Docosahexaenoic acid is both a product of and a precursor to tetracosahexaenoic acid in the rat

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Abstract  Tetracosahexaeoic acid (THA; 24:6n-3) is thought to be the immediate precursor of DHA in rodents; however, the relationship between THA and DHA metabolism has not been assessed in vivo. Here, we infused unesterified 2H5-THA and 13C22-DHA, at a steady state, into two groups of male Long-Evans rats and determined the synthesis-secretion kinetics, including daily synthesis-secretion rates of all 20-24 carbon n-3 PUFAs. We determined that the synthesis-secretion coefficient (a measure of the capacity to synthesize a given fatty acid) for the synthesis of DHA from plasma unesterified THA to be 134-fold higher than for THA from DHA. However, when considering the significantly higher endogenous plasma unesterified DHA pool, the daily synthesis-secretion rates were only 7-fold higher for DHA synthesis from THA (96.3 ± 31.3 nmol/d) compared with that for THA synthesis from DHA (11.4 ± 4.1 nmol/d). Furthermore, plasma unesterified THA was converted to DHA and secreted into the plasma at a 2.5-fold faster rate than remaining as THA itself (26.2 ± 6.3 nmol/d), supporting THA’s primary role as a precursor to DHA.

In conclusion, using a 3 h infusion model in rats, we demonstrate for the first time in vivo that DHA is both a product and a precursor to THA.—Metherel, A. H., R. J. S. Lacombe, R. Chouinard-Watkins, and R. P. Bazinet. Docosahexaenoic acid is both a product of and a precursor to tetracosahexaenoic acid in the rat. J. Lipid Res. 2019. 60: 412–420.

Supplementary key words  synthesis-secretion • Sprecher pathway • kinetics • fatty acid/metabolism • fatty acid/biosynthesis • omega-3 fatty acids • mass spectrometry

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Abbreviations: ALA, α-linolenic acid; DPAn-3, n-3 docosapentaenoic acid; GC/FID, gas chromatography-flame ionization detection; PFB, pentafluorobenzyl; Smax, maximum first derivative; THA, tetracosaheaxenoic acid; TLE, total lipid extract; TPAn-3, n-3 tetracosapentaenoic acid.

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feeding (6) are remarkably similar, despite much higher plasma levels of DHA. In addition, animals fed a high-ALA diet resulted in higher plasma THA levels compared with a low-ALA diet, but animals fed a high-versus low-DHA diet showed no change in plasma THA, suggesting that THA is indeed a precursor to DHA (8). These studies suggest some potentially unique metabolic differences between DHA and THA, despite their close relationship in the biosynthetic pathway. The ability to determine synthesis-secretion rates of the least abundant n-3 PUFA in living animals lends to the possibility of performing 3 h infusions with these low-abundance molecules to determine actual rates of synthesis between THA and DHA.

In the present study, we perform 3 h steady-state infusions of 2H5-THA and 13C22-DHA into Long-Evans rats by the method developed by Rapoport et al. (9) and modified by our laboratory (10). This is the first study to infused a THA or DHA tracer into a living animal to determine synthesis rates of DHA and THA, respectively. Briefly, we determined that plasma unesterified THA is converted to DHA, but also demonstrated that plasma unesterified DHA is converted to THA with the predominant rate of the reaction being for DHA synthesis from THA. In addition, we showed that THA is preferentially converted to DHA compared with acylation into complex lipids, a novel finding compared with other n-3 and n-6 PUFA assessed via the same animal models.

MATERIALS AND METHODS

Animals

All experimental procedures were performed in agreement with the policies set out by the Canadian Council on Animal Care and were approved by the Animal Ethics Committee at the University of Toronto. Eight 18-day-old male nonlittermate Long-Evans pups with dam were ordered from Charles River Laboratories (St. Constant, QC, Canada). Following arrival at the University of Toronto, the dam and pups were acclimated for 3 days and then weaned at 21 days old. The dams were placed on a standard chow diet (Teklad 2918, Harlan, Madison, WI) immediately upon arriving at the University of Toronto, and the pups were placed on the same diet for 11–12 weeks following weaning until 14–15 weeks of age. During this time and prior to cannulation, rats were handled frequently and housed in pairs (7).

