

Linoleic and α -linolenic acid as precursor and inhibitor for the synthesis of long-chain polyunsaturated fatty acids in liver and brain of growing pigs

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Studies suggested that in human adults, linoleic acid (LA) inhibits the biosynthesis of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), but their effects in growing subjects are largely unknown. We used growing pigs as a model to investigate whether high LA intake affects the conversion of n-3 LC-PUFA by determining fatty acid composition and mRNA levels of Δ 5- and Δ 6 desaturase and elongase 2 and -5 in liver and brain. In a 2 × 2 factorial arrangement, 32 gilts from eight litters were assigned to one of the four dietary treatments, varying in LA and α -linolenic acid (ALA) intakes. Low ALA and LA intakes were 0.15 and 1.31, and high ALA and LA intakes were 1.48 and 2.65 g/kg BW^{0.75} per day, respectively. LA intake increased arachidonic acid (ARA) in liver. ALA intake increased eicosapentaenoic acid (EPA) concentrations, but decreased docosahexaenoic acid (DHA) (all P < 0.01) in liver. Competition between the n-3 and n-6 LC-PUFA biosynthetic pathways was evidenced by reductions of ARA (>40%) at high ALA intakes. Concentration of EPA (>35%) and DHA (>20%) was decreased by high LA intake (all P < 0.001). Liver mRNA levels of Δ 5- and Δ 6 desaturase were increased by LA, and that of elongase 2 by both ALA and LA intakes. In contrast, brain DHA was virtually unaffected by dietary LA and ALA. Generally, dietary LA inhibited the biosynthesis of n-3 LC-PUFA in liver. ALA strongly affects the conversion of both hepatic n-3 and n-6 LC-PUFA. DHA levels in brain were irresponsive to these diets. Apart from Δ 6 desaturase, elongase 2 may be a rate-limiting enzyme in the formation of DHA.

Keywords: linoleic acid, linolenic acid, liver, brain, enzymes

Implications

Docosahexaenoic acid (DHA; c22:6 n-3) and eicosapentaenoic acid (EPA; c22:5 n-3) are important n-3 fatty acids to improve human health. DHA is important in brain for cognitive development. This study has been carried out with growing pigs as model for humans. The n-3 fatty acid such as α -linolenic acid (ALA; c18:3 n-3) is a precursor for DHA and EPA via enzymatic steps. EPA can be increased in the liver by dietary ALA. Feeding of linoleic acid (LA; c18:2 n-6), a precursor for a competitive pathway, inhibits the synthesis of EPA. The potential for increasing brain DHA via an increase in dietary ALA is limited.

Introduction

Arachidonic acid (ARA; c20:4 n-6), eicosapentaenoic acid (EPA; c20:5 n-3) and docosahexaenoic acid (DHA; c22:6 n-3) are long-chain polyunsaturated fatty acids (LC-PUFA) and

well known to play important regulatory functions in the immune, nervous and cardiovascular system (Innis, 2007; Calder, 2009; Calder and Yaqoob, 2009; Russo, 2009). The n-6 LC-PUFA can be synthesized from linoleic acid (LA; c18:2 n-6) and those of the n-3 family from α -linolenic acid (ALA; c18:3 n-3). Synthesis of EPA and DHA from ALA is achieved by a sequence of desaturation and chain elongation steps, controlled by the enzymes Δ 5- (FADS1) and Δ 6 desaturase (FADS2) and presumably elongase 2 (ELOVL2) and -5 (ELOVL5) (see Figure 1). DHA can be generated from EPA that requires an additional chain-shortening step (i.e. β -oxidation) in the peroxisome (Sprecher, 2000; Igarashi *et al.*, 2007a). The conversion of n-6 LC-PUFA from LA (resulting in the production of ARA) sharing the same enzymes resulting in enzymatic competition between the biosynthetic pathways of n-3 and n-6 LC-PUFA.

The requirement of LC-PUFA for brain growth and development is of current interest. LC-PUFA, in particular DHA and ARA, accumulate rapidly during the brain growth spurt, the most critical stage of brain development that takes place during the later part of gestation and early postnatal life.

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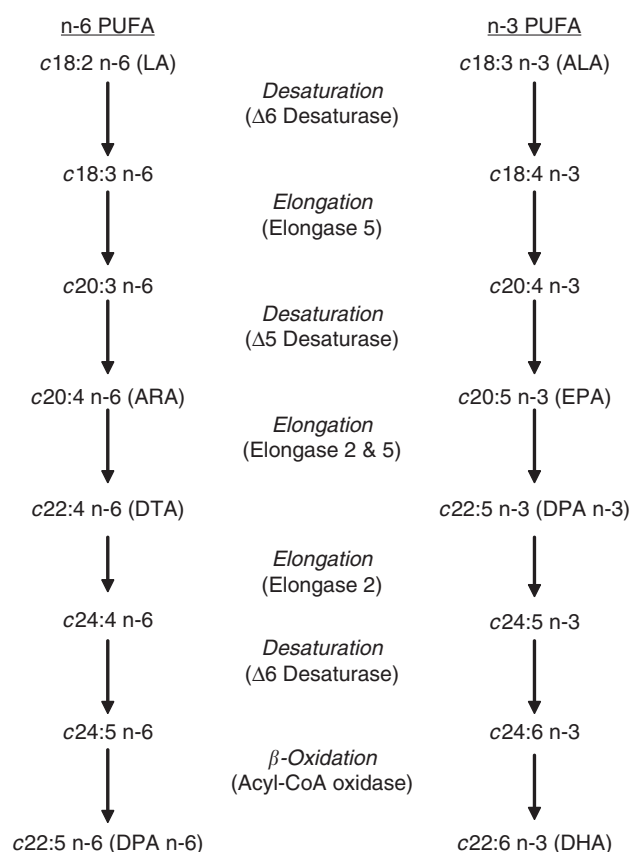


