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Acute injection of a DHA triglyceride emulsion after hypoxic-ischemic brain injury in mice increases both DHA and EPA levels in blood and brain

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Abstract

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Contributions

All the experiments listed in the manuscript were performed at Columbia University Medical Center, New York, NY, US. DK and HZ performed all the experiments. ZL did the GC-MS analyses. CJ and CNS performed the lipidomics analyses. DK wrote the first draft of the manuscript. RJD, VST, RR, HZ and CNS advised on this study and on data analyses and revised the manuscript. RJD, DK and HZ conceived the study, designed the experiments, coordinated the experiments and wrote the final version of the manuscript.

AUTHORSHIP STATEMENT

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Prostaglandins, Leukotrienes and Essential Fatty Acids*.

Authorship contributions

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

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Disclosures

Dr. Deckelbaum is a founding scientist and member of the scientific advisory board of DeckTherapeutics, Inc. (DT), a company that plans to use diglyceride lipid emulsions to prevent tissue death after ischemic brain injury. The plans for DT do not overlap with any of the data presented in this paper. The other authors have no conflicts of interest to declare.

We recently reported that acute injection of docosahexaenoic acid (DHA) triglyceride emulsions (tri-DHA) conferred neuroprotection after hypoxic-ischemic (HI) injury in a neonatal mouse stroke model. We showed that exogenous DHA increased concentrations of DHA in brain mitochondria as well as DHA-derived specialized pro-resolving mediator (SPM) levels in the brain. The objective of the present study was to investigate the distribution of emulsion particles and changes in plasma lipid profiles after tri-DHA injection in naïve mice and in animals subjected to HI injury. We also examined whether tri-DHA injection would change DHA- and eicosapentaenoic acid (EPA)-derived SPM levels in the brain. To address this, neonatal (10-day-old) naïve and HI mice were injected with radiolabeled tri-DHA emulsion (0.375 g tri-DHA/kg bw), and blood clearance and tissue distribution were analyzed. Among all the organs assayed, the lowest uptake of emulsion particles was in the brain (<0.4% recovered dose) in both naïve and HI mice, while the liver had the highest uptake. Tri-DHA administration increased DHA concentrations in plasma lysophosphatidylcholine and non-esterified fatty acids. Additionally, treatment with tri-DHA after HI injury significantly elevated the levels of DHA-derived SPMs and monohydroxy-containing DHA-derived products in the brain. Further, tri-DHA administration increased resolvin E2 (RvE2, 5S,18R-dihydroxy-eicosa-6E,8Z,11Z,14Z,16E-pentaenoic acid) and monohydroxy-containing EPA-derived products in the brain. These results suggest that the transfer of DHA through plasma lipid pools plays an important role in DHA brain transport in neonatal mice subjected to HI injury. Furthermore, increases in EPA and EPA-derived SPMs following tri-DHA injection demonstrate interlinked metabolism of these two fatty acids. Hence, changes in both EPA and DHA profile patterns need to be considered when studying the protective effects of DHA after HI brain injury. Our results highlight the need for further investigation to differentiate the effects of DHA from EPA on neuroprotective pathways following HI damage. Such information could contribute to the development of specific DHA-EPA formulations to improve clinical end points and modulate potential biomarkers in ischemic brain injury.

Graphical abstract

Keywords

DHA; EPA; n-3 fatty acids; DHA brain transport; stroke; specialized pro-resolving mediators

1. Introduction

Docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (n-3 PUFA), is a structural and functional constituent of cellular membranes with especially high levels in the central nervous system [1–3]. In addition to its own molecular properties, DHA can be converted to a diverse set of bioactive metabolites, including DHA-derived specialized pro-

resolving mediators (SPMs), ethanolamines, docosahexaenoyl amides of amino acids or neurotransmitters, and branched DHA esters of hydroxy fatty acids (FA) [4–6]. SPMs, considered highly potent lipid mediators, counteract eicosanoid-induced inflammation by activating local resolution processes [7,8], and likely contribute to the beneficial effects of DHA. With a neuroprotective role, DHA has been used as treatment in experimental models of ischemic brain injury, epilepsy and neurodegenerative diseases [9–13]. Blood brain barrier (BBB) crossing is critical for providing DHA to the brain and different lipid species within the circulation contribute to the brain distribution of DHA [14–16]. In particular, recent studies have shown that plasma DHA is carried into the brain as non-esterified fatty acid (NEFA) or by lysophosphatidylcholine (LPC) [17,18].

Our studies and those of others have described therapeutic benefits of DHA in experimental stroke models [19–23]. Thus, in animals subjected to ischemic injury, DHA administration inhibited immune cell infiltration, reduced the release of pro-inflammatory factors, restored mitochondrial function, and prevented excitotoxic glutamate release [12,24–26]. We reported and emphasized that acute post-ischemic administration of triglyceride (TG) emulsions containing only DHA (tri-DHA) conferred neuroprotection against hypoxic-ischemic (HI) injury in neonatal mice [22,23]. Our findings were not associated with increases of DHA levels in the whole brain, but did show increases of DHA in isolated mitochondrial membranes as well as increases in DHA-derived SPMs in brain tissue after tri-DHA administration [23].

