Aging decreases rate of docosahexaenoic acid synthesis-secretion from circulating unesterified α -linolenic acid by rat liver

Fei Gao · Ameer Y. Taha · Kaizong Ma ·
Lisa Chang · Dale Kieseewetter ·
Stanley I. Rapoport

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Abstract Docosahexaenoic acid (DHA, 22:6n-3), an n-3 polyunsaturated fatty acid (PUFA) found at high concentrations in brain and retina and critical to their function, can be obtained from fish products or be synthesized from circulating α -linolenic acid (α -LNA, 18:3n-3) mainly in the liver. With aging, liver synthetic enzymes are reported reduced or unchanged in the rat. To test whether liver synthesis-secretion of DHA from α -LNA changes with age, we measured whole-body DHA conversion coefficients and rates in unanesthetized adult male Fischer-344 rats aged 10, 20, or 30 months, fed an eicosapentaenoic acid (EPA, 20:5n-3)- and DHA-containing diet. Unesterified [U - ^{13} C] α -LNA bound to albumin was infused intravenously for 2 h, while [^{13} C]-esterified n-3 PUFAs were measured in arterial plasma, as were unlabeled unesterified and esterified PUFA

concentrations. Plasma unesterified n-3 PUFA concentrations declined with age, but esterified n-3 PUFA concentrations did not change significantly. Calculated conversion coefficients were not changed significantly with age, whereas synthesis-secretion rates (product of conversion coefficient and unesterified plasma α -LNA concentration) of esterified DHA and n-3 DPA were reduced. Turnovers of esterified n-3 PUFAs in plasma decreased with age, whereas half-lives increased. The results suggest that hepatic capacity to synthesize DHA and other n-3 PUFAs from circulating α -LNA is maintained with age in the rat, but that reduced plasma α -LNA availability reduces net synthesis-secretion. As unesterified plasma DHA is the form that is incorporated preferentially into brain phospholipid, its reduced synthesis may be deleterious to brain function in aged rats.

F. Gao · A. Y. Taha · K. Ma · L. Chang · S. I. Rapoport
Brain Physiology and Metabolism Section, National
Institute on Aging, National Institutes of Health,
Bethesda, MD 20892, USA

D. Kieseewetter
Laboratory of Molecular Imaging and Nanomedicine,
National Institute of Biomedical Imaging and
Bioengineering, National Institutes of Health,
Bethesda, MD 20892, USA

F. Gao (✉)
Center for Experimental Therapeutics and Reperfusion
Injury, Brigham and Women's Hospital,
Harvard Medical School,
77 Avenue Louis Pasteur,
Boston, MA 02115, USA
e-mail: fgao1@partners.org

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Abbreviations

α -LNA	α -Linolenic acid
AA	Arachidonic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
LA	Linoleic acid
PFB	Pentafluorobenzyl
PUFA	Polyunsaturated fatty acid

Introduction

Docosahexaenoic acid (22:6n-3, DHA), a long-chain n-3 polyunsaturated fatty acid (PUFA), is enriched in brain and retina phospholipids, where it modulates membrane fluidity, regulates functions of membrane-associated proteins (Stubbs and Smith 1984; Salem et al. 2001; Kim 2007), and participates in calcium-independent phospholipase A₂ mediated neurotransmission (Strokin et al. 2003). DHA may be obtained directly from dietary sources, such as fish or fish products, or it can be synthesized from its nutritionally essential plant-derived precursor, α -linolenic acid (18:3n-3, α -LNA), through a series of elongation and desaturation steps (Sprecher 2000). The major site of DHA synthesis is the liver, whereas brain or heart synthesis is much less (Scott and Bazan 1989; Demar et al. 2005; Igarashi et al. 2007; Igarashi et al. 2008; Rapoport et al. 2010).

The brain obtains DHA directly from the plasma unesterified fatty acid pool, which is maintained in part by hydrolysis of circulating esterified fatty acids synthesized and secreted by the liver, and by adipose triglyceride lipolysis (Demar et al. 2005; Gavino and Gavino 1992; Chen et al. 2008; Rahman et al. 2010a; Song et al. 2010; Gao et al. 2011). Newly synthesized DHA in liver is secreted into the plasma in triglyceride and phospholipid-rich lipoproteins, which can undergo lipolysis by a DHA-selective endothelial lipase (Chen and Subbaiah 2007). The resulting unesterified DHA can enter the brain to replace DHA lost due to metabolism (DeMar et al. 2004). In one human study, incorporation of orally-administered ¹³C-DHA into plasma esterified and unesterified fatty acid pools increased with age (Plourde et al. 2011), suggesting age-related changes in whole-body including liver DHA metabolism and changes in the liver's capacity to maintain plasma esterified and unesterified DHA concentrations.