Figure 1 General metabolic pathway of n-6 and n-3 fatty acids (adapted from Igarashi *et al.*, 2007a); LA = linoleic acid; ALA = α -linolenic acid; ARA = arachidonic acid; EPA = eicosapentaenoic acid; DTA = docosatrienoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

ARA is essential for normal brain growth, playing a role in synaptic transmission (Bazan, 2003; Sang and Chen, 2006). Changes in brain DHA concentrations have been demonstrated to be positively correlated with cognitive development (Innis, 2007). In addition, the dietary supplementation of DHA could improve the cognitive dysfunction due to aging (McCann and Ames, 2005; Cole and Frautschy, 2006). The regulatory mechanisms of the conversion of LC-PUFA into ARA, EPA and DHA, and the consequences of LA- and ALA-rich diets consumed during childhood remain to be elucidated. In adult humans, it has been estimated that the β -oxidation of ALA is $\pm 20\%$ to 30% , which is much lower than that of LA, which was estimated to be 12% to 19% (DeLany *et al.*, 2000). The oxidative losses may partly explain why the conversion of ALA to EPA and DHA in adults is low (Burdge and Calder, 2005). In addition, human food sources such as cereals, sunflower and soybean oil are rich in LA ($>50\%$ of the fat) and have been dominant in the Western diet over the last decades. Consumption of LA-rich diets could also result in inadequate conversion of ALA to DHA and reduced concentrations of DHA in the developing brain (Russo, 2009). Dietary studies about the effect of LA are mostly carried out at an isocaloric basis. LA is then replaced by other energy sources such as oleic acid (18:1 n-9). However, it has been reported recently that

oleic acid itself can reduce $\Delta 6$ -desaturase activity (Portolesi *et al.*, 2008).

This study focuses on the interactions between the n-3 and n-6 LC-PUFA biosynthetic pathways in liver and brain using young, growing pigs as a model for human infancy. This animal model has three major advantages over rodents when studying lipid metabolism. First, brain anatomy and morphology and the timing of the brain growth spurt in pigs and humans are similar (Pond *et al.*, 2000; Duhaime, 2006). Second, the anatomy of the digestion system, including liver, stomach and intestine, and also many of the pathways of lipid metabolism in pigs are rather similar to human (Moughan *et al.*, 1991; Innis, 1993). Third, the risk for obesity in young, growing pigs is low, enabling large study contrasts in absolute intakes of LC-PUFA precursors, rather than exchanging for other nutrients to maintain isocaloric intake. Here, we have fed young, growing pigs either low or high amounts of LA and ALA, with equal difference between low and high in the intake within the respective fatty acid, and investigated the effect on the fatty acid composition in liver and brain. In the same tissues, we have determined the mRNA levels of $\Delta 6$ - and $\Delta 5$ desaturase and ELOVL2 and ELOVL5 by quantitative PCR.

Material and methods

Animals and housing

In all, 32 female pigs were selected from eight litters. From each litter, four gilts were allotted to one of the four dietary treatments. On arrival, pigs had a body weight (BW) of 16 kg (s.d. = 1.8). The pigs were housed individually during an experimental period of 4 weeks. They were weekly weighed and feed intake was recorded daily. The experimental protocol was approved by the Animal Experimental Committee of Wageningen University, The Netherlands.

Diets

This study was carried out as a 2×2 factorial design with daily intakes of LA and ALA as independent variables. Differences between low and high intakes were designed to be identical for LA and ALA: low ALA and LA intakes were 0.16 and 1.32, and high ALA and LA intakes were 1.48 and 2.65 g/kg BW^{0.75} per day, respectively. The dietary energy percentage from LA was 3.4, 3.3, 6.6 and 6.4 and those of ALA was 0.4, 3.7, 0.4 and 3.5 for the diets low LA/low ALA, low LA/high ALA, high LA/low ALA and high LA/high ALA, respectively. The size of the experimental contrasts was, in part, based on recent studies with humans showing an effect of dietary LA on EPA in plasma phospholipids (Goyens *et al.*, 2006). In addition, the low LA and ALA intakes were slightly above minimal requirement figures for pigs (National Research Council, 1998; Schellingerhout, 2002). The addition of LA and ALA was on top of a basal diet. The intakes of fatty acids are presented in Table 1. With the exception of LA and ALA, the intakes of SFA (saturated fatty acids) and MUFA (monounsaturated fatty acids) were kept constant. This was done by optimizing the dosages of analyzed sunflower oil, linseed oil, high-oleic acid

Table 1 Experimental design: average intakes of DE, starch, ileal digestible lysine, fat and fatty acids¹ in g/kg BW^{0.75} per day