While DHA is the most abundant n-3 PUFA in the brain, eicosapentaenoic acid (EPA), also a bioactive n-3 PUFA, is present in much lower amounts [27]. DHA and EPA are biologically distinct molecules with specific tissue distributions as well as differential effects on membrane structure and lipid dynamics, rates of lipid oxidation, and signal transduction pathways [28,29]. However, DHA and EPA are reported to have interlinked metabolism. As an example, recent results from Bazinet's laboratory indicate that DHA supplementation not only increased blood and tissue DHA concentrations, but also EPA levels. In rats fed a DHA-enriched diet for 12 weeks, liver EPA concentrations significantly increased [30]. Further, DHA supplementation for 12 weeks significantly increased plasma EPA concentrations in humans [31]. While these studies focused on chronic supplementation with n-3 PUFA, no studies have been performed assessing the early changes in EPA profiles in blood and tissues after acute administration of DHA.

The aim of the present study is to characterize DHA brain transport and metabolism after injecting tri-DHA emulsion in HI injury. Our results showed that the amount of DHA entering the brain through whole emulsion particle uptake was very low, and that increased levels of plasma LPC-DHA and non-esterified DHA (NE-DHA) indicated that other potential DHA brain transport pathways might contribute to its brain uptake. Furthermore, tri-DHA administration increased levels of DHA-derived SPMs, monohydroxy-containing DHA-derived products and pathway markers in the brain. Remarkably, increased levels of EPA in blood as well as EPA-derived SPM, monohydroxy-containing EPA-derived products and pathway marker in brain were detected following tri-DHA administration, providing evidence for interlinked metabolic pathways between these n-3 PUFA.

2. Materials and methods

2.1. Ethics Statement

All research studies were carried out according to protocols approved by the Columbia University Institutional Animal Care and Use Committee (IACUC, Protocol Number AAAS6464) in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines (AAALAC).

2.2. Materials

We used purified (<99%) tri-DHA (Nu-Chek Prep, Inc., Elysian, MN) and egg yolk phosphatidylcholine (Avanti Polar-Lipids, Inc., Alabaster, AL) as starting material for emulsion preparation. The FA composition of egg yolk phosphatidylcholine as described by the producer was: 14:0 - 0.2%, 16:0 - 32.7%, 16:1 - 1.1%, 18:0 - 12.3%, 18:1 - 32%, 18:2 - 17.1%, 20:2 - 0.2%, 20:3 - 0.3%, 20:4 - 2.7%, 22:6- 0.4%, unknown- 1.0%. Radiolabeled [³H]-cholesteryl hexadecyl ether was purchased from PerkinElmer (Boston, MA) ([³H]Cet) (NET 85900).

2.3. Lipid emulsions

Tri-DHA emulsions (10% by TG weight/100 mL emulsion) were made in our laboratory with DHA-TG oil and egg yolk phospholipids (PL) by sonication as previously detailed [22]. To prepare radiolabeled emulsions, [³H]Cet was added to the TG-PL mixture before sonication (0.04 mCi/100 mg TG) [32]. The emulsions were analyzed for the amount of TG and PL using commercial kits (Wako Chemicals USA, Inc., Richmond, VA). The TG:PL mass ratio was 5.0 ± 1.0 , similar to VLDL-sized particles, and their molar ratio was 3.8. The incorporation of [³H]Cet into the emulsion particles were determined by capillary tube centrifugation and emulsion floatation as described previously [33].

2.4. Animal procedures

2.4.1. Unilateral cerebral hypoxic-ischemic injury—Three-day-old C57BL/6J neonatal mice were purchased from Jackson Laboratories (Bar Harbor) with their birth mother. At 10 days of age (p10), mice were subjected to HI insult as previously described [22]. Briefly, HI brain injury was induced by permanent ligation of the right common carotid artery. After 1.5 h of recovery, mice were exposed to hypoxic (humidified 8% O₂/ 92% N₂, Tech Air Inc., NY) insult for 15 min, at $37 \pm 0.3^{\circ}\text{C}$. After hypoxia, at the beginning of reperfusion, mice were returned to a clean home cage to the care of their mothers and were kept at a controlled temperature of 32°C during the reperfusion. For the sham group, animals were anesthetized and the carotid artery exposed without ligation of the artery and hypoxic insult.

2.4.2. Blood clearance and tissue uptake of emulsions in neonatal mice—P10 neonatal naïve, sham and HI mice were injected with a radiolabeled tri-DHA emulsion (0.375 g tri-DHA/kg bw, intraperitoneal (i.p.), single injection). We used a single bolus injection to assess blood clearance and tissue distribution of emulsion particles based on previously established protocols from our laboratory [32,33]. In mice subjected to HI injury, the emulsion was i.p. injected immediately after hypoxia, which we labeled as 0 h

reperfusion. Further, naïve and sham-injured animals were also i.p. injected with the tri-DHA emulsion. The animals were sacrificed at 2 and 8 h after injection of the radiolabeled emulsion. Peritoneal fluid and blood were collected and emulsion clearance assessed by measuring the level of [^3H]CET. Organs and tissues were dissected and removed after perfusion with 0.9% NaCl-containing heparin (2 units/mL). Tissues and organs were homogenized using a Polytron Tissue Disruptor (Omni TH, Kennesaw, GA). Brains were separated into left (contralateral) and right (ipsilateral) hemispheres and the radioactivity measured by liquid scintillation spectrometry [33].