The issue of maintaining sub-optimal tissue DHA status during aging has been debated (Bourre 2004). With age, levels of DHA in rat and human brain have been reported to decrease in some but not all studies (Soderberg et al. 1990; Lopez et al. 1995; Favrele et al. 2000; Giusto et al. 2002), and in humans, a low plasma DHA concentrations was associated with age-related cognitive impairment (Conquer et al. 2000). One explanation for these observations is that the liver's capacity to synthesize DHA from its dietary available precursor α -LNA is reduced because

of reduced activity of some enzymes involved in DHA biosynthesis (Horrobin 1981; Bordoni et al. 1988; Bourre and Piciotti 1992). However, reported changes in hepatic desaturase activities during aging vary according to species (Bourre 2004). They are decreased in mice (Bourre and Piciotti 1992), but are slightly modified (Bourre et al. 1990; Dinh et al. 1995) or unchanged (Bezard et al. 1994) in rats. Thus, there is a lack of agreement on liver synthesis of n-3 PUFAs with aging in rodents.

We reported a method to estimate rat liver synthesis-secretion coefficients and rates of DHA and other n-3 PUFAs from unesterified circulating α -LNA, by intravenously infusing [U-¹³C] α -LNA in unanesthetized rats for 2 h (Gao et al. 2009). These rates may be regulated by a variety of physiological and pathophysiological factors, such as dietary fatty acid content (Gao et al. 2011), aging, hormone status, or liver disease (Brenner 1981). We hypothesized that using this method would allow us to directly quantify in unanesthetized rats the liver's capacity to synthesize long-chain n-3 PUFAs, including DHA, from circulating unesterified α -LNA, in relation to age.

Accordingly, in the present study, we quantified whole-body (mainly liver) conversion (synthesis-secretion) coefficients and rates of α -LNA to DHA and other n-3 PUFAs, using this method in unanesthetized rats aged 10, 20, or 30 months. Unesterified albumin-bound [U-¹³C] α -LNA was infused intravenously for 2 h in a rat, and concentrations of esterified [¹³C]-labeled n-3 PUFAs and unesterified unlabeled plasma α -LNA were measured in arterial plasma at different times during infusion. Knowing synthesis-secretion coefficients and rates of n-3 PUFA at different ages should elucidate age-related changes in n-3 PUFA metabolism and have clinical implications for addressing n-3 PUFA dietary requirements in human aging populations.

Materials and methods

Materials

[U-¹³C] α -LNA (Spectra Stable Isotopes, Columbia, MD, USA) at >95% purity was used for infusion. Free heptadecanoic acid (17:0), α -LNA, eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3), and DHA standards, and pentafluorobenzyl (PFB) bromide and diisopropylamine, were purchased

from Sigma-Aldrich (St. Louis, MO, USA). Diheptadecanoate phosphatidylcholine (di-17:0 PC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Other chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific.

Animals

The protocol was approved by the Animal Care and Use Committee of the Eunice Kennedy Shriver National Institute of Child Health and Human Development and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 80-23). Fischer-344 (CDF) male rats aged 10, 20, and 30 months were obtained from rodent colonies at the National Institute on Aging (Bethesda, MD) and were maintained on an NIH-31 diet. The diet contained soybean oil and fishmeal and had 4% by weight crude fat (Demar et al. 2005). As reported, saturated and mono-unsaturated fatty acids contributed 20.1% and 22.5%, respectively, to its total fatty acid content. The n-3 PUFAs α -LNA, EPA, and DHA contributed 5.1%, 2.0%, and 2.3%, respectively, whereas the n-6 PUFAs linoleic acid (LA, 18:2n-6) and arachidonic acid (AA, 20:4n-6) contributed 47.9% and 0.02%, respectively. The animals were acclimated for 1 week prior to experimentation.