	Low LA		High LA	
	Low ALA	High ALA	Low ALA	High ALA
c18:2 n-6 (LA)	1.32	1.31	2.64	2.67
c18:3 n-3 (ALA)	0.15	1.49	0.16	1.47
c12:0	0.03	0.03	0.03	0.03
c14:0	0.02	0.02	0.02	0.02
c16:0	0.69	0.66	0.64	0.60
c16:1	0.01	0.01	0.01	0.01
c18:0	0.13	0.17	0.17	0.22
c18:1 n-9	1.34	1.32	1.31	1.31
c18:1 n-7	0.03	0.04	0.04	0.05
c20:0	0.01	0.01	0.02	0.02
c20:1 n-9	0.01	0.01	0.01	0.01
c22:0	0.02	0.01	0.03	0.03
c24:0	0.01	0.01	0.01	0.01
SFA	0.90	0.91	0.92	0.92
MUFA	1.39	1.38	1.37	1.38
Total fat	4.00	5.42	5.42	6.85
DE (MJ/kg BW ^{0.75} per day) ²	1.24	1.30	1.30	1.35
Ileal digestible lysine ²	0.89	0.89	0.89	0.89
Starch ²	34.7	34.7	34.7	34.7

DE = digestible energy; LA = linoleic acid; ALA = α -linolenic acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid.

¹Based on analyzed values.

²Based on calculated values (CVB Table Pigs, 2007).

sunflower oil and palm oil. The diets did not contain n-3 or n-6 LC-PUFA other than LA and ALA. The composition of the basal diet and the inclusion rates of the oils in the treatments are presented in Table 2. The low LA/low ALA diet was formulated to contain sufficient ileal digestible amino acids in relation to energy (CVB Table Pigs, 2007).

As the LA and ALA increments were not exchanged for other nutrients but dosed on top of the basal diet, digestible energy intake varied among experimental treatments between 2.6 and 2.8 \times maintenance energy requirement. The diets were fed twice daily as mash. To allow pigs to express at least some natural foraging behavior, straw (20 g/day) was provided daily, which has been demonstrated to alleviate the stress of individual housing (De Jong *et al.*, 1998; Chaloupková *et al.*, 2007). Water was provided *ad libitum*.

Sampling and fatty acid analysis

At the start of the experimental period, blood sample was taken from each pig at 2.5 h after feeding. At the end of the experimental period, pigs were anesthetized 2.5 h after feeding with an administration of ketamine (10 mg/kg BW) and xylazine (1 mg/kg BW). Blood sample was collected using cardiac puncture before euthanasia was performed by an intracardiac administration of pentobarbital (100 mg/kg BW). Brain and liver were immediately removed after euthanasia. The total brain, frontal cortex and hippocampus were weighed. For gene expression measurements, samples (~1 mg) of liver

Table 2 Dietary ingredients (in g/kg, relative to the low LA/low ALA diet) of the dietary treatments with differences in LA and ALA intakes

	Low LA		High LA	
	Low ALA	High ALA	Low ALA	High ALA
Basal diet ¹	967.4	967.4	967.4	967.4
Sunflower oil	10.5	3.36	40.5	33.6
Linseed oil	1.92	34.1	1.85	34.2
HOSF oil	10.3	6.61	3.76	–
Palm oil	9.89	6.07	4.05	–
Total ²	1000	1018	1018	1035

LA = linoleic acid; ALA = α -linolenic acid; HOSF = high-oleic sunflower oil.

¹The basal diet contained (g/kg, as fed) corn starch, 160.1; wheat, 160.5; barley, 321.0; wheat bran, 53.5; molasses, 32.1; soybean meal, 64.2; peas, 53.5; potato protein, 53.5; wheat gluten, 53.5; limestone, 14.1; monocalcium phosphate, 13.7; salt, 5.4; vitamin-mineral mix, 10.7; L-lysine HCl 3.0; L-Threonine, 0.32; tryptophan, 0.32; DL-methionine, 0.54.

²The sum of feed ingredients adds up to 1000 g/kg for the low LA/low ALA diet only. A total exceeding 1000 reflects an increased feed intake of the respective treatment group as explained in the text.

and hippocampus were rapidly frozen in liquid nitrogen and stored at -80°C until analysis. Other parts of the collected tissues were stored at -20°C pending fatty acid analysis. Total lipids were extracted from diets, liver, frontal cortex and hippocampus with a chloroform:methanol (2:1, v/v) Folch mixture and then saponified and methylated to determine fatty acid composition by gas chromatography as described previously (Smink *et al.*, 2008).

Quantitative PCR

Total RNA was isolated from the hippocampus and liver tissue using TRIzol reagent (Invitrogen, Bleiswijk, The Netherlands). Reverse transcription of 1 μg of total RNA was performed in a 20- μl reaction using Superscript III reverse transcriptase (Invitrogen), dNTPs (Roche) and random hexamer primers (Roche, Almere, The Netherlands) for 1 h at 50°C according to the manufacturer's protocol (Invitrogen). Real-time PCR was performed on a LightCycler 2.0 Real-Time PCR System by using FastStart DNA Master SYBR Green I reagents (Roche). The primers used are presented in Table 3. All primer pairs, except for 18S RNA, were designed in such a way that they span an intron of their corresponding genomic sequence or that its sense or reverse primer anneals on an exon-intron junction. Templates were amplified after a preincubation for 10 min at 95°C , followed by amplification for 40 cycles (10 s at 95°C , 5 s at 60°C , 5 s at 72°C). PCR efficiencies for the genes were established to be between 97% and 100%. Expression levels of FADS1, FADS2, ELOVL2 and ELOVL5 were normalized using the corresponding values of 18S RNA. All reactions revealed a single product as determined using melting curve analysis and specificity of the primer sets were verified by sequencing of the generated amplicons.