Diets

The diet contained 6.2% fat, 18.6% protein, and 44.2% carbohydrates by weight. The fatty acid composition (% weight in total fatty acids) was determined by gas chromatography-flame ionization detection (GC/FID) to be 6.17 ± 0.01% (n = 3, mean ± SEM) ALA, 11.83 ± 0.04% 16:0, 3.18 ± 0.001% 18:0, 1.09 ± 0.001% 18:1n-7, 19.97 ± 0.02% 18:1n-9, and 53.53 ± 0.08% 18:2n-6, equaling more than 95% of all fatty acids in the diet.

Surgery and 2H5-THA, 13C22-DHA, and 2H5-ALA infusion

At 11–12 weeks postweaning, 14- to 15-week-old rats were subjected to surgery to implant a catheter into the jugular vein and the carotid artery, as previously described in detail (10). Baseline plasma unesterified and total fatty acid concentrations were measured in plasma and drawn from the carotid artery 1 day after surgery and 2 days prior to stable isotope infusion. Modified from the method of Rapoport et al. (9), the first group of animals (n = 4) was infused into the jugular vein with 0.070 μmol/100 g body weight of 2H5-THA (purity >95% confirmed by GC/FID and GC/MS; n = 3; Cayman Chemical Co., Ann Arbor, MI). Due to the higher endogenous unesterified plasma ALA and DHA pools, significantly more tracer was used to try to better match these endogenous levels. The second group of animals (n = 4) was infused with 0.843 μmol/100 g body weight of 13C22-DHA (Cambridge Isotope Laboratories, Inc., Tewksbury, MA). A single rodent from the second group of animals was coinfused with 0.843 μmol/100 g body weight of 2H5-ALA (Cambridge Isotope Laboratories) to compare with previous reports as a positive control to confirm that the infusions were being administered properly. Infusate preparation, 3 h steady-state infusions, and blood collections from the carotid artery (0, 30, 60, 90, 120, 150, and 180 min) were performed as previously described in detail (10). All blood samples were centrifuged for 15 min at 500 g (PC-100 microcentrifuge; Diamex, ON, Canada) under refrigeration, and the serum was collected and stored at −80°C.

Tracer purity determination

At the end of a 3 h infusion, 10 μl aliquots of the 2H5-THA infusate and 125 μl aliquots of the 2H5-ALA + 13C22-DHA (n = 3) infusate were collected and extracted by a method modified from Folch, Lees, and Sloane Stanley (11). Briefly, lipids were extracted with 2:1:0.75 chloroform:methanol:0.88% potassium chloride (v/v/v) containing 5 μg of heptadecanoic acid (17:0; Nu-Chek Prep Inc., Elysian, MN) as internal standard. The mixtures were vortexed and centrifuged at 500 g for 10 min, and the lower, chloroform lipid-containing layer was pipetted into a new test tube. The total lipid extract (TLE) was then dried down under a stream of nitrogen, hydrolyzed, derivatized to pentafluorobenzyl (PFB) esters, and run on the GC/MS for identification of any contaminant tracers that would yield false positives for metabolism of infused tracers. For example, in the 2H5-ALA + 13C22-DHA infusate, it was important to confirm the absence of other 13C22-n-3 PUFAs (i.e., 13C22-DPAn-3, 13C22-TPAn-3, and 13C22-THA) to confirm that all 13C22-n-3 PUFAs were derived from the metabolism of 13C22-DHA.

Determination of plasma volume

 Plasma volume was determined using the method of Schreiber, Hair and Stepp and modified by our lab (12, 13). Briefly, a known amount of Evans Blue dye was injected into the jugular vein of a separate group of rats (n = 5). Fifteen minutes following injection, 1 ml blood was drawn from the carotid artery, twice. The plasma was collected as described above, and 100 μl of plasma was diluted into 1 ml of saline. Absorbance of plasma in saline was determined at 604 nm with a Nanodrop 1000 and compared with a standard curve, and the concentration of the Evans Blue dye was determined. Concentration of the dye was then used to determine plasma volume.

