Leptin levels in rat offspring are modified by the ratio of linoleic to α-linolenic acid in the maternal diet

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Abstract **The supply of polyunsaturated fatty acids (PUFA) is important for optimal fetal and postnatal development. We have previously shown that leptin levels in suckling rats are reduced by maternal PUFA deficiency. In the present study, we evaluated the effect of maternal dietary intake of (n-3) and (n-6) PUFA on the leptin content in rat milk and serum leptin levels in suckling pups. For the last 10 days of gestation and throughout lactation, the rats were fed an isocaloric diet containing 7% linseed oil (n-3 diet), sunflower oil (n-6 diet), or soybean oil (n-6/n-3 diet). Body weight, body length, inguinal fat pad weight, and adipocyte size of the pups receiving the n-3 diet were significantly lower during the whole suckling period compared with n-6/n-3 fed pups. Body and fat pad weights of the n-6 fed pups were in between the other two groups at week one, but not different from the n-6/n-3 group at week 3. Feeding dams the n-3 diet resulted in decreased serum leptin levels in the suckling pups compared with pups in the n-6/n-3 group. The mean serum leptin levels of the n-6 pups were between the other two groups but not different from either group. There were no differences in the milk leptin content between the groups. These results show that the balance between the n-6 and n-3 PUFA in the maternal diet rather than amount of n-6 or n-3 PUFA per se could be important for adipose tissue growth and for maintaining adequate serum leptin levels in the offspring.**—Korotkova, M., B. Gabrielsson, M. Lönn, L-Å. Hanson, and B. Strandvik. **Leptin levels in rat offspring are modified by the ratio of linoleic to α-linolenic acid in the maternal diet. *J. Lipid Res.* 2002.** 43: **1743–1749.**

Supplementary key words lactation • leptin mRNA • arachidonic acid • diet • adipose tissue • and perinatal period

An adequate supply of polyunsaturated fatty acids (PUFA) during pregnancy and lactation is crucial for optimal fetal and postnatal development. However, neither the role of nor the requirements for individual PUFA are yet established (1). Over the last 20 years the consumption of saturated fat from animal food and n-6 PUFA from oil seeds has been substantially increased, while the dietary intake of n-3 PUFA from plants and marine products has declined in industrialized countries (2, 3). As a consequence, the ratio n-6/n-3 PUFA in the diet has raised to 10:1–15:1 (3, 4). Since the maternal diet is the most important variable determining milk fatty acid composition (5), this shift in dietary intake of PUFA results in rising concentrations of n-6 PUFA and reduction of n-3 PUFA levels in human milk (3). The ratio n-6/n-3 PUFA in the milk is of importance, as n-6 and n-3 PUFA compete for the synthesis and the incorporation of long-chain PUFA into the cell membranes and they also have different functional roles (4). In animals and man, variations of the ratio n-6/n-3 PUFA in the milk are followed by changes in growth (6, 7), neural and retinal development (2, 8), and immune responsiveness (9) of the offspring and might also have additional effects.

We have previously shown that the maternal deficiency of PUFA in rats affects the serum leptin levels in their offspring (10) and alters milk leptin concentration (11). Leptin is an adipose tissue-derived hormone that regulates food intake and energy expenditure, and is involved in several physiological and pathological processes (12). Moreover, possible developmental roles of leptin in the perinatal period have been suggested (13, 14). During early development, leptin is produced by the placenta and by fetal and neonatal adipose tissues (14, 15), and is also provided via maternal milk (16). In humans, leptin levels in early life predict weight gain later in infancy (17, 18). These studies suggest that circulating leptin levels during the perinatal period could be important for normal development and health.

The aim of the present study was to investigate the effects of maternal dietary intake of n-6 and n-3 PUFA on

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Abbreviations: FA, fatty acids; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; USI, unsaturation index.

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the serum leptin levels in rat pups during the suckling period and on the leptin content in rat milk.

MATERIAL AND METHODS

Animals

Pregnant Sprague-Dawley rats (BK Universal, Stockholm, Sweden) were received on day 7 of gestation and kept in our animal facility under constant conditions of humidity (70–80%), temperature (22–25°C), and light (12-h light and dark cycle). The rats were housed individually in plastic cages with food and water available ad libitum. Ten days before delivery, the rats were assigned to one of three groups (n = 9–10 in each group) receiving either a diet containing both n-6 and n-3 essential fatty acids (n-6/n-3), a n-3 essential fatty acids-enriched (n-3) diet, or a n-6 essential fatty acids-enriched (n-6) diet. Litter size was adjusted to 10 pups per litter. Pups (n = 10–16) randomized from each litter were used at each time point. Body weight and length of pups were recorded every week. The animals were killed by decapitation in the morning (09 AM–11 AM) at 1 or 3-weeks-of-age. Truncal blood was collected and sera were kept frozen (−20°C) until analyses of leptin, glucose, protein, cholesterol, and triglycerides. Pairs of subcutaneous (inguinal) fat pads were removed, weighed, and kept frozen (−20°C) until analyses of fatty acid (FA) composition of total lipids and phospholipids. Fat pads from parallel sets of animals were placed into the RNAlater™ (Ambion, Austin, TX) and stored at −20°C until analysis by RT-PCR.