Tritium in blood and organs was assayed by liquid scintillation counting. The samples were suspended in scintillation fluid (Ultima Gold scintillation fluid, PerkinElmer, Boston, MA), mixed and ^3H dpm assayed in a PerkinElmer Tri-Carb liquid scintillation spectrometer 5110 TR. Tissue uptake was expressed as percent of total recovered dose/organ for all the organs analyzed [33].

2.4.3. Analysis of FA composition of plasma lipids—To analyze the changes in FA profile of plasma lipids, samples were collected from neonatal naïve and HI mice injected with tri-DHA emulsion (0.375 g tri-DHA/kg bw, two injections, 1 h apart) at 2, 4 and 8 h after the first injection of tri-DHA emulsion. The tri-DHA treatment regimen, proven effective and safe, was based on previous studies from our laboratory [22,23]. Total lipids were extracted as described previously [34]. Plasma PL, NEFA, TG and cholesteryl esters (CE) were separated by thin layer chromatography (TLC) using the solvent system hexane:diethyl ether:acetic acid (70:30:1) [34]. Plasma LPC were separated by one-dimensional TLC employing the solvent system chloroform:methanol:acetic acid:acetone:water (40:25:7:4:2) [35].

FA composition of lipids in different TLC band regions were determined by GC-MS as previously detailed with modifications [36]. After TLC separation, regions corresponding to individual lipid classes were scraped from the silica plate followed by direct transesterification of lipids from the silica gel. Briefly, the derivatization from FA to FA methyl ester (FAME) consisted of a two-step procedure, involving saponification and methylation with BF_3 -methanol. TLC samples were mixed with 1 mL of sodium hydroxide (NaOH)-methanol (0.4 M concentration) solution and heated at 85°C for 10 min. After cooling to room temperature, another 2 mL of BF_3 -methanol solution (13%) was added to the sample mixture tube and heated at 85°C for an additional 20 min. FAME were extracted with n-hexane. The organic phase was transferred and dried under a stream of N_2 . The samples were reconstituted with 500 μL hexane before GC-MS analysis. DB-23 column (30m $\times$ 0.25mm, 0.25 μm , Agilent Technologies, Inc.) was selected for separating 38 species of FAME and internal standard in a 20 min run time. Agilent 7890B system equipped with a MS detector (Agilent 5977B, EI sources) was used. The system was operated by MassHunter GC/MS acquisition software and the data analysis and quantitation was done using MassHunter Quantitative software (v 07.04).

2.4.4. Assessment of brain DHA and EPA metabolomes—Concentrations of DHA, EPA and its metabolome were measured in HI mice treated with tri-DHA (0.375 g tri-DHA/kg bw, 2 injections, 1 h apart) or control saline at 2 h of reperfusion. We adopted the

same tri-DHA treatment regimen, as previously described [22,23], to promote neuroprotection. All samples for LC-MS-MS-based lipidomics were assayed after solid-phase extraction as described previously [37]. Prior to sample extraction, d8-5-HETE, d5-RvD2, d5-LXA₄, d4-LTB₄, d4-PGE₂ internal standards (500 pg each) in ice-cold methanol were added to the tissue to facilitate quantification and calculation of recoveries. After a gentle dispersion of the tissue in methanol with a glass Dounce, samples were held at -20°C for 45 min to allow protein precipitation. Samples were centrifuged at 1000 *g* for 10 min at 4°C. SPMs were extracted from the supernatant by solid phase extraction using an Extrahera automated extractor (Biotage, Uppsala, Sweden). Samples were acidified to an apparent pH 3.5, loaded onto 3 mL Isolute SPE 100 mg cartridges (Biotage), and rapidly neutralized with double-distilled water. SPMs were eluted from the cartridges with methyl formate. The solvent was then brought to dryness under a gentle stream of nitrogen gas using an automated evaporation system (TurboVap LV, Biotage) and SPMs were immediately resuspended in a methanol-water mixture (50:50, v/v). Extracted samples were analyzed by a liquid chromatography-tandem mass spectrometry system consisted of a QTrap 5500 (AB Sciex, Framingham, MA, USA) equipped with a Shimadzu LC-20AD HPLC (Tokyo, Japan). A Poroshell 120 EC-18 column (100 mm × 4.6 mm × 2.7 μm; Agilent Technologies, Santa Clara, CA, USA) was maintained in a column oven at 50°C, and SPMs were eluted from this HPLC column with a gradient of methanol/water/acetic acid from 55:45:0.01 (v/v/v) to 100:0:0.01 at 0.5 mL/min flow rate. To monitor and quantify the levels of SPMs, targeted multiple reaction monitoring (MRM) and enhanced product ion (EPI) in a negative mode (ion spray voltage: -4000 V) were used. The limits of detection were ~ 0.1 pg. Optimized and reported criteria were used for identification of each lipid mediator, including tandem mass spectrometry (MS/MS) matching to at least 6 diagnostic ion fragments, and a matching retention time to authentic synthetic standards. For each lipid mediator, linear calibration curves were obtained using authentic synthetic standards with *r*² values of at least 0.98.