Statistical analysis

The trial was conducted as a 2×2 factorial arrangement of treatments with eight replicates per treatment. The individually

Table 3 Quantitative PCR primers

Gene	Forward primer	Reverse primer
$\Delta 5$ desaturase	CAGGATGCTACGGATCCCTTT	GCTGCTCCGGAGACAGTTCT
$\Delta 6$ desaturase	CAGCACGATTACGGCCATCT	AGTTGGCAGAGGCACCCTTT
ELOVL2	TGACGCTGGTCATCCTGTTC	CGGCACGTCCGTATCTTTCT
ELOVL5	CCTCTCGGCTGGCTGTACTT	GAGAGGCCCTTCTTGTG
18S RNA	GTTCAAAGCAGGCCCGAG	CGCCGCCGATCGCCA

Table 4 BW at the start and at the end of the trial and the effect of LA and ALA intakes on brain weight in growing pigs

	Low LA		High LA		Pooled s.e.m.	P-values for effect		
	Low ALA	High ALA	Low ALA	High ALA		LA	ALA	LA \times ALA
BW start (kg)	16.3	16.4	16.1	16.4	0.68	0.85	0.75	0.85
BW end (kg)	28.5	28.8	29.3	29.7	0.63	0.20	0.61	0.96
Brain (g/BW end)	2.14	2.13	2.00	1.99	0.051	0.012	0.78	0.97
Frontal cortex (g/BW end)	0.48	0.48	0.46	0.44	0.019	0.13	0.56	0.67
Hippocampus (g/BW end)	0.076	0.073	0.076	0.070	0.002	0.37	0.046	0.63

BW = body weight; LA = linoleic acid; ALA = α -linolenic acid.

housed pig served as the experimental unit. The results were analyzed by ANOVA using the software program SAS version 9.1 (Statistical Analysis Systems Institute Inc., Cary, NC, USA). The fatty acid concentrations in all tissues and enzyme RNA concentrations were tested using the following model:

$$Y = \mu + LA_i + ALA_j + (LA \times ALA)_{ij} + \text{litter}_k + e_{ijk},$$

where LA = daily LA intake i (i = low or high); ALA = daily ALA intake j (j = low or high); litter k (k = 1, ..., 8).

For fatty acid concentrations in blood plasma, the initial concentrations, measured at the start of the experiment were included as a covariate.

Homogeneity of variance was tested using the Shapiro–Wilk test. When model residuals were not distributed normally ($P < 0.05$), the statistical analysis was performed on transformed data. The level of statistical difference was preset at $P < 0.05$.

Results

All pigs remained healthy throughout the study and rates of BW gain were within the normal range. The effects of LA and ALA intakes on BW and brain weight are presented in Table 4. The average BW at slaughter was 29 kg and did not significantly differ among treatments. Fresh brain weights (g/kg BW) were significantly ($P = 0.012$) lower for the pigs fed the high LA diet. The hippocampus weight was lower ($P = 0.046$) for the high ALA groups.

The effects of LA and ALA intakes on the content of liver fatty acids are presented in Table 5. The results of the dietary treatments on the composition of plasma fatty acids largely resembled the effects on liver fatty acid composition (data not shown). Nearly all fatty acid contents were affected

by LA and ALA intakes. High LA pigs had a significant ($P < 0.0001$) higher content of LA and ARA in liver. The content of n-3 fatty acids such as ALA, docosapentaenoic acid (DPA; $c22:5$ n-3) and DHA were decreased ($P < 0.01$) in the high LA pigs. A high ALA intake resulted in an increased ($P < 0.0001$) ALA and EPA contents in liver. The proportion of DHA, however, was decreased ($P = 0.01$) by high intake of ALA. High ALA pigs had increased content of LA in the liver but ARA and docosatetraenoic acid (DTA; $c22:4$ n-6) contents decreased ($P < 0.0001$) by approximately 50%.

The fatty acid profiles of the frontal cortex and the hippocampus are presented in Tables 6 and 7, respectively. LA and ALA were hardly present in both brain tissues. ARA (9%), $c22:4$ n-6 (3% to 4%) and DHA (7% to 8%) were the main PUFA in the two brain tissues. High LA intake tended ($P < 0.1$) to increase the ARA content and significantly ($P < 0.05$) increased the proportion of $c22:4$ n-6 in both the hippocampus and frontal cortex. High LA intake resulted in a significant ($P < 0.001$) decrease of DPA and a tendency ($P = 0.09$) of a decreased DHA content in the frontal cortex. Increased ALA intake did not affect the ARA and $c22:4$ n-6 contents but lowered $c22:5$ n-6 in the frontal cortex. High ALA increased DPA, but did not affect DHA contents in the frontal cortex significantly. High LA intake resulted in a decreased content of DPA but did not affect that of DHA in the hippocampus. A high ALA intake significantly decreased all n-6 LC-PUFA, but increased the LA content in the hippocampus. High ALA intake increased the DPA content, but did not affect other n-3 fatty acids.