Milk samples were collected from dams at 3 weeks of lactation. After separation from the pups for 30 min, dams were anaesthetized i.p. with pentobarbital (35 mg/kg body weight) and injected ip with 4 IU of oxytocin (Sigma Chemical Co., St. Louis, MO) to stimulate milk flow. Milking was initiated 5 min after oxytocin injection and milk was collected by hand expression. The milk samples were stored at −20°C until analyses of leptin and of FA composition of total lipids.

The study was approved by the Animal Ethics Committee of Göteborg University.

Diets

The dams were fed one of three experimental pellet diets (Morinaga Milk Industry Co. LTD, Tokyo, Japan) for the last 10 days of gestation and throughout lactation. The diets differed only by lipid composition: 7% soybean oil (diet contains both n-6 and n-3 PUFA), sunflower oil (n-6 PUFA-enriched, n-6 diet), or linseed oil (n-3 PUFA-enriched, n-3 diet). The composition of the three diets is given in **Table 1**. The data on major components, salt, and vitamins have been obtained from the manufacturer. The FA composition was determined in our laboratory with the method described below. The ratio n-6/n-3 fatty acids in the n-6/n-3 diet was 9 and in the n-6 and n-3 diets, 216 and 0.4, respectively. The total metabolizable energy of the diets was 13.9 MJ/kg.

TABLE 1. Composition of the diets

Diet	n-3 Diet	n-6/n-3 Diet	n-6 Diet
		%	
Casein	20.0	20.0	20.0
Potato starch	54.0	54.0	54.0
Glucose	10.0	10.0	10.0
Cellulosal flour	4.0	4.0	4.0
Mineral mix[a]	4.0	4.0	4.0
Vitamin mix[b]	1.0	1.0	1.0
Linseed oil	7.0		
Soybean oil		7.0	
Sunflower oil			7.0
Fatty acids		*mol%*	
16:0	14	12	8
16:1	0.3	0.1	0.1
18:0	6.1	3.9	4.2
18:1	32	21	22
18:2	14	56	65
20:0	0.3	0.4	0.3
18:3	33	6.2	0.3

[a] Salt mixture containing (wt%): KH_2PO_4 (34.1); $CaCO_3$ (35.9); KCl (2.5); NaCl (18); $MgSO_4 \times H_2O$ (5.1); $FeC_6H_5O_7 \times {}^5H_2O$ (3.3); MnO (0.27); $Cu_2C_6H_4O_7 \times {}^{2.5}H_2O$ (0.06); $ZnCO_3$ (0.04); $CoCl_2 \times {}^6H_2O$ (0.002); $KAl(SO_4)_2 \times {}^2H_2O$ (0.008); NaF (0.025); KIO_3 (0.009); $Na_2B_4O_7 \times {}^{10}H_2O$ (0.002); $Na_2SeO_3 \times {}^5H_2O$ (0.001); $Na_2MoO_4 \times {}^2H_2O$ (0.001).

[b] Vitamin mixture containing: Vit A 11.9 IU/g; Vit D_3 1.5 IU/g; Vit B_1 4 μg/g; Vit B_2 12 μg/g; Vit B_6 5 μg/g; Ca-pantotenate 45% 11 μg/g; Niacin 40 μg/g; Vit B_{12} 0.02 μg/g; Vit K_3 7.75 μg/g; Biotin 2% 3 μg/g; Vit C 500 μg/g; Inositol 30 μg/g; Vit E 42 μg/g; Choline chloride 50% 1 mg/g; Folic acid 0.5 μg/g.

Fatty acid analysis

Total lipids of white adipose tissue, representing mainly triglycerides, and milk were extracted according to Folch et al. (19). The total lipids from adipose tissue were fractionated on a single SEP-PAK aminopropyl cartridge (Waters Corp., MA) and the fraction of phospholipids was analyzed. Milk total lipids were not fractionated. The FA methyl esters were separated by capillary gas-liquid chromatography in a Hewlett-Packard 6890 gas chromatograph according to the method described previously (10). The separation was recorded with HP GC Chem Station software (HP GC, Wilmington, DE). The FA 21:1 was used as internal standard and the FA methyl esters identified by comparison with retention times of pure reference substances (Sigma Aldrich Sweden AB, Stockholm, Sweden). The unsaturation index (USI) was calculated as ratio Σ (mol% each unsaturated FA × number of double bonds of the same FA)/saturated fatty acids (SFA).

Analysis of leptin

Leptin concentrations in serum and milk were measured by a rat leptin radioimmunoassay (RIA; Linco Research Ltd., St. Charles, MO) and all samples from one experiment were analyzed in duplicates in the same assay. The intra-assay coefficient of variation (CV) at 0.25 ng/ml was 2.4%, and at 20 ng/ml 1.6%. Milk samples were thawed at 37°C and vortexed vigorously before pipetting. The samples were diluted in assay buffer (1:2 to 1:5) before sonication (five bursts, five s/burst with cooling on ice between each burst, 80% power) to ensure homogenous samples. To control for possible matrix effects in the individual milk samples, each sample was divided into three tubes and a standard addition procedure was employed by adding 1 ng and 2 ng leptin, respectively, to the second and third tube. The original leptin content was then calculated using linear regression. An effect of non-specific background in the milk on the leptin data was evaluated by comparing added leptin with measured leptin. There was a matrix effect in the milk in both the diet groups. Therefore, we used the leptin values calculated from the standard addition procedure.