Lipid extractions from baseline serum

TLEs were obtained from 50 μl of serum by the extraction method described earlier. Aliquots of one-fifth of the TLE for total fatty acids and four-fifths for unesterified fatty acid determinations were taken. TLEs for total fatty acid determinations were stored for hydrolysis as described later. TLEs for unesterified fatty acid determinations were evaporated under N2 gas and reconstituted in 50 μl chloroform, plated on a silica 60 G plate (Merck Millipore, Billerica, MA), and developed using TLC with 60:40:2 heptane:diethyl ether:glacial acetic acid for separation of unesterified fatty acids, as previously described (14). Unesterified fatty acids are a precursor and product of THA

DHA
acids FAs were scraped from TLC plates and extracted from the silica by the method described earlier. Isolated unesterified fatty acids were stored until derivatization to PFB esters.

**Lipid extractions from stable isotope-infused plasma**

TLE were obtained from 80 µl of stable isotope-infused plasma by the Folch method as described earlier containing 5 µg of heptadecanoic acid (NuChek Prep Inc.) as internal standard. Three-quarters of the TLE was stored for hydrolysis and stable isotope enrichment of total fatty acids, and the remaining one-quarter of the TLE was used for isolation of isotopically labeled unesterified fatty acid by the TLC method described earlier. The isolated labelled unesterified fatty acids were stored for later derivatization to PFB esters.

**Hydrolysis of total endogenous and isotopically labeled total fatty acid pools**

The TLE from baseline serum and infused plasma used to determine endogenous and labeled fatty acid concentrations, respectively, was evaporated under nitrogen, and the lipids were hydrolyzed in 2 ml of 10% potassium hydroxide in methanol (v:v), as previously described (8, 14, 15). Hydrolyzed fatty acids from the total lipid pool were collected and stored at −80°C for later derivatization to PFB esters.

**PFB esterification**

The plasma unesterified fatty acids and hydrolyzed fatty acids from total lipid pools were dried under nitrogen and derivatized to PFB esters, as previously described and modified by our lab (7, 15). Briefly, 100 µl of acetonitrile:diisopropylamine:PFB bromide (1,000:10:1, vol:vol) was added and heated at 60°C for 15 min. The reagents were then evaporated under nitrogen, realiquoted in 40 µl of hexane, and analyzed for endogenous and labeled fatty acids by GC/MS.

**Endogenous and labeled fatty acid determination by GC/MS**

Fatty acid PFB esters were analyzed on an Agilent 5977A quadrupole mass spectrometer coupled to an Agilent 7890B gas chromatograph (Agilent Technologies, Mississauga, ON, Canada) in negative chemical ionization mode, as described previously (15) and recently modified by our lab (8). The fatty acid PFB esters were injected via an Agilent 7693 autosampler into a DB-FFAP 30 m × 0.25 mm inner diameter × 25 µm film thickness capillary column (J&W Scientific from Agilent Technologies) interfaced directly into the ion source with helium as the carrier gas and 5.0 ml/min. The ion source temperature was 230°C, the quadrupole temperature was 150°C, and the entrance and exit ionizer energies were set to 70 eV.

**Equations**

To determine the synthesis rates of n-3 PUFAs from THA and DHA, the appearance of [1H]-PUFA and [13C]PUFA, respectively, in the plasma-esterified pool were measured and fit to a Boltzmann sigmoidal curve ([1H]-[13C]PUFA) × plasma volume vs. time) using nonlinear regression (12) (GraphPad Prism Version 4.0, La Jolla, CA). In addition, the retroconversion of [13C]DHA to [15C]EPA and [15C]DPAn-3 was included in our calculations. At any point on the curves, the slope (S) is determined by the ability of the body to synthesize [2H]-PUFA from [2H]-THA or [15C]-PUFA from [15C]-DHA and the ability of the periphery to uptake [2H]-PUFA (equation 1) or [15C]-PUFA (equation 2), respectively.