2.5. Statistical analysis

Values are mean ± SEM for each experimental group. For emulsion distribution studies, 6-7 animals per group were used at each time-point. For GC-MS analysis, each sample was a pool from 4 different animals and the sample size was n=3-4 in each group. For LC-MS analysis, each sample was a pool of either right or left hemispheres from 2 animals and the sample size was n=5-9 in each group. One-way ANOVA, followed by post-hoc two-tailed t-tests, were used to compare groups.

3. Results

3.1. Peritoneal fluid clearance and tissue uptake of tri-DHA emulsion

We determined the distribution of radiolabeled tri-DHA emulsion particles in neonatal mice after i.p. injection. Tri-DHA emulsion particles were efficiently cleared from the peritoneal cavity (Table 1) with no significant differences between naïve and HI animals. At 2 h after the injection, over 1/4 of the injected emulsion remained in the peritoneal cavity in naïve and HI mice. By 8 h, the emulsion was completely cleared with only 1% remaining in the peritoneal cavity in both groups.

Organ and tissue uptake of tri-DHA emulsion particles is shown in Table 2. Lowest uptakes of emulsion particles were in the brain (<0.4% of the recovered dose of radiolabeled emulsion) in both naïve and HI animals, with no significant differences observed between ipsilateral and contralateral brain hemispheres. We noted significantly higher heart uptake of emulsion particles in HI mice compared to naïve animals at 2 h ($p<0.05$) and 8 h ($p<0.001$). Emulsion uptake was low in the lungs (1.3-2.1%) in both naïve and HI groups. Over time, liver radioactivity levels steadily increased while blood radioactivity levels decreased in naïve and HI animals. HI mice showed significantly higher liver uptake of the emulsion particles at 2 h ($p<0.05$) compared to the naïve group. Spleen uptake of emulsion particles was also significantly higher in HI vs naïve animals at 8 h ($p<0.05$). The muscle uptake of emulsion particles was higher at 2 h compared to the 8 h time-point in both groups. The distribution of emulsion particles in the sham-injured animals was similar to naïve mice at 2 h after emulsion injection (data not shown) and hence sham groups were not included in further studies. These results show that, after tri-DHA injection, brain uptake of emulsion particles is low, while the liver plays a major role in emulsion distribution.

3.2. FA composition of plasma lipids

We analyzed changes in DHA and EPA profile of different plasma lipids after tri-DHA administration. In naïve animals, tri-DHA administration had no effect on DHA concentration in LPC fractions (Figure 1A). In contrast, in the HI group, tri-DHA injection significantly increased DHA concentrations in plasma LPC at 2 h ($p<0.05$) and 8 h ($p<0.05$) after injection compared to saline (Figure 1B). DHA concentration in NEFA pools significantly increased in naïve (Figure 2A) animals at 2 h ($p<0.001$), 4 h ($p<0.01$) and 8 h ($p<0.05$) after tri-DHA administration compared to saline-treated littermates. Similarly, DHA concentration significantly increased in NEFA pools in HI animals (Figure 2B) at 2 h ($p<0.05$) and 4 h ($p<0.01$) after injection. Further, TG fractions showed a significant increase of DHA concentration in both naïve (Figure 3A) and HI (Figure 3B) mice. DHA concentration was increased in PL pools in naïve and HI animals only at 2 h after tri-DHA administration compared to the control, while no changes were observed in DHA profile of CE pools at any time-point (data not shown).

Of note, with tri-DHA injection, there was a tendency towards substantial increases in EPA concentration in plasma NEFA pools at 2 h and 4 h in naïve (Figure 4A) and HI (Figure 4B) animals, though only one of the four comparisons reached statistical significance ($p<0.05$). In naïve animals, EPA level in TG pools increased at 2 h and 4 h ($p<0.001$) and decreased at 8 h ($p<0.01$) after tri-DHA injection compared to saline (Figure 5A). In HI animals, tri-DHA injection significantly increased EPA concentration in TG pools at 4 h ($p<0.05$) and 8 h ($p<0.01$) after injection (Figure 5B). LPC-EPA was not detected in plasma at any time-point. No changes were evident in the EPA profile of PL and CE pools at 2 h, 4 h and 8 h after tri-DHA injection (data not shown). Collectively, these results show that acute tri-DHA injection not only rapidly changes DHA, but also EPA profiles in plasma lipid pools.

3.3. Brain lipid mediator - lipidomics

We reported earlier that tri-DHA administration significantly increased brain levels of DHA-derived SPMs, in particular D-series resolvins and neuroprotectins, after HI injury [23]. We

now determined the effect of tri-DHA administration on maresins and monohydroxy-containing DHA-derived products. Since we observed an increase in plasma EPA levels after tri-DHA treatment, we also assessed the effect of tri-DHA administration on EPA-derived SPMs and monohydroxy-containing EPA-derived products. The levels of maresin isomers (7S,14S-diHDHA, 7S,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid; 4S,14S-diHDHA, 4S,14S-dihydroxy-docosa-5E,7Z,10Z,12E,16Z,19Z-hexaenoic acid) significantly increased in tri-DHA-treated mice compared to saline-treated littermates (Figure 6A), while maresin 1 (7R,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid) itself did not change in neonatal HI mice administered with tri-DHA emulsion (data not shown). Resolvin E2 (RvE2, 5S,18R-dihydroxy-eicosa-6E,8Z,11Z,14Z,16E-pentaenoic acid) levels were significantly increased in ipsilateral ($p<0.05$) and contralateral ($p<0.01$) brain hemispheres of HI mice treated with tri-DHA emulsion (Figure 6B).