Analysis of glucose, protein, cholesterol, and triglyceride levels in serum

Serum glucose was determined by a quantitative glucose oxidase/PAP assay (ABX Diagnostics, Parc Evromedicine, Montpellier, France). Serum protein was determined by a quantitative

colorimetric assay (Biuret reaction) (ABX Diagnostics). Serum cholesterol was determined by a quantitative enzymatic colorimetric assay (Infinity™ cholesterol reagent, Sigma Diagnostics Inc., St. Louis, MO). Serum triglycerides were determined by a quantitative GPO (glycerol peroxidase)/PAP assay (ABX Diagnostics).

RNA extraction and analysis by RT-PCR

Total RNA was isolated from the adipose tissue of each individual rat with the RNeasy Mini Kit (QIAGEN, Valencia, CA) and used for analysis of leptin mRNA (mRNA) by RT-PCR. The RNA samples were treated with DNase (DNA-free™, Ambion, Austin, TX) according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically (OD_{260}) and its integrity was verified by agarose gel electrophoresis, with visualization by ethidium bromide (EtBr) staining.

Synthesis of cDNA was performed in a volume of 30 μl using 0.7 μg of total RNA and 3.3 μM random hexamers (Pharmacia Biotech, Uppsala, Sweden) in a solution containing 1× First strand buffer (Life Technologies, Gaitherburg, MD), dNTPmix (0.5mM each of dATP, dGTP, dCTP, and dTTP; Ultrapure dNTP Set, Pharmacia Biotech), RNase-inhibitor (1 U/μl, rRNasin, Promega, Madison, WI, USA) and Reverse Transcriptase (13.3 U/μl, Superscript™II RT, Life Technologies). The mixture was incubated at room temperature for 10 min and at 42°C for 60 min followed by 10 min at 70°C. The cDNA was stored at −70°C.

Multiplex relative RT-PCR was used for the analysis of differences in mRNA abundance. The leptin gene was co-amplified with invariant endogenous control. The cDNA was amplified by PCR using specific primers for the rat leptin cDNA (20). The primer pairs: 5′ CCT GTG GCT TTG GTC CTA TCT G 3′ (nucleotides 87–108. GenBank accession number D4582) and 5′ AGG CAA GCT GGT GAG GAT CTG 3′ (nucleotides 310–330) generated a single 244 base pair (bp) product. QuantumRNA 18S internal standard (Ambion) was used as internal control and generated a single 489 bp product. Linear range and optimal ratio of 18S primers/competimers were determined. The PCR reaction was carried out in a final volume of 50 μl with 2 μl of cDNA product, 1× PCR buffer, 2.0 mM $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each dNTPs, and 1.25 U AmpliTaqGold (Applied Biosystems, Foster City, CA). PCR was performed using the GeneAmp PCR System 9600 (Applied Biosystems) and following conditions: 94°C (12 min) for 1 cycle, 94°C (30 s), 60°C (30 s), 72°C (30 s) for 30 cycles, 72°C (7 min). The negative control consisted of omission of the reverse transcriptase for each sample, which resulted in no bands after RT-PCR.

The PCR products were separated on 2% EtBr agarose gel and were subsequently visualized and quantified using IPLab Gel Scientific Image processing (Signal Analitics, Vienna, VA). The intensity obtained for leptin amplicon was related to that of 18S in each individual sample.

Adipocyte cell size and number

Adipocyte and stroma-vascular (S-V) fractions were prepared following the procedure outlined by Smith et al. (21). Briefly, about 0.5 g adipose tissue (n = 4 in each group) was cut into smaller pieces and transferred to a plastic vial containing prewarmed 10 ml Parker medium 199 (SBL, Stockholm, Sweden) supplemented with 4% BSA and 0.8 mg/ml collagenase type A (Roche Diagnostics, Bromma, Sweden). The vials were incubated for 1 h at 37°C in a shaking water bath. The cells were filtered through a 250 μm nylon mesh and the adipocytes were allowed to float to the surface for 5 min before aspiration of the medium. The adipocytes were washed twice with 5 ml medium, allowing the cells to float to the surface each time. After the final wash, the adipocytes were resuspended in fresh medium (20% cells and 80% medium) to yield the final cell suspension. The suspension was gently mixed before placing 3–4 drops on a glass slide onto which two layers of adhesive tape had been attached to form a small chamber and a cover slip was placed on top. Cell diameter was measured with a Zeiss microscope at 10× magnification (Axioplan2 imaging, Carl Zeiss, Göttingen, Germany). Digital images were captured with a video camera mounted on the microscope and transferred to a computerized image analysis system, KS400 (Carl Zeiss). By introducing conditions on the roundness of the cell areas as well as smoothness of the contours, the program could identify healthy fat cells and automatically calculated the diameter.