The change in mRNA expression levels of desaturase and elongase enzymes were measured in liver and hippocampus (Table 8). In liver, both LA and ALA intakes significantly ($P < 0.05$) increased the transcript level of ELOVL2. On the contrary, ELOVL5 mRNA was not significantly increased.

Table 5 Effect of LA and ALA intakes on the fatty acid profile (% of total fatty acids) of the liver in growing pigs

Fatty acids	Low LA		High LA		Pooled s.e.m.	P-values of effects		
	Low ALA	High ALA	Low ALA	High ALA		LA	ALA	LA × ALA
c16:0	15.1	11.7	13.0	10.5	0.33	<0.0001	<0.0001	0.18
c17:0	1.31	1.17	1.29	1.03	0.040	0.49	0.07	0.55
c18:0	23.5	26.7	24.6	24.0	0.43	0.08	<0.01	<0.0001
SFA	40.3	39.6	38.9	35.6	0.40	<0.0001	<0.001	<0.01
c16:1	0.53	0.43	0.38	0.26	0.040	<0.001	0.013	0.75
c18:1 n-7	1.48	1.26	1.11	1.20	0.043	<0.001	0.17	<0.01
c18:1 n-9	13.5	10.8	10.7	9.51	0.31	<0.01	<0.001	0.79
MUFA	15.7	12.5	12.2	11.1	0.36	<0.0001	<0.0001	<0.01
c18:2 n-6 (LA)	16.4	16.8	21.2	24.3	0.33	<0.0001	<0.0001	<0.001
c20:3 n-6	0.81	0.77	0.68	0.80	0.047	0.28	0.38	0.10
c20:4 n-6 (ARA)	17.4	8.86	19.3	11.1	0.33	<0.0001	<0.0001	0.57
c22:4 n-6 (DTA)	0.73	0.13	1.02	0.19	0.036	<0.0001	<0.0001	<0.01
PUFA n-6	35.4	26.6	42.3	36.4	0.36	<0.0001	<0.0001	<0.001
LC n-6	19.0	9.77	21.0	12.1	0.32	<0.0001	<0.0001	0.62
c18:3 n-3 (ALA) ¹	0.48	5.09	0.44	4.18	0.030	<0.01	<0.0001	<0.05
c20:3 n-3	ND	0.87	ND	0.98	0.066	–	–	–
c20:5 n-3 (EPA)	0.58	8.13	0.28	5.28	0.155	<0.0001	<0.0001	<0.0001
c22:5 n-3 (DPA n-3)	2.42	3.23	1.67	2.66	0.079	<0.0001	<0.0001	0.26
c22:6 n-3 (DHA)	2.63	2.33	2.10	1.71	0.123	0.0001	0.010	0.07
PUFA n-3	6.16	19.7	4.50	14.8	0.25	<0.0001	<0.0001	<0.0001
LC n-3	5.67	14.6	4.06	10.6	0.181	<0.0001	<0.0001	<0.0001
c16:1/c16:0	0.035	0.037	0.029	0.024	0.0028	<0.01	0.56	0.24
c18:1 n-9/c18:0	0.58	0.40	0.44	0.40	0.019	<0.001	<0.001	<0.01

LA = linoleic acid; ALA = α -linolenic acid; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; ARA = arachidonic acid; DTA = docosatetraenic acid; PUFA = polyunsaturated fatty acids; LC = long chain (fatty acids with 20 or 22 C-atoms); EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

¹P-value and s.e.m. are obtained from $Y = \log(1 + x)$ function.

Table 6 Effect of LA and ALA intakes on the fatty acid profile (% of total fatty acids) of the frontal cortex in growing pigs

Fatty acids	Low LA		High LA		Pooled s.e.m.	P-values of effects		
	Low ALA	High ALA	Low ALA	High ALA		LA	ALA	LA × ALA
c16:0	20.0	19.4	19.2	19.3	0.234	0.06	0.30	0.20
c18:0	18.2	17.9	17.8	17.8	0.184	0.24	0.58	0.32
SFA	39.4	38.5	38.2	38.3	0.406	0.13	0.33	0.25
c16:1	0.89	0.94	0.90	0.92	0.018	0.76	0.07	0.26
c17:1	1.48	1.60	1.72	1.59	0.099	0.27	0.93	0.23
c18:1 n-7	4.91	4.93	5.03	5.02	0.097	0.30	0.97	0.23
c18:1 n-9	16.8	17.4	17.7	17.1	0.482	0.56	0.96	0.18
MUFA	24.9	25.8	26.3	25.4	0.733	0.48	0.97	0.89
c18:2 n-6 (LA)	0.69	0.80	0.79	0.90	0.030	<0.01	<0.01	0.91
c20:4 n-6 (ARA)	8.85	8.40	8.92	9.28	0.237	0.06	0.85	0.10
c22:4 n-6 (DTA)	3.30	3.04	3.39	3.36	0.086	0.027	0.10	0.21
c22:5 n-6 (DPA n-6)	1.18	0.59	1.02	0.61	0.094	0.46	<0.0001	0.34
PUFA n-6	14.5	13.4	14.6	14.7	0.330	0.015	0.17	0.08
LC n-6	13.8	12.6	13.8	13.8	0.330	0.07	0.09	0.08
c18:3 n-3 (ALA)	0.62	0.68	0.72	0.63	0.066	0.70	0.72	0.10
c22:5 n-3 (DPA n-3)	0.24	0.56	0.21	0.46	0.015	<0.001	<0.0001	<0.05
c22:6 n-3 (DHA)	7.77	8.14	6.81	7.69	0.393	0.09	0.13	0.52
PUFA n-3	8.56	9.39	7.68	8.74	0.356	0.044	0.015	0.74
LC n-3	8.16	8.91	7.20	8.30	0.388	0.06	0.027	0.65

LA = linoleic acid; ALA = α -linolenic acid; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; ARA = arachidonic acid; DTA = docosatetraenic acid; DPA = docosapentaenoic acid; PUFA = polyunsaturated fatty acids; LC = long chain (fatty acids with 20 or 22 C-atoms); DHA = docosahexaenoic acid.