Surgery

A rat was anesthetized with 1–3% halothane. Polyethylene catheters (Becton Dickinson, Sparks, MD) filled with heparinized saline (100 IU/ml) were surgically implanted into a right femoral artery and vein (Gao et al. 2009), after which the skin was closed and treated with 1% lidocaine for pain control. The rat was loosely wrapped in a fast-setting plaster cast that was taped to a wooden block, and allowed to recover from anesthesia for 3–4 h. Arterial blood pressure and heart rate were monitored after recovery. Body temperature was maintained at 36–38°C using a feedback-heating element (YSI Indicating Temperature Controller, Yellow Springs Instruments, Yellow Springs, OH, USA). Surgery, which took ~20 min, was performed between 10:00 a.m. and 12:00 noon. Animals were provided food overnight.

Stable-isotope tracer infusion

Each rat was infused via the femoral vein catheter with 3- μ mol/100 g body weight [U - ^{13} C] α -LNA, dissolved

in 5-mM 4-(2-hydroxy-methyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) containing 50-mg/ml fatty acid-free bovine serum albumin (BSA), at a constant rate of 0.021 ml/min. During the 2-h infusion, body temperature was maintained at 36–38°C using the feedback-heating element (YSI Indicating Temperature Controller), and 2 ml of normal saline was injected subcutaneously to prevent dehydration. Arterial blood (130 μ l) was collected in centrifuge tubes (polyethylene-heparin lithium fluoride-coated; Beckman, Brea, CA, USA) at 0, 0.5, 1, 2, 3, 5, 8, 10, 20, 30, 60, and 90 min of infusion. At 120 min, 500 μ l blood was removed, and the rat was euthanized by an overdose of sodium pentobarbital (100 mg/kg i.v.). The blood samples were centrifuged at 13,000 rpm for 1 min, and plasma was collected and kept at –80°C until use.

Extraction, PFB derivatization, and GC/MS analysis of n-3 PUFAs in plasma

Appropriate amounts of internal standards (free 17:0 and di-17:0 PC) were added to plasma prior to lipid extraction. Plasma non-esterified and esterified fatty acids were extracted by a basic partition system containing hexane/10% KOH in methanol (Yi et al. 2006; Han et al. 2011) and dried under a stream of N_2 . The dried residue of the esterified lipids was subjected to further hydrolysis with 1 ml of methanolic KOH (10% w/v) at 75°C for 1 h, followed by acidification with 1 ml of 12 N HCl, addition of 1-ml distilled water to dissolve the formed salts, extraction of the hydrolyzed fatty acids into hexane, and evaporation to dryness. A freshly made PFB derivatizing reagent (pentafluorobenzylbromide/diisopropylamine/acetonitrile, 10/100/1,000, by volume) was added to the sample residue of hexane extraction (non-esterified and esterified parts) and shaken for 15 min at room temperature. The sample was evaporated again to dryness under N_2 and redissolved in 100 μ l of hexane. The n-3 PUFA-PFB esters from the plasma samples were separated and quantified using a TRACE gas-chromatography (GC) system equipped with a Finnigan TRACE DSQ mass spectrometer (MS; Thermo Electron, Austin, TX) as described (Gao et al. 2009). The PFB esters were injected in splitless mode onto a DB-FFAP capillary column (30 m $\times$ 0.25 mm ID, 0.25- μ m film thickness; J & W Scientific, Folsom, CA) interfaced into the negative chemical ionization (NCI) source

using methane as a reagent gas and helium as a carrier gas. The GC column temperature was increased from 80°C to 185°C at a ramp of 20°C per min, then to 240°C at 10°C per min, and held at 240°C for 30 min. The temperature of the injector port and transfer line from the GC to the MS was maintained at 240°C and 220°C, respectively. The retention times of the [¹³C]-labeled fatty acids were determined by running unlabeled n-3 PUFA standards. [¹³C]-labeled and unlabeled n-3 PUFA (α-LNA, EPA, DPA, and DHA) masses were monitored by selected ion mode (SIM) of the base peak (M-PFB). The concentration of each n-3 PUFA was quantified by relating its peak area to the area of the internal standard, using an experimentally determined response factor for each fatty acid.

Kinetic calculations

The pulse-labeling equations for determining the in vivo kinetics of α-LNA conversion to longer chain n-3 PUFAs by the whole body, after 2-h intravenous infusion of stable-isotope labeled α-LNA, have been published (Gao et al. 2009; Gao et al. 2010). To estimate steady-state rates of secretion, we fit concentration × plasma volume data plotted against infusion time for esterified PUFA *i* with sigmoidal curves (S curves) by non-linear least squares (Origin 8.0, Originlab, Northampton, MA).