Statistical analysis

Values are presented as mean ± SD. The data were analyzed by one-way ANOVA (Fisher's PLSD). Differences within individuals were determining using paired *t*-test. When the number of observations was limited, non-parametric statistical methods were used (Kruskal-Wallis test). A value of $P < 0.05$ was considered statistically significant.

RESULTS

FA composition of total milk lipids

There was no difference in the total amount of PUFA or USI in milk from rats fed the different diets (**Table 2**). The levels of SFA were higher in the milk from dams fed the n-3 diet, while the levels of monounsaturated fatty acids (MUFA) were higher in the milk of the dams fed the n-6/n-3 diet. Feeding the n-6 diet to the dams generated decreased levels of α-linolenic 18:3(n-3) and docosahexoenoic 22:6(n-3) acids in the milk lipids, whereas the levels of linoleic 18:2(n-6) and γ-linolenic 18:3(n-6) acids

TABLE 2. The fatty acid composition of the milk total lipids from the rats fed different diets at 3 weeks of lactation

Fatty Acids	n-3	n-6/n-3	n-6
		mol%	
12:0	12.9 ± 1.1[a]	10.3 ± 2.1[b]	13.4 ± 1.9[a]
14:0	12.6 ± 1.4[a]	8.0 ± 2.5[b]	10.8 ± 2.6[a]
16:0	18.9 ± 1.2[a]	19.1 ± 2.0[a]	16.6 ± 1.9[b]
16:1(n-7)	0.68 ± 0.26[a]	1.1 ± 0.4[b]	0.63 ± 0.34[a]
18:0	3.7 ± 0.3[a]	4.7 ± 0.5[b]	4.2 ± 0.4[c]
18:1(n-9)	14.9 ± 1.4[a]	19.4 ± 2.0[b]	15.8 ± 2.4[a]
18:2(n-6)	9.4 ± 0.8[a]	31.1 ± 2.7[b]	35.1 ± 2.3[c]
18:3(n-6)	0.04 ± 0.02[a]	0.10 ± 0.02[a]	0.23 ± 0.11[b]
18:3(n-3)	26.3 ± 1.6[a]	3.1 ± 0.4[b]	0.0 ± 0.0[c]
20:2(n-6)	0.14 ± 0.04[a]	0.60 ± 0.12[b]	0.57 ± 0.13[b]
20:4(n-6)	0.31 ± 0.06[a]	1.6 ± 0.4[b]	1.4 ± 0.3[b]
24:0	0.05 ± 0.01[a]	0.08 ± 0.03[a]	0.13 ± 0.02[b]
24:1(n-9)	0.02 ± 0.02[a]	0.53 ± 0.14[b]	0.44 ± 0.22[b]
22:6(n-3)	0.45 ± 0.1[a]	0.62 ± 0.1[b]	0.24 ± 0.13[c]
20:4(n-6)/22:6(n-3)	0.7 ± 0.1[a]	2.5 ± 0.6[a]	8.1 ± 4.7[b]
n-6	9.8 ± 0.9[a]	33.4 ± 2.9[b]	37.3 ± 2.3[c]
n-3	26.7 ± 1.6[a]	3.75 ± 0.3[b]	0.2 ± 0.1[c]
n-6/n-3	0.4 ± 0.0[a]	8.9 ± 0.7[a]	206.3 ± 101.5[b]
SFA	48.1 ± 2.3[a]	42.3 ± 4.2[b]	45.3 ± 4.8[b]
MUFA	15.6 ± 1.6[a]	21.0 ± 2.4[b]	16.8 ± 2.9[a]
PUFA	36.6 ± 2.0[a]	37.1 ± 3.0[a]	37.6 ± 2.5[a]
USI	2.5 ± 0.2[a]	2.5 ± 0.4[a]	2.4 ± 0.7[a]

SFA, saturated fatty acids; MUFA monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USI, unsaturation index. Values given as mean ± SD (n = 7–8).

[a,b,c]Values with unlike superscript letters are significantly different ($P < 0.05$).

TABLE 3. The fatty acid composition of white adipose tissue total lipids and phospholipids of the pups at 3-weeks-of-age