At 2 h of reperfusion, brain concentration of DHA did not change in either ipsilateral or contralateral samples of tri-DHA group compared to saline (Figure 7A). However, we observed significant increases of monohydroxy-containing DHA-derived products (7-HDHA, 7-hydroxy-docosa-4Z,8E,10Z,13Z,16Z,19Z-hexaenoic acid; 4-HDHA, 4-hydroxy-docosa-5E,7Z,10Z,13Z,16Z,19Z-hexaenoic acid) and SPM pathway markers (17-HDHA, 17-hydroxy-docosa-4Z,7Z,10Z,13Z,15E,19Z-hexaenoic acid; 14-HDHA, 14S-hydroxy-docosa-4Z,7Z,10Z,12E,16Z,19Z-hexaenoic acid) in ipsilateral and contralateral brain hemispheres in tri-DHA-treated mice (Figure 7B). Similar to DHA, we detected no changes in EPA levels in brain hemispheres after tri-DHA injection (Figure 8A). However, when treated with tri-DHA emulsion, HI mice showed a significant boost of monohydroxy-containing EPA-derived products (15-HEPE, 15-hydroxy-eicosa-5Z,8Z,11Z,13E,17Z-pentaenoic acid; 12-HEPE, 12-hydroxy-eicosa-5Z,8Z, 10E,14Z,17Z-pentaenoic acid; 5-HEPE, 5-hydroxy-eicosa-6E,8Z,11Z,14Z,17Z-pentaenoic acid) and SPM pathway marker (18-HEPE, 18-hydroxy-eicosa-5Z,8Z,11Z,14Z,16E-pentaenoic acid) levels in both brain hemispheres compared to saline-treated littermates (Figure 8B). Taken together, these results indicate that tri-DHA administration markedly enhances the levels of both DHA- and EPA-derived SPMs, pathway markers as well as monohydroxy-containing DHA- and EPA-derived products in the brain.

4. Discussion

Our previous results demonstrated that exogenous DHA increased DHA content in brain mitochondria and DHA-derived neuroprotectin D1 (NPD1) and D-series resolvins in cerebral tissue, and these changes likely contributed to mediating neuroprotection after ischemic injury [22,23]. In this study, we further found that liver is a major organ involved in tri-DHA emulsion metabolism, and that LPC and NEFA may represent the key carriers for DHA transport to the brain. Additionally, our results show that there is an interlinked metabolism between DHA and EPA after acute administration of DHA.

In our earlier study, n-3-rich TG emulsions induced transient increases of blood TG levels within a short time period (1.5 h) in neonatal mice after i.p. injection [22], indicating that the emulsion was systemically absorbed after the injection. We confirm herein that tri-DHA

emulsion is efficiently cleared from the peritoneal cavity after i.p. injection, and the liver is responsible for taking up and repackaging TG-rich emulsion particles. If injected emulsion fuses or aggregates in blood, large particles or oil droplets could be deposited in lungs [38]. In contrast, we observed low uptake of tri-DHA in lung in our experiments, indicating the absence of emulsion aggregation in blood. In stroke pathophysiology, emerging studies have provided evidence for interactions between the brain and other peripheral organs which impact their metabolic functions [39,40]. We found significant differences between naïve and HI conditions in the uptake of emulsion particles in heart, liver and spleen, confirming that ischemic brain injury affects the pathways of emulsion clearance and uptake in peripheral organs. We also observed an increase of radioactivity in muscle in both naïve and HI groups, but the reason for this high recovery at an early time-point after emulsion injection is not fully clear. Previous studies have shown that TG uptake in skeletal muscle is related to high lipoprotein lipase (LPL) activity [41,42]; however, further studies are required to assess whether tri-DHA administration changes LPL activity and emulsion uptake specifically in muscle. With regard to uptake of emulsion by the brain, ischemic stroke disrupts the BBB and continually elevates BBB permeability [43,44], influencing the passage of drugs and various metabolites into the injured brain in a more robust way [45]. In contrast, we showed no differences between naïve and HI mice in the uptake of emulsion particles in the brain hemispheres, suggesting that a “leakier” BBB after ischemic injury has little or no effect on whole emulsion particle transport to the brain.