The first derivative of this “S” curve was determined for each esterified PUFA *i* in each rat as a function of time. Its maximum value, $S_{\max,i}$ nmol/min, occurring when steady-state liver secretion is best approximated, was used to calculate the steady-state synthesis-secretion (conversion) coefficient, k_i^* (min⁻¹),

$$k_i^* = \frac{S_{\max,i}}{C_{\text{FA,unes}}^* \times V_{\text{plasma}}} \quad (1)$$

where k_i^* is proportional to the net rate of elongation and desaturation reactions and is independent of plasma

volume, thus a marker of net synthesis efficiency or capacity.

The synthesis-secretion rate (nmol/min) J_i , or the daily secretion rate (μmol/day), of unlabeled esterified PUFA *i* derived from unlabeled unesterified precursor, equals,

$$J_i = k_i^* \times C_{\text{FA,unes}} \times V_{\text{plasma}} \quad (2)$$

where $C_{\text{FA,unes}}$ is the unesterified unlabeled plasma precursor (α-LNA in this study) concentration.

Because plasma concentration $C_{i,\text{es}}$ (nmol/ml) of esterified unlabeled PUFA *i* is essentially unchanging during infusion, turnover F_i (min⁻¹) and half-life $t_{1/2,i}$ (min) due explicitly to synthesis-secretion equal, respectively,

$$F_i = \frac{J_i}{C_{i,\text{es}} \times V_{\text{plasma}}} \quad (3)$$

and

$$t_{1/2,i} = \frac{0.693}{F_i} \quad (4)$$

Statistical analysis

Data are expressed as means ± SD ($n=5$ per age group). Data were analyzed by a one-way ANOVA followed by Bonferroni's post-hoc test. Different superscripts indicate significant differences between the means. $p<0.05$ was taken as statistically significant. All analyses were completed with SPSS software, version 17 (SPSS, Chicago, Ill., USA).

Results

Physiological parameters

Table 1 presents the physiological parameters of the rats at different ages. Mean body weight and arterial

Table 1 Physiological parameters of rats at different ages

Data are means ± SD ($n=5$). Data were analyzed by a one-way ANOVA. Different superscripts indicate significant differences between the means ($p<0.05$), as determined by the Bonferroni post-hoc test.

Parameter	Age (months)		
	10	20	30
Body weight (g)	452 ± 27 ^a	453 ± 23 ^a	365 ± 26 ^b
Plasma volume (ml)	11.8 ± 0.9	11.4 ± 0.8	12.1 ± 0.5
Mean arterial blood pressure (mm Hg)	145 ± 5 ^a	135 ± 5 ^{ab}	130 ± 9 ^b
Heart rate (beats/min)	371 ± 30	393 ± 33	389 ± 30

blood pressure were reduced significantly with advancing age. Plasma volume and heart rate were not changed. The values agree with those previously reported (London et al. 1981).

Plasma endogenous n-3 PUFA concentrations

Table 2 presents the plasma concentrations and ratios of endogenous unesterified and esterified n-3 PUFAs. Compared to 10-month-old rats, unesterified n-3 PUFA concentrations decreased significantly with age, whereas the mean esterified PUFA concentration was not changed significantly. The ratios of unesterified to esterified n-3 PUFA concentrations ($R_{\text{unes/es}}$) declined significantly with age. The unesterified α -LNA concentration was 20.8 ± 5.7 , 14.3 ± 3.2 , and 9.39 ± 4.15 nmol/ml plasma for 10-, 20-, and 30-month-old rats, respectively ($p < 0.05$).

^{13}C -labeled unesterified α -LNA plasma concentration during constant-rate infusion

Once the constant-rate intravenous infusion of ^{13}C - α -LNA was started, the unesterified plasma concentration of ^{13}C - α -LNA increased rapidly and reached a steady state after about 8 min for rats at each age (Fig. 1). There was no significant difference in the steady-state plasma concentration or half-life of unesterified ^{13}C - α -LNA among 10-, 20-, and 30-month-old rats. Concentrations of unesterified ^{13}C - α -LNA at the end of infusion were 2.5 ± 0.2 , 2.2 ± 0.2 , and 2.0 ± 0.3 nmol/ml plasma for 10-, 20-, and 30-month-old rats, respectively.