	Total Lipids			Phospholipids		
Fatty Acids	n-3	n-6/n-3	n-6	n-3	n-6/n-3	n-6
			mol %			
12:0	9.2 ± 0.8^a	9.2 ± 1.0^a	9.4 ± 1.0^a	0.1 ± 0.1^a	0.2 ± 0.2^a	0.2 ± 0.2^a
14:0	13.3 ± 0.7^a	11.5 ± 0.5^b	11.8 ± 0.9^b	2.1 ± 0.4^a	1.9 ± 0.2^a	1.9 ± 0.3^a
16:0	28.5 ± 1.0^a	25.6 ± 1.2^b	25.2 ± 1.5^b	27.9 ± 1.4^a	26.9 ± 1.8^{a,b}	26.1 ± 1.1^b
16:1(n-7)	1.9 ± 0.4^a	1.8 ± 0.3^a	1.7 ± 0.5^a	0.7 ± 0.2^a	0.5 ± 0.1^b	0.5 ± 0.1^b
18:0	3.2 ± 0.3^a	3.3 ± 0.3^a	3.3 ± 0.3^a	19.8 ± 1.7^a	19.3 ± 2.2^a	20.0 ± 2.0^a
18:1(n-9)	15.7 ± 0.9^a	17.5 ± 0.7^b	16.9 ± 1.2^b	8.7 ± 0.8^a	7.4 ± 0.8^b	7.0 ± 1.0^b
18:2(n-6)	8.9 ± 1.0^a	25.8 ± 1.3^b	27.9 ± 1.9^c	11.9 ± 1.0^a	12.9 ± 1.0^{a,b}	14.0 ± 1.9^b
18:3(n-6)	0.10 ± 0.00^a	0.28 ± 0.04^b	0.32 ± 0.04^c	0.03 ± 0.01^a	0.1 ± 0.0^b	0.06 ± 0.05^c
18:3(n-3)	16.9 ± 0.7^a	2.1 ± 0.2^b	0.5 ± 0.5^c	1.8 ± 0.2^a	0.2 ± 0.1^b	0.1 ± 0.1^b
20:2(n-6)	0.24 ± 0.05^a	0.64 ± 0.08^b	0.64 ± 0.07^b	0.25 ± 0.05^a	0.51 ± 0.09^b	0.54 ± 0.1^b
22:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.71 ± 0.07^a	0.90 ± 0.07^b	0.96 ± 0.12^b
20:4(n-6)	0.57 ± 0.18^a	1.7 ± 0.3^b	1.8 ± 0.2^b	16.1 ± 2.5^a	24.0 ± 1.7^b	24.5 ± 2.0^b
20:5(n-3)	0.74 ± 0.12^a	0.12 ± 0.06^b	0.0 ± 0.0^c	4.9 ± 0.7^a	0.3 ± 0.1^b	0.1 ± 0.1^b
24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.2^a	1.6 ± 0.1^a	1.8 ± 0.2^b
24:1(n-9)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.2^a	1.1 ± 0.2^b	0.9 ± 0.2^c
22:6(n-3)	0.50 ± 0.12^a	0.42 ± 0.08^a	0.29 ± 0.11^b	1.7 ± 0.1^a	1.8 ± 0.2^a	1.1 ± 0.3^b
20:4(n-6)/ 22:6(n-3)	1.1 ± 0.1^a	4.1 ± 0.5^b	6.9 ± 2.2^c	9.2 ± 1.4^a	13.8 ± 1.7^b	22.9 ± 6.5^c
n-6	9.8 ± 1.1^a	28.4 ± 1.2^b	30.7 ± 2.0^c	28.2 ± 2.5^a	39.2 ± 0.9^b	37.5 ± 1.1^c
n-3	18.2 ± 0.7^a	2.6 ± 0.2^b	0.83 ± 0.62^c	8.4 ± 0.9^a	2.2 ± 0.2^b	1.3 ± 0.2^c
n-6/n-3	0.5 ± 0.1^a	11.1 ± 1.2^a	48.2 ± 19.8^b	3.4 ± 0.6^a	17.3 ± 1.7^b	32.5 ± 6.8^c
SFA	54.2 ± 2.8^a	49.7 ± 1.3^b	49.7 ± 2.4^b	51.0 ± 1.2^a	49.8 ± 1.2^b	49.7 ± 1.5^b
MUFA	17.7 ± 0.9^a	19.4 ± 0.6^b	18.8 ± 1.4^b	10.7 ± 1.1^a	8.9 ± 0.8^b	8.4 ± 1.1^b
PUFA	36.6 ± 1.9^a	39.7 ± 1.2^b	40.4 ± 1.0^b	28.0 ± 1.5^a	30.9 ± 1.2^b	31.5 ± 1.7^b
USI	1.8 ± 0.1^a	1.8 ± 0.1^a	1.8 ± 0.2^a	2.7 ± 0.2^a	2.9 ± 0.2^b	2.8 ± 0.2^{a,b}

Values given as mean ± SD (n = 10).
^{a,b,c}Values with unlike superscript letters are significantly different within head columns ($P < 0.05$).

were enhanced compared with those in the dams fed the n-6/n-3 diet. Feeding the n-3 diet to lactating animals induced marked changes in the total milk lipid FA composition. The contents of 18:2(n-6), eicosadienoic acid 20:2(n-6), and arachidonic 20:4(n-6) acids were significantly lower compared with the other diets, while the levels of 18:3(n-3) were markedly higher in the n-3 group compared with the n-6/n-3 group ($P < 0.05$). The content of 22:6(n-3) was reduced in the n-3 group compared with the n-6/n-3 group.

FA composition of total lipids and phospholipids in white adipose tissue from the pups

Total lipid FA composition of white adipose tissue in the suckling pups at 3-weeks-of-age generally reflected that of the total milk lipids (Table 2 and 3). White adipose tissue total lipid FA composition of pups suckling dams on the n-6/n-3 and on the n-6-diet was similar, except the significantly lower levels of (n-3) PUFA in the n-6-group (**Table 3**). The ratios of 20:4(n-6) to 22:6(n-3), and of n-6 to n-3 FA in the n-6-group were higher compared both to the n-3 and n-6/n-3 groups.