As the amount of DHA entering the brain through whole emulsion particle uptake is low, additional mechanisms most likely are operating in the transport of exogenous DHA to the brain. A previous study showed that, in adult mice intravenously injected with radiolabeled DHA complexed to albumin, liver rapidly took up DHA, which was then esterified and released into plasma in different lipid pools, facilitating DHA transport to the brain [46]. Also, liver produces large amounts of unsaturated LPC and is the primary source of this lipid in plasma [47,48]. Our results on higher uptake of tri-DHA whole emulsion particle by the liver, followed by elevated levels of LPC-DHA and NE-DHA in plasma, indicate that the liver facilitates DHA transport to the brain. Plasma NE-DHA enters the brain through passive diffusion or active pathways mediated by FA-binding proteins and FA transporters [16], while LPC-DHA is transported to the brain through the transporter major facilitator superfamily domain containing 2a (Mfsd2a) found in the BBB [15,49]. Hung et al. initially reported that the metabolic effects of sn-2 LPC-DHA are different from those of sn-1 LPC-DHA; the sn-2 isomer was more potent in reducing inflammation in mice compared to sn-1 LPC-DHA, and this was ascribed to the more effective absorption of sn-2 LPC-DHA *in vivo* [50]. However, more recent studies have shown that both sn-1 and sn-2 isomers of LPC-DHA are absorbed and incorporated into the brain to the same extent [18,49,51]. Thus, in our study, we did not differentiate the two isomers. Furthermore, a recent study has shown that LPC metabolism is affected in stroke [52]. In line with these findings, our results show reduction in LPC-DHA levels in HI saline animals compared to naïve saline, confirming the modulation of plasma LPC profiles by ischemic insult. However, when HI animals were treated with tri-DHA emulsion, LPC-DHA levels were restored similar to the naïve group, indicating that LPC might represent a target carrier for DHA following injection of exogenous DHA.

SPMs are highly potent molecules with bioactivity in picomolar to nanomolar concentrations and endogenously synthesized in murine and human brains and other organs [5]. Conventional anti-inflammatory agents, such as cyclooxygenase-2 inhibitors, are non-specific inhibitors of eicosanoid pathways and thereby prevent active resolution processes, while SPMs promote resolution of inflammatory responses through multiple mechanisms at the tissue level [53]. Thus, SPMs block neutrophil and eosinophil infiltration, stimulate non-phlogistic recruitment of monocytes, induce macrophage uptake of apoptotic neutrophils and increase the exit of phagocytes from the inflammatory site to the lymphatics [54]. In earlier studies, our laboratory found that acute administration of tri-DHA emulsion after HI injury increased brain levels of protectins (NPD1) and D-series resolvins [23]. Other studies have also shown that RvE2 and 7S,14S-diHDHA reduced polymorphonuclear leukocyte infiltration and displayed anti-inflammatory properties in murine models of inflammation [55,56]. The elevated levels of 7S,14S-diHDHA, 4S,14S-diHDHA, isomers of maresin 1 within the maresins family, and also RvE2, observed in this study, confirm a potential enhancement of anti-inflammatory and proresolving responses by tri-DHA treatment to counteract ischemic injury.

The actions of SPMs are stereochemically selective, reflecting their routes of biosynthesis and their ability to activate tissue-specific receptors and molecular pathways. Thus, alcohol chirality and double bond geometry are important parameters in defining the conformation of each lipid mediator and their related bioactivity [57]. While cytochrome P450 (CYP) monooxygenases participate in producing both *S* and *R* conformation alcohols in PUFA [58,59], in our system we focused mainly on lipid mediators derived by specific lipoxygenases and cyclooxygenases, which synthesize mainly *S* conformation molecules. Hydroxylation of DHA and EPA leads to the generation of hydroxydocosahexaenoic acids and hydroxyeicosapentaenoic acids [60]; some of these mediators from DHA and EPA are pathway markers for the synthesis of SPMs, and their production serves as an index of activation of anti-inflammatory and pro-resolving downstream pathways [61,62]. In addition, these pathway markers themselves have been shown to exhibit anti-inflammatory and pro-resolving actions [63,64]. In the present study, we found significant increases in brain levels of pathway markers from DHA and EPA, that, together with SPMs, might contribute to the marked neuroprotection observed with tri-DHA treatment after HI injury [23]. Of note, brain levels of DHA and EPA themselves remained unchanged after tri-DHA injection, suggesting a rapid conversion of these PUFA to their metabolites in the brain.

In a separate pilot study, when lipidomic profiles after tri-DHA injection were analyzed in spleen, we found markedly elevated levels of both DHA- and EPA-derived SPMs, pathway markers and monohydroxy-containing DHA- and EPA-derived products in HI mice compared to saline-injected mice (data not shown). These preliminary data prompt to further investigate whether SPMs and pathway markers produced in tissues other than the brain also contribute to tri-DHA neuroprotective effects after HI injury.

DHA supplementation significantly increases blood and tissue levels of EPA in rodent models and in humans [30,31,65,66]. However, these changes are observed when DHA is provided via oral route and also after long time periods of supplementation (days/weeks). We now show that acute injection of tri-DHA emulsion rapidly induces increases in EPA and

EPA-derived SPMs and pathway markers in plasma and brain within a few hours. The increase in EPA levels after oral DHA administration has been associated with DHA slowing EPA turnover, resulting in a longer half-life of EPA, without significant changes in retroconversion contributing to this effect [30,31,67,68]. Also, DHA reduces expression of Elovl5 and Elovl2, enzymes involved in EPA elongation into docosapentaenoic acid (DPA_n-3) [69], and may also downregulate β -oxidation and/or other pathways involving EPA catabolism [30]. These factors may have contributed to the increased levels of EPA metabolomes observed in this study, as high EPA availability leads to its conversion to monohydroxy-containing EPA-derived products and E-series resolvins. Our results provide a basis for future studies on how acute DHA administration rapidly changes EPA metabolic pathways. Although our previous study reported no effects on infarct size after tri-EPA administration in HI injury [22], other reports have shown EPA to be beneficial against depression, cardiovascular diseases and inflammation [70–72]. Hence, our results strongly suggest that the metabolic pathways derived from DHA and EPA are interlinked and should be taken into consideration when interpreting the protective mechanisms of these PUFA.