Fig. 1 Time course of arterial plasma concentrations of unesterified ^{13}C - α -LNA during 2-h infusion of rats at 10, 20, and 30 months of age. Data are means $\pm$ SD ($n=5$)

^{13}C -labeled esterified n-3 PUFAs in plasma during 2-h infusion

As illustrated in Fig. 2, esterified ^{13}C -labeled n-3 PUFAs (α -LNA, EPA, DPA, and DHA) appeared in plasma about 30 min after infusion of ^{13}C - α -LNA was initiated. The net amount of esterified ^{13}C - α -LNA (concentration $\times$ plasma volume) in plasma then increased linearly, whereas net amounts of other labeled esterified n-3 PUFAs (EPA, DPA, and DHA) initially increased rapidly and then approached a plateau, consistent with a sigmoidal change represented by Eq. 2. There was no significant difference in the amount of esterified ^{13}C -n-3 PUFAs among different age groups (data not shown).

Sigmoidal (S) curves were determined using Origin software by fitting Eq. 2 to labeled plasma esterified

Table 2 Plasma concentrations and ratios of unesterified to esterified concentrations of n-3 PUFAs in rats at different ages

n-3 FA	Unesterified (nmol/ml plasma)			Esterified (nmol/ml plasma)			$R_{\text{unes/es}}$		
	10 months	20 months	30 months	10 months	20 months	30 months	10 months	20 months	30 months
α -LNA	20.8 ± 5.7^a	14.3 ± 3.2^{ab}	9.39 ± 4.15^b	41.1 ± 16.9	53.5 ± 28.3	52.5 ± 16.1	0.56 ± 0.21^a	0.34 ± 0.18^b	0.17 ± 0.11^b
EPA	11.1 ± 4.2^a	4.81 ± 0.75^b	3.02 ± 1.70^b	198 ± 68	128 ± 21	175 ± 65	0.061 ± 0.034^a	0.038 ± 0.008^{ab}	0.014 ± 0.005^b
DPA	15.2 ± 3.9^a	7.00 ± 1.22^b	5.60 ± 5.18^b	82.8 ± 20.6	77.7 ± 32.6	90.8 ± 25.3	0.20 ± 0.09^a	0.10 ± 0.04^b	0.048 ± 0.021^b
DHA	21.1 ± 3.1^a	12.6 ± 2.4^b	7.5 ± 2.7^c	412 ± 128	402 ± 124	435 ± 132	0.081 ± 0.035^a	0.033 ± 0.010^b	0.016 ± 0.007^b

Data were analyzed by a one-way ANOVA. Different superscripts indicate significant differences between the means ($p < 0.05$), as determined by the Bonferroni post-hoc test.

α -LNA alpha-linolenic acid (18:3n-3), EPA eicosapentaenoic acid (20:5n-3), DPA docosapentaenoic acid (22:5n-6), DHA docosahexaenoic acid (22:6n-3)

Fig. 2 Sigmoidal curves were determined using Origin software by fitting Eq. 2 to labeled plasma esterified concentrations $C_{i,es}^*$ × plasma volume plotted against infusion time, for **A** α -LNA, **B** EPA, **C** DPA, and **D** DHA in rats 10, 20, and 30 months of age. “S” curves were used to fit the data by the non-linear least squares method except for the precursor, ^{13}C - α -LNA, whose concentrations were fitted linearly. Data are means $\pm$ SD (n=5)

concentration $C_{i,es}^*$ × plasma volume plotted against infusion time, for the different PUFAs in rats at 10, 20, and 30 months of age (Fig. 2b–d). These “S” curves were fit to the data for EPA, DPA, and DHA by a non-linear least squares method. However, the data for esterified $[\text{U-}^{13}\text{C}]\alpha$ -LNA were fit with a linear equation (Fig. 2a).

DHA and other n-3 PUFAs' conversion coefficients and rates from α -LNA

To estimate steady-state synthesis-secretion (conversion) coefficients k_i^* (min^{-1}) of unesterified α -LNA to the longer chain n-3 PUFAs, first derivatives of best-fit “S” curves for unesterified labeled EPA, DPA, and DHA (Fig. 2b–d) were calculated. Figure 3 is one example in a 10-month-old rat. The maximum value of the first derivative, $S_{\max,i}$ for ^{13}C -labeled esterified n-3 PUFA i , was used to calculate k_i^* according to Eq. 1. Because the plasma concentration of esterified $[\text{U-}^{13}\text{C}]\alpha$ -LNA did not start to level off during the 2-h infusion (Fig. 2), we estimated $S_{\max}$ for α -LNA from peak slopes of the linear best-fit curves (Gao et al.