There were significant changes in the FA composition of white adipose tissue total lipids in the pups of the dams on the n-3 diet compared with those in the n-6/n-3 group. The contents of (n-6) PUFA were significantly decreased, with an increase in the levels of myristic 14:0, palmitic 16:0, 18:3(n-3) and 20:5(n-3) acids, while the levels of 22:6(n-3) were unchanged. The level of SFA was higher and the levels of MUFA and PUFA were lower in the total lipids of white adipose tissue in the pups fed the dams on the n-3 diet.

Phospholipids of white adipose tissue from the pups of the dams on the n-6 and n-6/n-3 diet contained similar FA levels, except reduced proportion of 22:6(n-3) in the n-6 group. Feeding the n-3 diet resulted in elevated levels of 18:3(n-3) and pronounced changes of the LC-PUFA levels in the phospholipids of the white adipose tissue in the pups compared with those in the pups in the n-6/n-3 diet group. A significant decrease in the levels of 20:4(n-6) was observed along with an accumulation of 20:5(n-3). The proportion of SFA and MUFA was increased and levels of PUFA were decreased significantly in the n-3 group compared with the n-6/n-3 group. The unsaturation index in the white adipose tissue phospholipids was slightly reduced in the n-3 group.

Body and white adipose tissue growth parameters of the pups

The mean body weight and length of the pups of the dams fed the n-3 diet were lower during the whole suckling period compared with the pups of the dams fed the n-6/n-3 diet or the n-6 diet (**Table 4**). The mean body weight of the pups fed the n-6 diet was lower at 1 week of age and did not differ at 3-weeks-of-age from those of the pups fed the n-6/n-3 diet. The mean body length of the pups receiving the n-6 diet did not differ from those of the n-6/n-3-fed pups at 1 week of age, but was reduced compared with the n-6/n-3 pups at 3-weeks-of-age.

The weight of the inguinal white adipose tissue in the n-6 and the n-6/n-3 fed pups increased compared with those in the n-3 group during whole suckling period (Table 4). The relative weight of white adipose tissue to body

TABLE 4. Body weight and length, and weight of the inguinal white adipose tissue (WAT) of the pups, at 1- and 3-weeks-of-age, of the dams fed different diets

	1 Week			3 Week		
	n-3	n-6/n-3	n-6	n-3	n-6/n-3	n-6
Weight, g	14 ± 1^b	19 ± 2^a	17 ± 3^c	37 ± 4^b	49 ± 5^a	46 ± 5^a
Length, cm	7 ± 0.3^b	8 ± 0.4^a	8 ± 0.3^a	11 ± 0.6^b	13 ± 0.4^a	12 ± 0.5^c
WAT, mg	117 ± 27^b	240 ± 59^a	161 ± 47^c	449 ± 143^b	719 ± 231^a	631 ± 150^a
WAT, mg/ body wt, g	8.3 ± 1.4^b	12.6 ± 2.4^a	9.4 ± 1.6^b	11.8 ± 2.7^b	14.6 ± 3.6^a	13.7 ± 2.4^a

Values given as mean ± SD (n-10, 1 week and n-16, 3 week).
[a,b,c] Values with unlike superscript letters are significantly different within head columns ($P < 0.05$).

weight in the n-6/n-3 fed pups increased compared with those in the pups of dams fed the n-6 and the n-3 diet at 1 week of age. The mean adipocyte cell size in the white adipose tissue of the n-6/n-3 fed pups was significantly higher (58.9 ± 5.6 μm) than in the pups of the dams fed the n-3 diet (51.3 ± 2.4 μm) ($P < 0.05$) and showed a trend toward higher values compared with the n-6 diet (53.9 ± 2.1 μm).

Serum glucose, protein, cholesterol, and triglyceride levels in the pups

The serum glucose levels of the suckling pups from the dams fed the different diets were similar (5.5 ± 0.5, 5.9 ± 1.0, and 5.6 ± 0.9 mmol/l in the n-3, n-6/n-3 and n-6 groups, respectively). Corresponding values of the serum protein levels in the offspring were 52.0 ± 7.0, 51.5 ± 5.2 and 52.6 ± 13.6 g/l, respectively. There were no significant differences between the pups in the serum cholesterol levels (153.4 ± 25.2, 159.0 ± 38.6, and 164.1 ± 50.8 mg/dl in the n-3, n-6/n-3 and n-6 groups, respectively). The serum triglyceride levels in the pups were not different in the three diet groups (135.1 ± 81.5, 172.1 ± 52.4, and 175.2 ± 144.0 mg/dl in n-3, n-6/n-3 and n-6 groups, respectively), though n-3 group showed a trend toward lower levels. The glucose, protein, cholesterol, and triglyceride levels in the serum of the dams fed the different diets were similar at 3 weeks of lactation (data not shown).

