25
22:5n3	FO-rich	1.32±0.06	1.92±0.23	ND
	control	ND	ND	ND
22:6n3	FO-rich	28.06±0.64***	28.62±3.39	ND
	control	17.54±0.43	22.75±1.03	ND

Levels are expressed as percent of total fatty acids
DMA: dimethylacetal; ND: nondetectable (under 0.5%)
Control vs. FO-rich: *denotes $p<0.05$, **denotes $p<0.01$, ***denotes $p<0.001$

	Control	FO-rich	Control + STZ	FO-rich + STZ
Glucose (mM)	7.4 ± 0.2	6.6 ± 0.3	23.8 ± 0.3 †††	23.5 ± 0.1 †††
FFA (mM)	0.35 ± 0.02	0.44 ± 0.06	0.94 ± 0.27 †	0.84 ± 0.21
Lactate (mM)	2.39 ± 0.13	1.29 ± 0.14 ***	2.22 ± 0.14	1.78 ± 0.16
TGL (mM)	3.78 ± 0.66	1.31 ± 0.27 *	10.70 ± 4.13	7.63 ± 2.92
Cholesterol (mM)	2.10 ± 0.10	2.02 ± 0.13	3.13 ± 1.10	2.35 ± 0.28
HDL-cholesterol (mM)	1.03 ± 0.04	1.21 ± 0.08	1.14 ± 0.18	1.10 ± 0.06
Leptin (ng/ml)	6.22 ± 0.83	2.84 ± 0.86 *	0.61 ± 0.19 ††	0.88 ± 0.21
Insulin (ng/ml)	5.32 ± 0.27	3.12 ± 0.39 **	0.51 ± 0.11 ††	1.12 ± 0.58 †
Adiponectin (µg/ml)	4.74 ± 0.26	5.10 ± 0.32	5.65 ± 1.07	5.24 ± 0.58
Body weight (g)	454 ± 7	362 ± 18 **	380 ± 25 †	336 ± 17

Control vs. FO-rich: *denotes $p<0.05$, **denotes $p<0.01$, ***denotes $p<0.001$
Non-STZ vs. STZ: †denotes $p<0.05$. ††denotes $p<0.01$, †††denotes $p<0.001$

Table 3 Effect of diet and streptozotocin (STZ) treatment on hormonal and metabolic parameters

4.5 month of age, animals were separated, and assessment of food intake and body weight was performed over 2 consecutive weeks. Thereafter, in the middle of light phase following an overnight fast, 1 randomly selected male from each of 8 FO-rich litters and 1 male from each 8 control litters were injected intraperitoneally with insulin (0.5 U/kg, Velosulin, Novo Nordisk). Before (t = −15 min) and after insulin injection (at t = 15, 30, 60, and 120 min), tail blood (ca. 100 µl by clip) was taken and transferred into cooled tubes containing heparin. Finally, 6–7 animals per group had their blood successfully sampled throughout the whole experiment. Samples were centrifuged (10 min, 2 600 *g*) and plasma was analyzed for concentration of glucose (enzymatic kit, Sigma).

All values are represented as means ± SEM. Differences between FO-offspring and control offspring were analyzed using ANOVA followed by student t-tests.

Results

The FO-supplemented and control foods were consumed in equal amounts by the female rats during the pregnant and postnatal stage. Pregnancies of FO-rich fed females were normal, but tended to be slightly, but not significantly, longer than control pregnancies. There were no differences in litter size and weights of newborns. All litters from FO-rich and control rats were reduced to 7 pups at day 10. At that age, membrane phospholipid incorporation from FO-rich and control pups differed among groups, particularly those derived from the long-chain 20+:n-6 and 20+:n-3 PUFAs (see **Table 2**). More specifically, phosphatidylethanolamine (PE) with 20+:n-3 fatty acids was more abundant in FO-rich offspring than in controls, whereas PE 20+:n-6 fatty acids was generally more abundant in control offspring. Concentrations of PE with 16:0 dimethylacetal (DMA), 18:0 DMA, and 18:1 DMA (plasmalogens; i. e., glycerophospolipids of neural membranes containing ether bonds [27] were lower in FO-rich offspring than in controls. Differences in concentrations of phosphatidylserine (PS) and phosphatidylcholine (PC) were generally in the same direction, but less pronounced.

At 3 months of age, male offspring from FO-rich pregnancies weighed 20% less than those from control pregnancies (see **Table 3**). There were no differences in body length. In addition, they had markedly and significantly lower plasma leptin (−54%), insulin (−41%), triglyceride (−65%), lactate (−46%) (see **Table 3**). STZ treatment caused frank diabetes in offspring of both dietary groups – as evidenced by elevated plasma levels of glucose and free fatty acids (FFA), and reductions in plasma levels of insulin and leptin – all initial differences in hormonal and metabolic parameters between control and FO-rich offspring were lost following STZ administration (**Table 3**).