As a limitation of our study, there is no direct evidence on the exact molecular transport pathways that carry DHA into the brain. Future studies on n-3 PUFA receptors/transporters employing inhibitors and knockout models will aim to characterize the potential mechanisms operating in DHA brain delivery after tri-DHA emulsion administration. Also, one could consider studies of EPA TG emulsions. EPA is a key molecule in the DHA synthetic pathway [73] and some studies have already reported that increasing EPA intakes can lead to increases in DHA levels in certain tissues [74,75]. Understanding how acute administration of DHA or EPA affects the levels and SPM-related products of the other will require more complex experimentation using differently labeled DHA and EPA, and compartmental analysis of the tracer kinetic data. Still, these limitations do not diminish the significance of our work, demonstrating early and rapid DHA-EPA interlinked metabolism as a key step in post-HI neuroprotection observed with tri-DHA treatment.

Increases in DHA, EPA and monohydroxy-containing DHA and EPA derived products following tri-DHA administration suggest a therapeutic potential for bolus administration of tri-DHA as strategy for rapid delivery of n-3 FA to tissues and organs. With the increasing evidence that DHA and EPA do have distinct effects on cardiovascular and other disease endpoints [29,76], it is important to explore whether DHA or EPA alone are sufficient to reach maximal cardiovascular benefits, or whether specific combinations of DHA and EPA would be more bioactive and advantageous as stroke therapeutics.

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Abbreviations:

[³H]CEt	[³ H] cholesteryl hexadecyl ether
DHA	docosaheptaenoic acid
EPA	eicosapentaenoic acid
FA	fatty acids
LPC	lysophosphatidylcholine
NEFA	non-esterified fatty acids
SPM	specialized pro-resolving mediator
TG	triglyceride
PL	phospholipids
PUFA	polyunsaturated fatty acids
7S,14S-diHDHA	7S,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid
4S,14S-diHDHA	4S,14S-dihydroxy-docosa-5E,7Z,10Z,12E,16Z,19Z-hexaenoic acid
Maresin 1	7R,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid
Resolvin E2	RvE2, 5S,18R-dihydroxy-eicosa-6E,8Z,11Z,14Z,16E-pentaenoic acid
17-HDHA	17-hydroxy-docosa-4Z,7Z,10Z,13Z,15E,19Z-hexaenoic acid
14-HDHA	14S-hydroxy-docosa-4Z,7Z,10Z,12E,16Z,19Z-hexaenoic acid
7-HDHA	7-hydroxy-docosa-4Z,8E,10Z,13Z,16Z,19Z-hexaenoic acid
4-HDHA	4-hydroxy-docosa-5E,7Z,10Z,13Z,16Z,19Z-hexaenoic acid
18-HEPE	18-hydroxy-eicosa-5Z,8Z,11Z,14Z,16E-pentaenoic acid
15-HEPE	15-hydroxy-eicosa-5Z,8Z,11Z,13E,17Z-pentaenoic acid
12-HEPE	12-hydroxy-eicosa-5Z,8Z,10E,14Z,17Z-pentaenoic acid
5-HEPE	5-hydroxy-eicosa-6E,8Z,11Z,14Z,17Z-pentaenoic acid

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Highlights

- DHA injected as triglyceride emulsion (tri-DHA) was associated with highest uptake in the liver while brain has the lowest
- Rapid increases in plasma LPC-DHA levels were observed in animals subjected to ischemic injury and acutely treated with tri-DHA
- Tri-DHA administration increased DHA derived SPMs in brain after ischemic injury
- Tri-DHA administration increased EPA and EPA derived SPMs and pathway markers in plasma lipid pools and in brain

Figure 1. DHA levels in plasma lysophosphatidylcholine (LPC).

DHA concentrations of plasma LPC in neonatal (p10) naïve (A) and hypoxic-ischemic (HI) (B) mice at 2, 4 and 8 h after tri-DHA injection (0.375 g tri-DHA/kg bw, two injections). Values are mean $\pm$ SEM, n=3-4. Each sample is a pool from 4 different animals. *p<0.05, compared to saline group. Data was analyzed by one-way ANOVA, followed by post-hoc two-tailed t-tests.

Figure 2. DHA levels in plasma non-esterified fatty acids (NEFA).

DHA concentrations of plasma NEFA in neonatal (p10) naïve (A) and hypoxic-ischemic (HI) (B) mice at 2, 4 and 8 h after tri-DHA injection (0.375 g tri-DHA/kg bw, two injections). Values are mean $\pm$ SEM, $n=3-4$. Each sample is a pool from 4 different animals. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to saline group. Data was analyzed by one-way ANOVA, followed by post-hoc two-tailed t-tests.

Figure 3. DHA levels in plasma triglyceride (TG).

DHA concentrations of plasma TG in neonatal (p10) naïve (A) and hypoxic-ischemic (HI) (B) mice at 2, 4 and 8 h after tri-DHA injection (0.375 g tri-DHA/kg bw, two injections). Values are mean $\pm$ SEM, $n=3-4$. Each sample is a pool from 4 different animals. * $p < 0.05$, compared to saline group. Data was analyzed by one-way ANOVA, followed by post-hoc two-tailed t-tests.