\[
S = k_{PUFA} \left( [^2\text{H}_5 - \text{THA}]_{\text{unest}} - k_{1,PUFA} [^2\text{H}_5 - \text{PUFA}]_{\text{unest}} \right) \quad (\text{Eq. 1})
\]

where \(k_{1,PUFA}\) is the steady-state synthesis-secretion coefficient for any PUFA, representing a measure for the amount of infused label that is converted to downstream products; \([^2\text{H}_5 - \text{THA}]_{\text{unesterified}}\) and \([15\text{C}]\text{PUFA}_{\text{esterified}}\) are the plasma concentrations of the infused; \(k_{1,PUFA}\) is the disappearance coefficient for any individual PUFA; and \([^2\text{H}_5 - [15\text{C}]\text{PUFA}]_{\text{esterified}}\) is the concentration of an individual PUFA in the plasma that has been synthesized from the respective infusate, packaged into lipoprotein, and secreted into the plasma.

The maximum first derivative (\(S_{\text{max}}\)) of the curves is assumed to be the time point when the uptake of esterified labeled PUFA from the periphery is negligible, i.e., 0 (equations 3 and 4).

\[
S_{\text{max}} = k_{1,PUFA} [^2\text{H}_5 - \text{ALA}]_{\text{unest}} - 0 \quad (\text{Eq. 3})
\]

**Statistics**

Differences in n-3 PUFA synthesis rates of downstream products from an individual tracer ([2H]-THA or [13C]-DHA) were assessed by one-way ANOVA followed by Tukey’s honest significant difference posthoc test. Student’s t test was used to compare kinetic parameters of an individual n-3 PUFA product between the two tracers (i.e., [2H]-THA vs. [13C]-THA). Statistical significance was set at \(P < 0.05\) for all tests. Normality was assessed by the Shapiro-Wilk test.
for normality, and in the case of nonnormally distributed groups, the data were log-transformed prior to statistical analysis. All data are presented as mean ± SEM.

RESULTS

Tracer purity

The $^{2}$H$_{5}$-THA infusate and the $^{2}$H$_{5}$-ALA + $^{13}$C$_{22}$-DHA infusate were assessed by GC/MS for contaminant isotopes that would result in false-positive determinations of the origin of n-3 PUFA metabolites, with representative chromatograms presented (Fig. 1). An external reference standard chromatogram is also presented (Fig. 1A). In the $^{2}$H$_{5}$-ALA + $^{13}$C$_{22}$-DHA infusate (Fig. 1B), no $^{13}$C$_{20,22}$-n-3 PUFA contaminants were detected for EPA, DPAn-3, TPAn-3, or THA, with $^{13}$C$_{22}$-DHA being the only $^{13}$C-n-3 PUFA identified. In the $^{2}$H$_{5}$-THA infusate (Fig. 1C), no $^{2}$H$_{5}$-n-3 PUFA contaminants were detected for ALA, EPA, DPAn-3, DHA, or TPAn-3. The only n-3 PUFA isotope detected was $^{2}$H$_{5}$-THA.

Plasma-labeled and unlabeled n-3 PUFA concentrations

Plasma unesterified ALA, DHA, and THA (Fig. 2A), unesterified $^{2}$H$_{5}$-ALA, $^{13}$C$_{22}$-DHA, and $^{2}$H$_{5}$-THA (Fig. 2B), and tracer/tracee ratios ($^{2}$H$_{5}$-ALA/ALA, $^{13}$C$_{22}$-DHA/DHA, and $^{2}$H$_{5}$-THA/THA) (Fig. 2C) were determined and are presented in Fig. 2. For ALA, DHA, and THA, respectively, endogenous unesterified fatty acid concentrations (nmol/ml ± SEM) were 4.4 ± 0.7, 2.8 ± 1.0, and 0.12 ± 0.03; labeled unesterified fatty acid infusates were 0.30 ± 0.06, 0.41 ± 0.12, and 0.052 ± 0.004; and tracer/tracee ratios were 0.08 ± 0.02, 0.17 ± 0.04, and 0.52 ± 0.12, respectively.