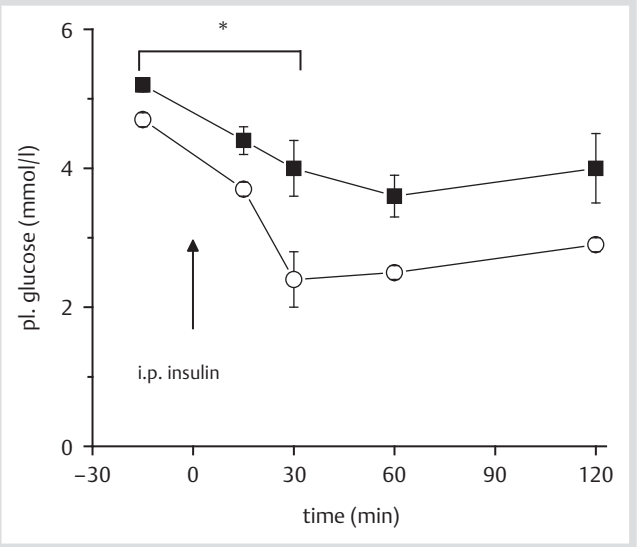

Fig. 1 Effects of i.p insulin injection on blood glucose levels in control offspring (closed squares) and offspring of females supplemented with fish-oil during pregnancy (open circles).

At 4.5 months of age, differences in mean body weight of male offspring from FO-rich (496 ± 10 g) and control pregnancies (531 ± 12 g) were less pronounced (6.6%) than at 3 months, but still significant ($p=0.02$). At that age, daily food intake of grouped FO-rich (2 or 3 rats per cage, 5 cages) offspring (first week: 29.8 ± 0.8 g; second week: 31.0 ± 1.1) was similar as that of grouped control (2 or 3 rats per cage, 6 cages) offspring (first week: 28.8 ± 0.5 g; second week: 29.2 ± 0.8 g). However, food intake per 100 g body weight was markedly higher in FO-rich offspring (first week: 5.92 ± 0.14 g, $p=0.005$; second week 6.05 ± 0.16 g, $p=0.04$) than in control offspring (first week: 5.39 ± 0.08 g; second week: 5.43 ± 0.21 g).

Following food intake measurements, FO-rich and control offspring were overnight food deprived, and injection of insulin caused reductions in blood glucose levels relative to baseline levels (see **Fig. 1**). ANOVA revealed an interaction effect of time and treatment when considering the first phase of the glucose response to insulin (from −15 to 30 min: $F(2,22)=3.99$, $p=0.033$). The glucose drop from baseline (assessed as delta) to the level found at t = 30 min was stronger ($p=0.03$) in FO-rich offspring (−49 ± 9%) than in controls (−24 ± 8%), and the slope of

the reduction of glucose levels in control offspring was delayed relative to FO-rich offspring. It may be possible that differences after 30 min following insulin injection are underestimated since the glucose levels in the FO-rich offspring, but not in the control offspring, more likely reached a hypoglycemic threshold of glucose-counterregulation [28].

Discussion

In the present study, pregnant female rats received dietary supplementation of fish oil (FO) between 10 days after conception and until 10 days post-partum. FO is known to contain high quantities of long-chain n-3 polyunsaturated fatty acids (lc PUFA), and in particular the endpoint biosynthesis product docosahexaenoic acid (DHA: 22:6 n-3). Although exact causality remains to be elucidated, this elevated dietary level was reflected by increased concentrations of phospholipids containing DHA in the CNS of 10-day-old pups of FO-supplemented mothers. The endpoint biosynthesis products of n-6 lcPUFAs, such as for example arachidonic acid (AA: 20:4 n-6), were reduced in CNS phospholipid fractions of FO-offspring. While FO supplementation was terminated at post-natal day 10 in the present study, these changes (at least in brain phospholipid content) are consistent with those observed by Amusquivar et al. [29] who treated female rats with a comparable FO-diet throughout pregnancy and lactation.

Besides being involved in CNS functioning [12] and neuronal homeostasis [30], it may be hypothesized that n-3 PUFAs are involved in metabolic programming during fetal development. Since fetal undernourishment [1,2] as well as feeding of a cafeteria diet [22] may predispose obesity and related metabolic diseases in the offspring, and because these perinatal conditions are associated with low maternal PUFA availability for the developing fetus [19–21], we hypothesized that elevated maternal PUFA supply would induces a leaner phenotype in the offspring. Consistent with this idea was the observation in the present study that male FO-offspring weighed 20% less at 3 months of age than control offspring, and these observations largely confirm the findings by Siemelink et al. [25]. Because plasma leptin is a correlate of total body adiposity [31,32], the finding that the plasma leptin was more than 50% lower in the FO-offspring than in control offspring is a strong indication that they stored less fat.