Leptin levels in serum and milk

The serum leptin levels in the suckling pups were significantly higher ($P < 0.05$) at week 1 compared with 3-weeks-of-age for all the diet groups (**Fig. 1**). Feeding the dams the n-3 diet resulted in decreased serum leptin levels in the suckling pups at 1- and 3-weeks-of-age compared with those in the pups of the dams on the n-6/n-3 diet. There were no significant differences in the leptin levels during the suckling period between the offspring of the dams on the n-6/n-3 or the n-6 diets.

The milk leptin levels in the lactating dams were not significantly different between the groups on the n-3, n-6/n-3, and n-6 diets (9.0 ± 2.8, 13.3 ± 3.4, and 10.7 ± 3.8 ng/ml, respectively), though the milk leptin tended to be higher in the n-6/n-3 group of dams.

Leptin mRNA expression in adipose tissue

The expression of leptin in the inguinal white adipose tissue was detected as a 244 bp band. The identity of the PCR product was verified by restriction enzyme digestion with Age I, which gave the expected pattern of two bands at 140 bp and 104 bp (data not shown). The leptin mRNA expression in the inguinal white adipose tissue of the pups of the dams on the different diets was similar at 1 week of age. At 3-weeks-of-age, however, the levels of the leptin mRNA were significantly lower in the white adipose tissue of the pups receiving the n-6/n-3 diet compared with those in the n-3 and n-6 groups ($P < 0.05$) (**Fig. 2**).

DISCUSSION

To our knowledge, this is the first study showing that serum leptin levels in the rat pups are affected by the maternal diet ratio of n-6 and n-3 PUFA. Increased maternal intake of n-3 PUFA led to a decreased growth rate, reduced adipose tissue mass, and lower serum leptin levels in the suckling pups. Furthermore, the ratio n-6/n-3 PUFA rather than the levels of n-6 PUFA in the maternal milk promoted body weight, growth of inguinal white adipose tissue, and adipocyte size in the offspring.

The involvement of PUFA in various physiological and pathological processes in the organism suggests that variation in maternal milk n-3 and n-6 PUFA might significantly affect the postnatal growth and development, and risk for development of chronic diseases in adulthood (22, 23). It is well established that the n-3 and n-6 PUFA

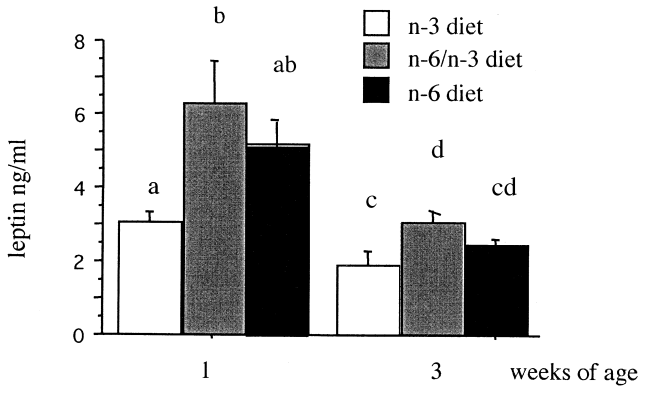

Fig. 1. The effect of the different diets on serum leptin levels in rat pups during the suckling period (each bar represents mean ± SE for 9–10 animals at each time point). Values with unlike letters were significantly different ($P < 0.05$).

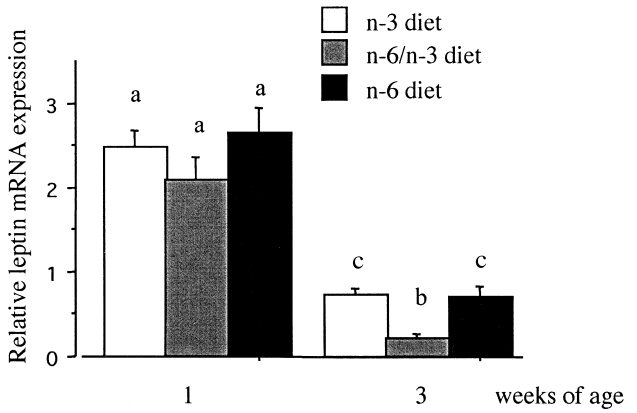

Fig. 2. The effect of the different diets on the relative expression of leptin mRNA in rat inguinal white adipose tissue (WAT) at 3-weeks-of-age (each bar represents mean ± SE for 9–10 animals). The expression level of leptin was normalised to that of 18S RNA. Values with unlike letters were significantly different ($P < 0.05$).

contents in the maternal diet define their levels in the maternal milk (5, 8). Feeding the different diets to rat dams gave the same ratios of n-6 to n-3 FA in the milk as in the diets. Supplementation with 18:3(n-3) lowered the levels of both long-chain PUFA 20:4(n-6) and 22:6(n-3) in the milk. Despite pronounced differences in the concentration of n-3 and n-6 PUFA, the total levels of PUFA in the maternal milk were similar between the diet groups.