Total plasma n-3 PUFA concentrations (nmol/ml ± SEM; data not shown) were also determined for turnover and half-life calculations in the $^{2}$H$_{5}$-THA and the $^{2}$H$_{5}$-ALA + $^{13}$C$_{22}$-DHA infused animals (data not shown). In the $^{2}$H$_{5}$-THA infused animals, total EPA, DPAn-3, DHA, and THA were 17.8 ± 3.5, 20.9 ± 4.1, 126 ± 31, and 1.88 ± 0.49, respectively, and in the $^{13}$C$_{22}$-DHA, total concentrations were 9.1 ± 0.8, 11.0 ± 1.7, 68.5 ± 14.4, and 0.84 ± 0.13, respectively. No significant differences ($P > 0.05$) were determined between the two infusate groups for any n-3 PUFA.

n-3 PUFA synthesis-secretion coefficients, rates, turnovers, and half-lives

Representative infusion curves, $S_{\text{max}}$, and representative GC/MS chromatograms for the synthesis secretion of THA and DHA from plasma unesterified DHA (Fig. 3A) and THA (Fig. 3B) are shown. Furthermore, a statistical comparison for the synthesis-secretion kinetic parameters of DHA from plasma unesterified THA to the synthesis secretion of THA from plasma unesterified DHA is presented (Table 1). Most strikingly, the synthesis-secretion coefficient for DHA from THA (0.539 ± 0.098, ml/min ± SEM) is 134-fold higher ($P < 0.01$) than for THA from DHA (0.004 ± 0.002); however, when determining daily synthesis-secretion rates (96 ± 31 vs. 11.4 ± 4.1, nmol/day ± SEM), this difference ($P = 0.036$) is reduced to only 7-fold. Similarly, the turnover and half-life of newly synthesized plasma DHA from unesterified THA is 18-fold lower ($P < 0.001$) and 15-fold higher ($P < 0.0001$), respectively, compared with newly synthesized THA from unesterified DHA.

A comprehensive assessment of synthesis-secretion kinetic parameters for all plasma n-3 PUFAs from plasma unesterified DHA and THA is presented (Table 1). In addition, synthesis-secretion kinetics from plasma unesterified ALA for two animals are presented as a positive control to demonstrate that the pathway is behaving within an acceptable range (supplemental Table S1). Most interestingly, the synthesis-secretion coefficient of total plasma DHA from plasma unesterified DHA compared with THA is not different ($P > 0.05$), despite a 45-fold difference ($P < 0.001$) in the daily synthesis-secretion rates. Furthermore, the synthesis-secretion coefficient and the daily synthesis-secretion rate for the synthesis of DHA from THA is 2- and 3-fold ($P < 0.05$) higher, respectively, compared with the synthesis of THA itself. In addition, the turnover of all measured n-3 PUFA was faster ($P < 0.05$) when from plasma unesterified DHA, except THA, which was not different ($P > 0.05$) between the two plasma sources. Finally, retroconversion of both DHA and THA to EPA and DPAn-3 were detected; however, these daily synthesis-secretion rates from DHA compared with THA were 60-fold and 18-fold higher ($P < 0.05$) for EPA and DPAn-3, respectively, with no differences ($P > 0.05$) in the synthesis-secretion coefficient. Importantly, labeled DPAn-3 was determined to be $^{13}$C$_{22}$DPAn-3 most likely via elongation of newly formed EPA, and direct conversion of DHA to DPAn-3 via the appearance of $^{13}$C$_{22}$DPAn-3 could not be detected (data not shown).