2010; Gao et al. 2011). No significant difference in conversion coefficients of each n-3 PUFA was observed among the three age groups (Fig. 4).

Using the unesterified unlabeled α -LNA plasma concentration that was measured before $[\text{U-}^{13}\text{C}]\alpha$ -LNA infusion, plasma volume, k_i^* , and daily whole-body secretion rates ($\mu\text{mol}/\text{day}$) of esterified α -LNA, EPA, DPA, and DHA were calculated by Eq. 2. Synthesis-secretion rates for the long-chain n-3 PUFAs were highest for DHA, followed by EPA and DPA, at all ages. However, mean secretion rates for α -LNA, DPA, and DHA, but not for EPA, were significantly reduced in rats with advancing age (Fig. 5). Since mean conversion coefficients did not differ among the four PUFAs, the reductions in conversion rates largely reflected reductions in the input (concentration) of unlabeled esterified plasma α -LNA (precursor) with age (Table 2).

Turnovers and half-lives of esterified n-3 PUFAs in plasma

Turnovers F_i of esterified n-3 PUFAs in plasma were calculated by Eq. 3 using $C_{i,es}$ in Table 1. F_i of

Fig. 3 First derivatives of plasma esterified concentration $\times$ plasma volume curves for EPA, DPA, and DHA in a 10-month-old rat

esterified α -LNA, DPA, and DHA was reduced significantly with advancing age (Fig. 6a), whereas corresponding half-lives were prolonged significantly (Fig. 6b).

Correlation between $R_{\text{unes/es}}$ of each n-3 PUFA and turnover in plasma

A Pearson correlation analysis showed that the turnover correlated significantly and positively with the ratio $R_{\text{unes/es}}$ of the unesterified/esterified plasma concentration (Table 2) for α -LNA ($r=0.720$, $p<0.01$), DPA ($r=0.788$, $p<0.001$), and DHA ($r=0.827$, $p<0.001$) (Fig. 7). With aging,

Fig. 4 Conversion coefficients k^* of unesterified α -LNA to esterified (α -LNA, EPA, DPA, and DHA) in rats aged 10, 20, and 30 months. Data are means $\pm$ SD ($n=5$). Data were analyzed by a one-way ANOVA. There was no significant difference between means

turnovers of esterified n-3 PUFAs other than EPA in plasma were reduced, as was the ratio of unesterified to esterified concentration.

Discussion

The aim of this study was to quantify whole-body (mainly liver) synthesis-secretion (conversion) coefficients and rates of unesterified unlabeled circulating α -LNA to esterified α -LNA, DPA, EPA, and DHA in unanesthetized rats of different ages. The coefficients and rates were determined by infusing [^{13}C]-labeled α -LNA intravenously for 2 h and applying validated operational equations to the experimental data (Gao et al. 2009; Gao et al. 2010; Gao et al. 2011).

One major finding of this study is that whole-body (mainly liver) efficiency for synthesis-secretion of circulating esterified n-3 PUFAs from unesterified circulating α -LNA, represented as the conversion coefficient k_i^* (Eq. 1), was not changed in rats at 20 or 30 months compared to 10 months of age. The conversion coefficient depends on the net rate of elongation and desaturation reactions in liver, as well as on packaging in very low density lipoproteins, independently of plasma volume (Gao et al. 2010; Gao et al. 2011). Changes in hepatic desaturase activity during aging vary according to species (Bourre 2004). They are reported to be decreased in mice (Bourre and Piciotti 1992), but slightly modified (Bourre et al. 1990; Dinh et al. 1995) or unchanged (Bezard et al. 1994) in rats, which is consistent with our results showing age invariance of the conversion coefficients.

Despite the unchanging plasma concentrations of esterified n-3 PUFAs with age, each of the respective unesterified concentrations declined (Table 2). Unesterified circulating n-3 PUFAs are produced mainly by hydrolysis by lipoprotein lipases from esterified PUFAs in circulating triglycerides, phospholipids, and cholesteryl esters within lipoproteins (Purdon et al. 1997) or from adipose tissue triglycerides by PUFA-selective lipases such as hormone sensitive lipase (Gavino and Gavino 1992; Goldberg 1996; Raclot et al. 2001). Tissue or plasma lipoprotein lipase and adipose tissue lipase activities are reported to be reduced with age (Carlile and Lacko 1985; Bey et al. 2001; Hamilton et al. 2001), which may account for the reduced plasma concentrations of unesterified n-3 PUFAs and reduced turnover of the esterified PUFAs