At week 1, the serum leptin levels in the suckling pups were significantly higher compared with the levels at 3-weeks-of-age for all diet groups. This early leptin surge, which is independent of fat mass and may signify a developmental leptin insensitivity, has been described during the early postnatal period in rodents (13, 24, 25) and at birth in humans (14, 15, 26, 27). However, the rise in serum leptin levels during the first week was 2-fold less in the n-3 fed pups compared with the n-6/n-3 group. Further increase in the dietary intake of n-6 PUFA did not elevate leptin levels in the n-6 fed pups compared with the n–6/n-3 group, but rather showed a trend of reduction. The magnitude of the leptin surge during the early life might represent a signal for postnatal development and future health. It precedes the establishment of adult levels of several hormones in mice (13) and in humans leptin levels in cord blood predicts rate of weight gain in infancy (17). In *fa/fa* rats, the development of the obese phenotype after weaning is preceded by a postnatal leptin rise that is 2.5-fold higher compared with normal rat pups (28). Furthermore, it was recently shown in mice by cross-fostering experiments that penetrance of complex genetic predisposal for diabetes and obesity in offspring was postnatally regulated by factors in the milk (29). Our data suggest that the dietary ratio of n-6/n-3 PUFA during the suckling period is important to maintaining adequate levels of circulating leptin, influencing leptin sensitivity and could affect weight gain later in life.

During the neonatal development, leptin levels might be determined by endogenous leptin production and/or by leptin derived from maternal milk (16, 24). Since there were no significant differences in milk leptin content between the three diet groups, the lower serum leptin levels in the n-3 fed pups probably reflected a lower production of endogenous leptin rather than contribution by milk leptin. At 3-weeks-of-age, the leptin mRNA expression was lower in the white adipose tissue of the n-6/n-3 pups compared with the other two diet groups. These data suggest that serum leptin levels in the pups at this age are determined by both adipose tissue mass and transcriptional regulation of the leptin gene.

Feeding dams the n-3 diet had several effects on adipose tissue homeostasis in the pups compared with those in both the n-6/n-3 and n-6 groups. The n-3 pups had lower fat pad weight, smaller adipocytes, and a trend toward lower serum triglyceride levels compared with pups from the other diet groups. Both the lower adipose tissue mass and adipocyte size could explain the lower serum leptin levels in the n-3 fed pups (30). Surprisingly, fat pad weight alone or related to body weight at one week of age were highest in the pups from dams on the n-6/n-3 diet. The growth of adipose tissue in the suckling rat is determined by an initial adipocyte hypertrophy and by a later hyperplasia (31). In adult rats, an n-3 PUFA enriched diet is associated with reduced adipocyte size and fat depot mass compared with an n-6 enriched PUFA diet (32, 33). The mechanisms involved are suggested to be suppression of adipocyte differentiation (34) and/or increase in the lipolytic response of the adipocytes (32). As arachidonic acid metabolites have opposite effects on adipogenesis (35), the balance between the n-6 and n-3 PUFA rather than amount of n-6 or n-3 PUFA per se seems to be important for adipose tissue growth.

The FA composition of white adipose tissue quickly adapts to that of the diet in adult rats (32). In the suckling pups, the total lipid FA composition of the inguinal white adipose tissue, which is mainly triglycerides, reflected very well the milk lipid FA composition at 3-weeks-of-age. Differently from the total lipids of adipose tissue, higher levels of long-chain PUFA were incorporated in the adipose tissue phospholipids. It is known that as dietary intake of 18:2(n-6) increases from zero, the tissue concentrations of 20:4(n-6) rise rapidly and then plateau (1). This was observed in the n-6 fed pups where adipose tissue phospholipid levels of 20:4(n-6) did not differ from that detected in n-6/n-3 pups despite the higher ratio of n-6/n-3 PUFA in the former diet. In contrast, the elevated levels of 18:3(n–3) in the milk induced a decrease of the 20:4(n-6) content in adipose phospholipids while 20:5(n–3) levels were increased compared with those in the n-6/n-3 group. Such alterations in phospholipid FA composition can affect membrane functions by modifying membrane fluidity, interactions with membrane proteins, or by altering the balance of synthesized eicosanoids (4). These observed pronounced differences in long-chain PUFA of adipose tissue phospholipids might lead to modification of adipocyte differentiation and to changes in production of active molecules (35).

Postnatal growth in humans and animals has been related to the levels of 20:4(n-6) in plasma phospholipids (6, 7). An increased body weight was observed in the rat pups

of the dams on the n-6/n-3 diet at 1 week of age compared with both the n-6 and n-3 diet groups. The balance between the n-6 and n-3 PUFA rather than the levels of the n-6 PUFA in the maternal diet promoted body growth in the early postnatal period. Human epidemiological data and animals studies evidence that both reduced and elevated body weight at birth or infancy are associated with a risk for development of diseases later in life (36, 37).

In conclusion, we found that the ratio of n-6 to n-3 PUFA in the maternal diet affected the leptin levels in the offspring. The magnitude of leptin levels during the early life might represent a signal for long-term development and predict the risk of disease in later life. Thus, a balanced supply of PUFA during this sensitive period seems to be of importance to attaining the leptin levels sufficient for the normal development that could have an impact on later health of the offspring.

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