Table 7 Effect of LA and ALA intakes on the fatty acid profile (% of total fatty acids) of the hippocampus in growing pigs

Fatty acids	Low LA		High LA		Pooled s.e.m.	P-values of effects		
	Low ALA	High ALA	Low ALA	High ALA		LA	ALA	LA × ALA
c14:0	0.46	0.44	0.45	0.43	0.0059	0.13	<0.05	0.90
c16:0	17.6	17.5	18.3	17.8	0.36	0.17	0.47	0.61
c17:0	0.21	0.21	0.21	0.20	0.0040	0.35	0.12	0.17
c18:0	18.5	18.4	18.6	18.6	0.14	0.42	0.60	0.73
SFA ¹	37.6	37.2	38.4	37.7	0.47	0.20	0.27	0.80
c16:1	0.83	0.86	0.80	0.52	0.011	<0.01	0.015	0.50
c17:1	1.15	1.28	1.06	1.17	0.073	0.17	0.12	0.91
c18:1 n-7	4.93	4.80	4.72	4.80	0.047	0.031	0.57	0.041
c18:1 n-9	18.5	19.0	17.6	18.3	0.54	0.15	0.29	0.80
c20:1 n-9	0.74	0.76	0.62	0.68	0.050	0.07	0.45	0.63
MUFA	26.5	27.0	25.0	26.0	0.72	0.12	0.31	0.74
c18:2 n-6 (LA)	0.53	0.70	0.56	0.94	0.10	0.19	0.010	0.29
c20:4 n-6 (ARA)	8.90	8.33	9.35	8.81	0.24	0.07	0.033	0.96
c22:4 n-6 (DTA)	4.18	3.87	4.42	4.12	0.098	0.018	<0.01	0.96
c22:5 n-6 (DPA n-6)	0.70	0.43	0.88	0.50	0.034	0.03	<0.0001	0.14
PUFA n-6	14.1	13.5	14.8	14.5	0.37	0.02	0.06	0.81
LC n-6	13.6	12.8	14.2	13.5	0.32	0.03	0.01	0.97
c18:3 n-3 (ALA) ¹	0.45	0.48	0.38	0.43	0.194	0.56	0.29	0.06
c20:3 n-3	0.04	0.04	0.03	0.06	0.027	0.86	0.59	0.82
c20:5 n-3 (EPA)	0.07	0.06	0.06	0.06	0.0051	0.13	0.25	0.67
c22:5 n-3 (DPA n-3)	0.29	0.67	0.26	0.54	0.011	<0.0001	<0.0001	<0.001
c22:6 n-3 (DHA)	6.38	6.65	6.96	6.93	0.31	0.18	0.71	0.63
PUFA n-3	7.23	7.90	7.69	8.02	0.29	0.32	0.10	0.55
LC n-3	6.78	7.42	7.31	7.58	0.31	0.29	0.17	0.56

LA = linoleic acid; ALA = α -linolenic acid; MUFA = monounsaturated fatty acids; ARA = arachidonic acid; DTA = docosatetraenic acid; DPA = docosapentaenoic acid; PUFA = polyunsaturated fatty acids; LC = long chain (fatty acids with 20 or 22 C-atoms); EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.

¹P-value and s.e.m. are obtained from an inverse function.

Table 8 Effects of LA and ALA intakes on the mRNAs of D5- and D6 desaturases and elongase 2 and -5 in liver and hippocampus of growing pigs

	Low LA		High LA		Pooled s.e.m.	P-values for effect		
	Low ALA	High ALA	Low ALA	High ALA		LA	ALA	LA × ALA
Liver								
ELOVL2	1.00	2.44	2.40	3.53	0.412	0.044	0.037	0.24
ELOVL5	1.00	1.13	1.20	1.24	0.108	0.11	0.27	0.92
Δ 5 desaturase	1.00	1.08	1.60	1.78	0.219	<0.01	0.57	0.81
Δ 6 desaturase	1.00	1.15	1.54	1.71	0.158	<0.01	0.33	0.96
Hippocampus								
ELOVL2	1.00	2.81	1.80	2.14	0.421	0.89	0.022	0.09
ELOVL5	1.00	1.59	1.02	1.22	0.248	0.50	0.13	0.43
Δ 5 desaturase	1.00	1.60	1.14	1.05	0.393	0.58	0.51	0.36
Δ 6 desaturase	1.00	1.73	1.00	1.14	0.264	0.27	0.12	0.27

LA = linoleic acid; ALA = α -linolenic acid.

mRNA levels were quantified by quantitative PCR and normalized to 18S ribosomal RNA. They are expressed relative to the Low LA/Low ALA diet group (1.00).