DISCUSSION

In the current study, we have for the first time using living animals through the infusion of $^{2}$H$_{5}$-THA over 3 h demonstrated that DHA is the primary n-3 PUFA product of THA. However, we have also shown for the first time in vivo the elongation of $^{13}$C$_{22}$DHA back to THA in Long-Evans rats, and a modified n-3 PUFA biosynthesis pathway is presented in Fig. 4. The predominant rate of flow between these two n-3 PUFAs is from THA to DHA compared with DHA to THA. Importantly, the high purity of our tracers strengthens our conclusions with the knowledge that the only source of labeled DHA could be THA and vice versa. Surprising to many might be that the hallmark study by Voss et al. (1) in 1991, which was the first to demonstrate the need for a second Δ6-desaturation followed by peroxisomal β-oxidation to DHA, demonstrated that DHA can be elongated in isolated rat liver microsomes. They showed that a small proportion of 75 µM $^{14}$C-DHA is chain elongated to THA, and, when compared with acylation of DHA into phospholipids, DHA elongation was insignificant. Their conclusions were reasonable based on the labeled precursors and products in isolation; however, our 3 h infusion model (12) determines daily synthesis-secretion rates by correcting the labeled precursor-product ratio.
(synthesis-secretion coefficient) by the endogenous unesterified PUFA precursor pool. Significantly, this correction narrows the difference between the two rates of travel from 134-fold when considering the tracers only to merely 7-fold for the daily synthesis-secretion rate. Furthermore, because the liver DHA-CoA pool might dilute the labeled DHA more than the THA-CoA would dilute the labeled THA (14), the THA synthesis-secretion rates are expected to be closer to the DHA synthesis-secretion rates. The elongation of DHA to THA is likely mediated by the elongation of very-long-chain 2 (Elovl2) enzyme that has a high affinity for 22C-PUFA (16–18), particularly in comparison to Elovl5.

Fig. 1. Representative GC/MS chromatograms for external standard (A), $^2$H$_5$-ALA + $^{13}$C$_{22}$-DHA infusate (B), and $^2$H$_5$-THA infusate (C). n.d., not detected.
DHA is a precursor and product of THA that has very low affinity for 22C-PUFA and high affinity for 20C-PUFA (16).

Our animals were fed a standard chow diet containing ALA as the only n-3 PUFA present. The inclusion of other n-3 PUFAs in the diet, particularly DHA, may result in even higher rates of THA synthesis-secretion from plasma-unesterified DHA. Previously, we fed animals a DHA-free diet or a 2% DHA in total fat diet for 8 weeks and found that serum THA levels did not change, whereas serum DHA levels were significantly higher when fed the 2% DHA diet (6, 8). Furthermore, plasma unesterified DHA increased 124-fold and THA only 24-fold in response to a 2% DHA diet (6); however, these data were not reported in our previous study. This suggests that when animals are fed DHA, the higher plasma unesterified DHA would result in daily THA synthesis-secretion rates that are similar to the rates determined for DHA from plasma unesterified THA. However, because our study is the very first to infuse labeled THA and labeled DHA into free-living animals for the determination of daily synthesis-secretion rates to DHA and THA, respectively, the effect of DHA feeding on these metabolic questions has never been tested, and future studies need to be performed to address these unknowns.

What we cannot conclude based on our study design and results is the presence (or absence) of a Δ4-desaturase step that directly desaturates DPAn-3 to DHA while bypassing the Sprecher pathway. As a result, we also cannot clearly state what the predominant precursor to THA is, DHA or TPAn-3. It is possible that the n-3 PUFA biosynthetic pathway behaves very differently in isolated liver microsomes or hepatocytes compared with in living mammals. In such a scenario, DPAn-3 could be desaturated directly to DHA by a Δ4-desaturase as evidenced in human cells (4, 5), further elongated to THA, and then quickly β-oxidized back to DHA in the peroxisome. Interestingly, cells grown in the presence of labeled DHA demonstrated an up to 5-fold higher retroconversion of DHA to EPA compared with elongation to THA (4). We showed a similar, albeit larger (15-fold), rate of retroconversion over elongation that may continue to suggest similarities between rodent and human models of n-3 PUFA metabolism. Although the bulk of the evidence in rodents supports the Sprecher pathway, future studies could aim to clarify remaining questions relating to the final steps in DHA synthesis. For instance, 3 h infusions of DPAn-3, TPAn-3, THA, and/or DHA tracers in combination with in vivo inhibition of peroxisomal β-oxidation (19, 20), Δ6-desaturase inhibition (21, 22), or knockout (23) would provide a wealth of additional information and has the potential for a more complete understanding of these steps in the pathway. This could now be explored even further in humans, as a recent study has validated the infusion method in two individuals (24).