Opposed to the findings of Amusquivar et al. [29] and Siemelink et al. [25], the FO-rich offspring in the present study did not have differences in body weight postnatally relative to controls. Difference in outcome among studies might be ascribed to differences between strains, differences in ratios of DHA and AA, but also in the timing of FO-supplementation (i.e., which was shorter in our study than in most others). In fact, large amounts of dietary fish oil during pregnancy and lactation have been shown to have adverse effects on body length and head circumference, which appears to be related to low AA levels [33]. Another concern with respect to the implementation of this treatment might be that AA is important for the brain's learning capability [17,34]. Thus, caution should be exercised with respect to timing as well as relative levels of supplementation of both n-3 and n-6 PUFAs during pregnancy (see also [35]).

Markers indicative of a leaner phenotype at adulthood with improved metabolic control were lower plasma levels of insulin (−41%), triglycerides (−65%), and lactate (−46%) in FO-offspring than in offspring of control females. Furthermore, the FO-offspring tended to have elevated plasma levels of HDL-cholesterol, which, in conjunction with the lower triglyceride levels may be considered as reduced risk for development of metabolic syndrome and the consequent cardiovascular and atherosclerotic diseases (see also [36]). In spite of the markedly reduced plasma levels of leptin in the FO-offspring relative to controls, plasma levels of another adipocyte hormone, adiponectin were not reduced in FO-offspring. A relatively high plasma level of adiponectin (i.e., in relation to the lower plasma leptin and presumably reduced level of body adiposity) would be considered agreeable since adiponectin is suggested to enhance insulin action, and to increase fatty acid oxidation in skeletal muscle and the liver [37,38]. In fact, the plasma adiponectin level has been found to be more closely associated to insulin-mediated glucose disposal than to body adiposity per sé [39]. The finding in the present study that intraperitoneal insulin injection caused a relatively stronger reduction in glucose levels in the FO-rich offspring, even from a significantly reduced baseline level, relative to the one observed in control offspring is consistent with this idea. To investigate whether perinatal FO supplementation offers resistance against experimentally-induced type 1 diabetes all parameters (except food intake) were also assessed 48 h after streptozotocin (STZ) treatment. In both offspring groups, STZ treatment led to full-blown diabetes as evidenced by similar hyperglycemia and low levels of plasma insulin and leptin. All initial differences between FO-, and control offspring were lost following STZ treatment, indicating that pancreatic B cell disruption and the resulting disturbances in fuel homeostasis are not affected by perinatal PUFA supplementation. These data are highly relevant in light of the fact that STZ-treatment is less effective in adult rodents that are actually subjected to feeding a diet enriched with PUFAs at that time [13–16]. Thus, although PUFA supplementation during the perinatal stage may clearly reduce risk for the development of obesity and cardiometabolic disease, this study demonstrates that PUFA supplementation does not offer resistance against the typical type 1 diabetic derangements in fuel homeostatis associated with pancreatic B cell destruction by STZ.

The mechanisms through which perinatal FO-supplementation affect energy balance and fuel homeostasis are yet to be discovered. It is known that PUFAs may accumulate in maternal fat depots and become available for placental transfer during late pregnancy when the fetal growth is maximal [40]. In addition, PUFAs may be provided in excess through the mother's milk to the offspring [41]. In turn, PUFAs may have numerous beneficial effects in offspring tissues, including the central nervous system [42]. Neuronal circuitry involved in the regulation of energy balance is mostly located in the hypothalamus, and contains a number of neuropeptides, which develop [43] around the same time when the brain requires large quantities of DHA for expansion [44,45]. It is possible that DHA can directly affect the development and functioning of these neuropeptidergic neurons [46]. Another potential mechanism is that eicosanoid factors (such as prostaglandins), which are synthesized from n-3 and n-6 lc PUFAs, are affected and regulate activity of neuropeptidergic systems later in life according to mechanisms for example described by Ericksson et al. [47]. Imprinting effects on these hypothalamic neuropeptide systems have previously been proposed by others (e.g., [48–50]), and may consequently affect signalling of peripheral factors related to the energetic status. It might be speculated that the FO-offspring in the present study

are more sensitive to leptin than the controls. Increased sensitivity to leptin in the neuronal circuitry controlling energy balance in FO-offspring would be consistent with their presumed increased metabolic rate, lower levels of triglyceride stores, but also with relatively high levels of plasma adiponectin [51]. Studies are currently performed in our laboratories to investigate these potential mechanisms.

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