In addition, both hepatic transcript levels of Δ 5- and Δ 6 desaturase were increased following high LA intake ($P < 0.01$). In hippocampus, high ALA intake significantly ($P = 0.022$) increased the mRNA expression of ELOVL2, but not of ELOVL5, Δ 6- and Δ 5 desaturase. In contrast, variation in LA intake had no effect on the mRNA expression of these four genes.

Discussion

The formulated diets contained LA and ALA and were free of other n-3 or n-6 LC-PUFA. Once consumed, LA and ALA can be converted to other LC-PUFA by desaturation and elongation. The route and assumed enzymes involved in the biosynthesis of LC-PUFA are given in Figure 1.

In this study, we supplied LA or ALA maintaining identical intakes of other nutrients, an approach that allows independent evaluation of the effects of LA and ALA in liver and brain. Importantly, we did not observe a difference in BW of the gilts among the experimental groups.

Liver

A high LA intake increased the content of LA and n-6 LC-PUFA such as ARA in liver lipid. This agrees well with other studies in pigs (Romans *et al.*, 1995; Schellingerhout, 2002). Increasing the ALA intake by 1.3 g/kg BW^{0.75} per day increased the EPA content in liver lipid from less than 1% to 8%. In contrast to other n-3 LC-PUFA, the DHA content was decreased by high ALA intake. Interestingly, a similar effect was seen on the content of ALA, EPA and DHA in plasma phospholipids in humans (Goyens *et al.*, 2006), indicating that the composition of these plasma lipids is a reflection of the lipid metabolism of the liver. Other earlier studies showed that plasma DHA level was not affected or marginally increased in humans by dietary ALA (Blank *et al.*, 2002; Burdge and Calder, 2005). In contrast to our study, these authors compensated the higher intake of ALA with a decrease in the intake of other fatty acids. We believe that caution should be taken in choosing this exchange approach, as there is evidence that other lipids such as LA, oleic acid and myristic acid (c14:0) can alter hepatic desaturase activity as well (Bézard *et al.*, 1994; Jan *et al.*, 2004; Portolesi *et al.*, 2008; Rioux *et al.*, 2008). These fatty acids may contribute to the observed opposite effects on plasma DHA in their studies.

This study shows that high LA intake increased levels of both $\Delta 5$ - and $\Delta 6$ -desaturase mRNA in liver. Similar effects have been found in rats (Bézard *et al.*, 1994) and piglets (Theil and Lauridsen, 2007). In contrast, in a human hepatoma cell line a suppression of $\Delta 6$ -desaturase mRNA content was seen after the addition of LA or ALA (Portolesi *et al.*, 2008). Increased ALA intake had no effect on $\Delta 5$ - and $\Delta 6$ desaturase. The rate-limiting enzyme $\Delta 6$ desaturase has been reported to have a higher affinity for ALA than for LA (Rodriguez *et al.*, 1998; Sprecher, 2002). Although not measured in our model, it is tempting to speculate that the surfeit of ALA in liver is preferentially converted above LA down the cascade (Figure 1) or alternatively catabolized (via β -oxidation) and that the LA-elevated expression of $\Delta 6$ (and $\Delta 5$)-desaturase may be necessary to metabolize the surplus of LA provided by the diet. Indeed, we found a strong increase in the EPA content in liver lipid in response to ALA intake, suggesting that the enzymatic activity of both desaturases and ELOVL5, at least for ALA, were not rate limiting.

In our study, high LA and high ALA intakes increased the transcript of ELOVL2 but not of ELOVL5 in the liver of growing piglets. These findings indicate that the conversion of ARA and EPA to longer elongation products became more efficient (see Figure 1). Indeed, we found an increased proportion of DPA and DTA in liver lipid in response to ALA and LA intakes, respectively. Unfortunately, we did not have the standards in order to establish whether this was paralleled by a positive effect on the content of c24:5 n-3 and c24:4 n-6

as well. Fatty acid-dependent changes in hepatic ELOVL2 expression have also been reported in rat and salmon (Igarashi *et al.*, 2007a; Morais *et al.*, 2009).

Interestingly, DHA levels were significantly reduced, whereas ELOVL2 mRNA expression was increased in response to ALA intake. This finding suggests a rate-limiting enzyme downstream of ELOVL2. As $\Delta 6$ desaturase has a higher affinity for ALA than for c24:5 n-3 (D'Andrea *et al.*, 2002; Portolesi *et al.*, 2007), it is conceivable that $\Delta 6$ desaturation of c24:5 n-3 is inhibited when ALA is abundant, preventing DHA synthesis. We would suggest that in the liver of piglets, $\Delta 6$ desaturase rather than ELOVL2 is the rate-limiting enzyme downstream of EPA in the biosynthesis of DHA. Whether ALA has any inhibitory effect on the peroxisomal β -oxidation of c24:6 n-3 remains to be elucidated.

Increased intake of LA decreased the proportion of total n-3 LC-PUFA and a high intake of ALA decreased the total n-6 LC-PUFA in liver lipid. This confirms the general concept that competition between ALA and LA occurs because of sharing of the same desaturation and elongation enzymes. In addition, this study illustrates that for EPA and DHA, the effects of LA intake are stronger at high ALA intake and the effects of ALA intake are stronger at low LA intake (interaction LA \times ALA for EPA, DHA, $P < 0.001$ and $P = 0.07$, respectively). The first concern is the effect of high LA as the Western diet is generally rich in LA and poor in n-3 fatty acids. High LA intake in our study decreased the proportion of EPA and DHA in liver fat by $>35\%$ and $>20\%$, respectively. A similar effect was also observed in blood plasma fat. On the other hand, high ALA intake drastically decreased the proportion of ARA by approximately 50%. This inhibitory effect provides a strong indication that ALA and LA competes for the same desaturases and elongases.