Interestingly, the rate of DHA synthesis from THA (96.3 ± 31.3 nmol/d) is very similar to our previously reported synthesis rates of DHA from ALA (47–157 nmol/d) (6, 7, 14, 25) and DHA from EPA (70–172 nmol/d) (6, 14) when feeding ALA as the only n-3 PUFA in the diet. In addition, these previously reported values are comparable, albeit somewhat lower, to the values presented in supplemental Table S1 and may be representative of the different diet used here. This further supports the recent conclusion from these studies that DHA synthesis rates are not as low as previously thought when considering ALA as the only substrate. DHA synthesis rates have frequently been reported to be very low and have been reviewed by our laboratory (26); however, if we consider for simplicity that DHA synthesis from ALA is 100 nmol/d, from EPA is 100 nmol/d, and from THA is 100 nmol/d, then DHA synthesis rates are at least three times higher than originally believed due to the inclusion of multiple DHA precursors.
Furthermore, it is plausible that when we consider the four additional DHA precursors (18:4n-3, 20:4n-3, 24:5n-3, and, in particular, DPAn-3) that DHA whole-body synthesis rates will be even higher, and additional studies should be performed to test this.

Plasma unesterified THA in the rodent is converted to DHA at a faster rate than remaining as THA. This is shown very clearly, as the daily synthesis-secretion rate for DHA from THA is approximately two-and-a-half-fold higher than for THA remaining as itself and acylated into more complex lipids. This is a novel finding when compared with all previous 3 h infusion studies from our laboratory, as each of ALA, EPA, DHA, linoleic acid (18:2n-6), and DPAn-6 (22:5n-6) (6, 7, 10, 12, 14, 25) were more rapidly acylated into complex lipids versus converted to other PUFAs. This is likely related to the very low and relatively stable plasma levels of THA seen in the rodents (8), suggesting that THA must be rapidly metabolized in order to remain at these levels. This is also supportive of the original conclusion of Voss et al. (27) that the role of both THA and TPAn-3 are as “obligatory intermediates in 22:6(n-3) (DHA) biosynthesis” (1), and it has been shown in isolated peroxisomes + microsomes that β-oxidation of THA to DHA predominates over THA acylation into complex lipids. Unesterified DHA is also rapidly acylated into more complex lipids at a rate that is 390-fold higher than for elongation to THA, further supporting a molecular requirement for the presence of DHA in the body. This seemingly required conversion of THA into complex lipids versus converted to other PUFAs.

### TABLE 1. Kinetic parameters for the whole-body synthesis secretion of n-3 PUFAs following infusion of $^{13}$C$_{22}$-DHA or $^2$H$_5$-THA

| Source | N-3 PUFA | Maximum First Derivative (nmol/min) | Synthesis-Secretion Coefficient (ml/min) | Daily Synthesis-Secretion Rate (nmol/day) | Turnover (turnover/day) | Half-life (days) |
|--------|----------|------------------------------------|----------------------------------------|------------------------------------------|------------------------|-----------------|
|       | DHA      | 0.016 ± 0.003$^a$                  | 0.058 ± 0.022$^a$                      | 179 ± 61$^a$                             | 2.40 ± 0.66$^a$       | 0.390 ± 0.136$^a$ |
|       | EPA      | 0.006 ± 0.002$^b$                  | 0.029 ± 0.010$^a$                      | 50.9 ± 10.7$^b$                          | 0.652 ± 0.165$^b$    | 1.28 ± 0.29$^b$   |
|       | DPAn-3   | n.d.                               | n.d.                                   | n.d.                                    | 1.74 ± 0.48$^{ab}$    | 0.468 ± 0.085$^a$  |
|       | THA      | 0.001 ± 0.0002$^{cd}$              | 0.004 ± 0.002$^{cd}$                    | 11.4 ± 4.1$^c$                           | 8.17 ± 2.2$^b$        | 0.102 ± 0.022$^b$ |
|       | DHA      | 0.030 ± 0.006$^{ab}$               | 1.32 ± 0.45$^a$                        | 4434 ± 1575$^b$                         | 0.016 ± 0.003$^{ab}$  | 48.8 ± 9.3$^b$    |
|       | TPAn-3   | n.d.                               | n.d.                                   | n.d.                                    | 1.80 ± 0.16$^b$       | 0.394 ± 0.037$^b$  |
|       | THA      | 0.008 ± 0.002$^{ab}$               | 0.157 ± 0.024$^{ab}$                    | 26.2 ± 6.3$^a$                           | 1.80 ± 0.16$^b$       | 0.394 ± 0.037$^b$ |
|       | DHA      | 0.027 ± 0.005$^{ab}$               | 0.539 ± 0.098$^{ab}$                    | 96.3 ± 31.3$^{ab}$                       | 0.093 ± 0.007$^{ab}$  | 7.55 ± 0.61$^{ab}$ |