Fig. 5 Daily secretion rates of esterified α -LNA, EPA, DPA, and DHA in rats aged 10, 20, and 30 months. Data are means $\pm$ SD ($n=5$). Data were analyzed by a one-way ANOVA. Different superscripts indicate significant differences between the means ($p<0.05$), as determined by the Bonferroni post-hoc test

with age. Other factors that may regulate plasma unesterified fatty acid concentrations are hormones such as insulin and leptin, reported to increase with age in some studies, and fatty acid uptake by muscle (Tucker and Turcotte 2003; Chowdhury et al. 2011; Gomez-Perez et al. 2011). In one rat study, insulin-stimulated unesterified fatty acid uptake by muscle was increased with age (Tucker and Turcotte 2003).

The conversion rate (J_i) of circulating unesterified α -LNA to esterified n-3 PUFAs is the product of the conversion coefficient k_i^* , the plasma concentration of unlabeled unesterified α -LNA, and plasma volume (Eq. 2). Overall, the decreased conversion rates to DHA and the other n-3 PUFAs with age were due to reduced precursor unesterified α -LNA availability in plasma (concentration), which, in turn, may have been due to age-related changes in lipoprotein or hormone levels. As the esterified PUFA concentrations did not change with age, turnovers were reduced, and half-lives

were prolonged (Fig. 6a, b). The results are consistent with a human study that showed that orally administered ^{13}C -DHA was retained longer in plasma esterified and unesterified fatty acid pools of elderly than young subjects (Plourde et al. 2011).

Calculated turnovers in this study reflect the contributions of conversion from unesterified circulating α -LNA, but exclude contributions from diet or synthesis from other precursors. Thus, they are lower bounds, while the respective calculated half-lives are overestimates. The turnovers positively correlated with the unesterified/esterified concentration ratios, except for EPA (Fig. 7), as reported (Gao et al. 2010). This observation suggests that unesterified circulating PUFAs are derived largely from their respective esterified circulating PUFAs, although increased muscle uptake with age of unesterified fatty acids, which depends on insulin, could make a contribution (Tucker and Turcotte 2003). Turnovers and unesterified concentrations declined with age, while esterified concentrations did not. The correlations in Fig. 7 suggest that the plasma unesterified/esterified concentration ratio can be used as a biomarker of esterified n-3 PUFA turnover in clinical studies.

Kinetic and concentration values in the 10-month-old rats of this study are similar to those reported in 4-month-old Fischer-344 (CDF) male rats fed the same diet (Gao et al. 2009), except for differences in some plasma fatty acids, time of steady-state for α -LNA (8 versus 3 min in the 2009 report) and time of appearance of labeled esterified fatty acids (60 vs. 30 min in the 2009 report). These differences may be age related since the controls used in the present study were 10 month old, or may reflect experimental variability between studies. In this study, plasma esterified n-3 PUFA concentrations did not change with age, whereas unesterified

Fig. 6 **A** Turnovers and **B** half-lives of n-3 PUFAs (α -LNA, EPA, DPA, and DHA) in rats aged 10, 20, and 30 months. Data are means $\pm$ SD ($n=5$). Data were analyzed by a one-way ANOVA. Different superscripts indicate significant differences between the means ($p<0.05$), as determined by the Bonferroni post-hoc test

Fig. 7 Correlation between the ratio of the plasma unesterified to esterified concentration ($R_{\text{unes/es}}$) of **A** α -LNA, **B** EPA, **C** DPA, and **D** DHA and turnover in rats aged 10 (*squares*), 20 (*circles*), and 30 (*triangles*) months. Pearson correlation analysis was done between $R_{\text{unes/es}}$ of each n-3 PUFA and turnover using SPSS 17th (SPSS, Chicago, Ill., USA)

concentrations decreased with age. In another study in Fischer-344 rats, total plasma PUFA percent composition increased with age (Engler et al. 1998). In humans, total unesterified fatty acid concentrations increased with age (Bonadonna et al. 1994), as did EPA and DHA concentrations in plasma total lipids (Balkan et al. 2002; Fortier et al. 2010). The inconsistency in study outcomes may relate to differences in DHA synthesis capacity between rodents and humans (Wang et al. 2006), diet, or the fact that most studies measured n-3 PUFA concentrations in plasma total lipid instead of esterified and unesterified fractions (Engler et al. 1998; Fortier et al. 2010).