Taken together, our experimental design allows a direct comparison of the effects of identical increments in daily intakes of LA and ALA (both 1.3 g/kg BW^{0.75}), which is rarely found in literature. The effect of LA both as a substrate of the n-6 chain and as an inhibitor of the n-3 chain was much lower in comparison with that of ALA. Generally, the effect of ALA on LC PUFA appeared to be four times stronger than that of LA. Our findings in growing piglets are in agreement with human intervention studies showing a strong positive correlation of ALA intake and blood EPA, but weaker between LA intake and blood ARA (Mantzioris *et al.*, 1995).

Brain

The timing of the pig brain growth spurt is similar to that in humans (Dobbing and Sands, 1979; Pond *et al.*, 2000). Several trials with young piglets were conducted to study the effect of dietary fat on brain fatty acid composition (Arbuckle *et al.*, 1991; De la Presa-Owens *et al.*, 1998; Arbuckle and Innis, 2003). These nutritional intervention studies were mainly focused on whole brain. In this study, we selected the frontal cortex, representing a rather large region of the brain, with primary functions (Ng and Innis, 2003), and in addition, the hippocampus, which is involved in the memory storage and retrieval. Although it has been reported that deprivation

of essential fatty acids causes a decrease in brain weight (Odutuga, 1981), we found to our surprise a decreased brain weight in response to increased LA intake, irrespective of ALA intake. This is in contrast with the study by Hrboticky *et al.* (1990), showing that higher LA at the expense of oleic acid did not affect brain weight in young piglets. In our study, an increased LA intake tended ($P < 0.1$) to increase ARA and significantly increased $c22:4$ n-6 in fat extracts of both hippocampus and frontal cortex. Moreover, high ALA intake increased DPA, but not DHA concentrations in the fat of those brain tissues. Earlier studies in piglets demonstrated an increase in brain DHA by dietary ALA (Arbuckle *et al.*, 1991). The difference in the effect on DHA between the latter and our study may be due to their use of younger piglets, knowing that younger piglets respond stronger to dietary PUFA (Cheon *et al.*, 2000). However, the lack of the effect on brain DHA was also found in adult rats in which ALA intake exceeded the low ALA groups in our study (Bourre *et al.*, 1993). The elongation of n-3 fatty acids in brain astrocytes did not extend beyond DPA (Innis and Dyer, 2002). Although high ALA intake drastically increased the ALA content in plasma lipids (an increase of the proportion from $\pm 1\%$ to 10%), which is a reflection of the increase of ALA content in liver (Table 4), the ALA and DHA contents in frontal cortex and hippocampus did not increase (Tables 5 and 6). Other studies in rat brain showed that ALA after uptake from the circulation across the blood-brain barrier will be mainly oxidized and only a small fraction is converted to DHA (DeMar *et al.*, 2005; Igarashi *et al.*, 2007b). The activity of desaturation and elongation are higher in liver in comparison with brain (Igarashi *et al.*, 2007a). This suggests that most DHA in the brain is derived from another source, such as liver, intestine (diet) and/or adipose tissue. Surprisingly, the DHA concentration in the pig liver lipid decreased with increasing ALA intake. High ALA intake resulted in a significant increase in ELOVL2 mRNA expression, which may relate to the strong increase in the content of DPA, a product of ELOVL2, in both frontal cortex and hippocampus. On the other hand, we did not observe an effect on mRNA expression of $\Delta 6$ and $\Delta 5$ desaturase and ELOVL5 in the hippocampus that agrees with studies in rat brain (Igarashi *et al.*, 2007a).

Similarly to liver, high dietary ALA intake increased the concentration of LA in fat extracts of both frontal cortex and hippocampus. The proportion of ALA, however, was not increased. The latter may be due to a high rate of oxidation in brain (DeMar *et al.*, 2005), or elongation and desaturation, resulting in increased concentrations of n-3 LC PUFA in the frontal cortex. The higher LA concentration in high ALA diets might be due to competition for enzymes and sparing of LA oxidation in the presence of ALA. The rate of oxidation of ALA is higher than that of LA (DeLany *et al.*, 2000).

In conclusion, the effect of LA and ALA are important both as precursor and inhibitor for the synthesis of LC-PUFA. LA as a substrate is increasing mRNA expression of $\Delta 6$ - and $\Delta 5$ desaturase and all n-6 PUFA in liver. High ALA intake did increase EPA in liver lipid but decreased the proportion

of DHA. This study supports the idea that high dietary LA intake inhibits the conversion of ALA into n-3 LC-PUFA and that ALA inhibits the conversion of LA into n-6 LC-PUFA. The magnitude of the effect of ALA in liver is higher than that of LA. This suggests that manipulation of ARA and EPA availability by dietary interventions should be optimized varying both dietary LA and ALA. DHA in brain tissue is hardly affected by both dietary LA and ALA. mRNA expression of ELOVL2 was upregulated by dietary treatment and was, apart from $\Delta 6$ desaturase, identified as a potentially rate-limiting step.

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