* Different superscript letters represent statistically different kinetic values between fatty acids and within a kinetic measure following a significant one-way ANOVA and Tukey’s posthoc test. Values are expressed as means ± SEM, n = 4. n.d., not detected.

* P < 0.05 [significantly different kinetic values between sources (THA vs. DHA) for a specific n-3 PUFA as determined by Student’s t-test].

# P < 0.05 (significantly different kinetic values for THA synthesis from DHA vs. DHA synthesis from THA as determined by Student’s t-test).
into DHA is also highlighted by the lower relative rates of THA compared with DHA retroconversion to EPA and DPAn-3, indicating a likely requirement for the conversion of THA to DHA prior to retroconversion (28). Previous studies have also demonstrated DHA retroconversion to both EPA and DPAn-3 (29–31); however, they were unable to determine whether the DHA was converted directly to DPAn-3 with the removal of a single double bond or whether DHA was converted to EPA prior to elongation to DPAn-3. To the best of our knowledge, we show for the first time that retroconversion to EPA is likely to occur first and is supportive of the idea that the metabolic conversion of THA to DHA does not include a DPAn-3 intermediate (28). However, the low rates of DHA elongation to THA compared with retroconversion are not necessarily indicative of an unimportant role for THA, and the low conversion rates and plasma levels of THA could be further explained by rapid entry into other metabolic pathways. As such, future studies should be designed to investigate additional metabolic products of THA such as fatty acid ethanalamides or novel lipid mediators. Recently, a new class of neuroprotective lipid mediators, elovanoids, derived from very-long-chain n-3 PUFAs of 28 or more carbons has been identified (32, 33) and lends to the possibility that THA-derived lipid mediators and other bioactive metabolites have the potential to mediate some of the effects of DHA observed following DHA administration.

The direct plasma THA turnover (THA from plasma unesterified THA) was slower than the direct turnover of plasma DHA (Table 1). In light of the previous paragraph’s discussion, this is somewhat of a surprising finding. In order for plasma THA levels to remain low and plasma DHA to remain high, an opposite effect would have been expected. One explanation might be the tracer/tracee ratios (labeled tracer/unesterified PUFA) of the $^{3}H_{2}$-THA tracer that is approximately three times higher than the $^{13}$C$\textsubscript{15}$-DHA tracer, meaning that 33% of all the unesterified THA present throughout the infusion is the $^{3}H_{2}$-THA. This may again support an “obligation” for THA conversion to DHA, as the higher than normal tracer/tracee ratio has potentially flooded the pathway to create a bottleneck at THA, which, when calculating turnover, would result in artificially slower values. This may necessitate lower infusion levels of $^{3}H_{2}$-THA for future studies while keeping infusate levels high enough so as to remain detectable by mass spectrometry.

In conclusion, we have shown for the first time in vivo that DHA is both a product and a precursor to THA (and vice versa) and determined daily synthesis-secretion rates for both n-3 PUFAs. Furthermore, although synthesis secretion of DHA from THA is significantly higher than THA from DHA, additional studies feeding DHA would be expected to yield more similar rates between the directions. In addition, the recent application of these infusion models to humans (24), the evidence from human cell lines suggesting the bypassing of the Sprecher pathway (4, 5), and the potentially very low $^{3}H_{2}$-THA tracer requirements make the implementation of human THA infusions an exciting future step. In particular, the comparison of DHA synthesis rates between individuals with peroxisomal disorders, such as Zellweger’s syndrome, would lead to an even more comprehensive understanding of such conditions.

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