Figure 4. EPA levels in plasma non-esterified fatty acids (NEFA).

EPA concentrations of plasma NEFA in neonatal (p10) naive (A) and hypoxic-ischemic (HI) (B) mice at 2, 4 and 8 h after tri-DHA injection (0.375 g tri-DHA/kg bw, two injections). Values are mean $\pm$ SEM, n=3-4. Each sample is a pool from 4 different animals. *p<0.05, compared to saline group. Data was analyzed by one-way ANOVA, followed by post-hoc two-tailed t-tests.

Figure 5. EPA levels in plasma triglyceride (TG).

EPA concentrations of plasma TG in neonatal (p10) naïve (A) and hypoxic-ischemic (HI) (B) mice at 2, 4 and 8 h after tri-DHA injection (0.375 g tri-DHA/kg bw, two injections).

Values are mean $\pm$ SEM, $n=3-4$. Each sample is a pool from 4 different animals. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to saline group. Data was analyzed by one-way ANOVA, followed by post-hoc two-tailed t-tests.

Figure 6. Specialized pro-resolving mediators derived from DHA.

Maresin 1 isomers (7S,14S-diHDHA, 4S,14S-diHDHA) (A) and resolvin E2 (RvE2) (B) content in ipsilateral (ipsi) and contralateral (contra) brain hemispheres of mice treated with saline or tri-DHA (0.375 g tri-DHA/kg bw, two injections). Values are mean $\pm$ SEM, $n=5-9$. Each sample is a pool from 2 different animals, * $p<0.05$, ** $p<0.01$ compared to saline group. Data was analyzed by one-way ANOVA, followed by post-hoc two-tailed t-tests.

Figure 7. DHA pathway markers.

Content of DHA (A) and monohydroxy-containing DHA derived products/ pathway markers (B) in ipsilateral (ipsi) and contralateral (contra) brain hemispheres of mice treated with saline or tri-DHA (0.375 g tri-DHA/kg bw, two injections). Values are mean $\pm$ SEM, n=9. Each sample is a pool from 2 different animals, *p<0.05, **p<0.01, ***p<0.001 compared to saline group. Data was analyzed by one-way ANOVA, followed by post-hoc two-tailed t-tests.

Figure 8. EPA pathway markers.

Content of EPA (A) and monohydroxy-containing EPA derived products/ pathway marker (B) in ipsilateral (ipsi) and contralateral (contra) brain hemispheres of mice treated with saline or tri-DHA (0.375 g tri-DHA/kg bw, two injections). Values are mean $\pm$ SEM, $n=9$. Each sample is a pool from 2 different animals, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to saline group. Data was analyzed by one-way ANOVA, followed by post-hoc two-tailed t-tests.

Table 1:

Clearance of radiolabeled tri-DHA emulsions from the peritoneal cavity in neonatal (p10) naïve and HI mice at 2 and 8 h after a single i.p. injection (0.375 g tri-DHA/ kg body weight)

Groups	% injected dose
Naïve 2 h	27.4 ± 2.3
HI 2 h	28.6 ± 3.7
Naïve 8 h	1.4 ± 0.2 *** ###
HI 8 h	1.2 ± 0.1 *** ###

Values are mean ± SEM, n=6-7

p<0.001, compared to naïve 2 h

p<0.001, compared to HI 2 h

Table 2:

Tissue uptakes (% of recovered dose) of radiolabeled tri-DHA emulsion in neonatal (p10) naïve and HI mice at 2 and 8 h after single i.p. injection (0.375 g tri-DHA/kg body weight)

	2 h		8 h	
	<i>Naïve</i>	<i>HI</i>	<i>Naïve</i>	<i>HI</i>
Brain contra	0.13 ± 0.02	0.12 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
Brain ipsi	0.15 ± 0.03	0.16 ± 0.03	0.13 ± 0.01	0.17 ± 0.02
Heart	6.04 ± 1.29	9.1 ± 0.49 [*]	5.35 ± 0.59	8.8 ± 0.41 ^{***}
Lung	1.76 ± 0.62	2.13 ± 0.72	1.36 ± 0.10	1.31 ± 0.09
Liver	25.6 ± 0.83	33.4 ± 3.19 [*]	65.7 ± 1.62 [#]	59.6 ± 1.3 ^{*,f}
Kidney	0.89 ± 0.09	1.06 ± 0.11	1.16 ± 0.11	1.24 ± 0.16
Spleen	0.89 ± 0.09	1.20 ± 0.14	0.82 ± 0.15	1.4 ± 0.15 [*]
Muscle	20.9 ± 4.15	15.6 ± 2.91	11.4 ± 0.84 [#]	10.1 ± 1.01
Blood	43.5 ± 3.74	37.1 ± 3.98	13.9 ± 0.96 [#]	17.1 ± 0.8 ^{*,f}

Values are mean ± SEM, expressed as % of total recovered dose from the organs/tissues listed above, n=6-7

Contra, contralateral hemisphere; ipsi, ipsilateral hemisphere

^{*}
p<0.05,

^{***}
p<0.001, naïve vs HI

[#]
p<0.05, naïve 2 h vs naïve 8 h

^f
p<0.05, HI 2 h vs HI 8 h