Most studies of plasma fatty acids measured the net concentration, but the present experiment highlights the importance of measuring unesterified and esterified concentrations separately. Knowing concentrations of unesterified α -LNA and LA is particularly important for understanding liver synthesis of longer chain n-3 (including DHA) and n-6 (including AA) PUFAs, since unesterified but not esterified fatty acids are selectively incorporated into brain (Purdon et al. 1997; Smith and Nagura 2001; DeMar et al. 2004; Rahman et al. 2010). Although this study focused on n-3 PUFA kinetics and concentrations, future studies should test the effects of

aging on n-6 PUFA liver kinetics. Rates of incorporation of unesterified circulating DHA and AA into brain reflect their respective rates of loss from brain due to metabolism, as the shorter chain PUFAs that enter brain largely undergo rapid oxidation (Demar et al. 2005; DeMar et al. 2006; Chen et al. 2009; Chen et al. 2011). As biomarkers of brain PUFA integrity, unesterified plasma DHA and AA concentrations may be more informative than the respective esterified concentrations (Kuriki et al. 2003; Rapoport et al. 2011).

A decreased liver synthesis-secretion rate of DHA and the corresponding reduction in plasma unesterified concentration may affect brain PUFA metabolism by decreasing brain DHA consumption and increasing vulnerability to neuroinflammation associated with upregulated AA metabolism, as reported in rats on a low n-3 PUFA diet, which also reduces plasma unesterified DHA concentration (DeMar et al. 2004; Rao et al. 2007; Kim et al. 2011a). In agreement with this suggestion, one study reported a decrease in brain mRNA expression of DHA-selective iPLA₂ in 24- and 30-month-old rats compared to 4-month-old animals and an increase in pro-inflammatory thromboxane A₂ concentration (Aid and Bosetti 2007), suggesting increased brain DHA half-life due to

reduced consumption and upregulated AA metabolism during aging, respectively (DeMar et al. 2004). The implication of altered brain AA and DHA metabolism with age on brain PUFA concentrations is controversial since some but not all studies reported age-related changes in PUFA composition in rodents and post-mortem human brain (Soderberg et al. 1990; Lopez et al. 1995; Favrele et al. 2000; Giusto et al. 2002). In humans, changes in brain PUFA (DHA or AA) metabolism do not correlate with plasma concentration changes (Igarashi et al. 2010; Kim et al. 2011b), suggesting that quantitative assessment of whole-body synthesis-secretion rates in vivo may serve as a predictive biomarker of disturbed brain and tissue PUFA metabolism.

In contrast to DPA and DHA, the synthesis-secretion rate of EPA was not reduced significantly with aging, despite a significant reduction in plasma unesterified EPA concentration in aged rats. One possible explanation for this discrepancy is increased tissue EPA uptake, consistent with the reported increases in insulin-stimulated free fatty acid uptake by muscle with age (Tucker and Turcotte 2003). It is possible that muscle uptake of the other n-3 PUFAs increased as well, corresponding to their reduced concentration. The decrease in plasma unesterified EPA also may be due to reduced adipose triglyceride lipolysis, which was reported to decrease with age (Caimari et al. 2008). Adipose lipolysis is regulated by hormone sensitive lipase and triglyceride lipase, which may be PUFA-selective (Gavino and Gavino 1992; Ahmadian et al. 2009; Jaworski et al. 2009). In one rat study, aging impaired fasting-induced increase in triglyceride lipase expression (Caimari et al. 2008).

In summary, whole-body (largely liver) synthesis-secretion coefficients and rates of unesterified circulating α -LNA to esterified DHA and other longer-chain n-3 PUFAs were quantified by infusing [U - ^{13}C] α -LNA intravenously for 2 h in unanesthetized male rats aged 10, 20, and 30 months. Calculated secretion-synthesis (conversion) coefficients were not changed significantly with age, suggesting no age difference in the liver's capacity to synthesize long chain n-3 PUFAs from α -LNA. However, daily secretion rates of DPA and DHA were reduced with advancing age, reflecting the decline in the unesterified unlabeled α -LNA concentration. Turnover of the esterified n-3 PUFAs also were reduced with aging, as were their unesterified plasma concentrations,

suggesting that the unesterified PUFAs were largely derived from the esterified circulating PUFAs. These age-related changes may limit brain DHA metabolism, since unesterified plasma DHA is the main source of brain DHA. Validation of our method with aging in the unanesthetized rats supports translating it to assess human liver PUFA synthesis with regard to aging as well.

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