IN VIVO APPROACHES TO QUANTIFYING AND IMAGING BRAIN ARACHIDONIC AND DOCOSAHEXAENOIC ACID METABOLISM

STANLEY I. RAPOPORT, MD

A novel in vivo fatty acid method has been developed to quantify and image brain metabolism of nutritionally essential polyunsaturated fatty acids (PUFAs). In unanesthetized rodents, a radiolabeled PUFA is injected intravenously, and its rate of incorporation into brain phospholipids is determined by chemical analysis or quantitative autoradiography. Results indicate that about 5% of brain arachidonic acid (20:4 n–6) and of docosahexaenoic acid (22:6 n–3) acid are lost daily by metabolism and are replaced from dietary sources through the plasma. Calculated turnover rates of PUFAs in brain phospholipids, due to deesterification by phospholipase A2 (PLA2) followed by reesterification, are very rapid, consistent with active roles of PUFAs in signal transduction and other processes. Turnover rates of arachidonate and docosahexaenoate are independent of each other and probably are regulated by independent sets of enzymes. Brain incorporation of radiolabeled arachidonate can be imaged in response to drugs that bind to receptors coupled to PLA2 through G proteins, thus measuring PLA2-initiated signal transduction. The in vivo fatty method is being extended for human studies using positron emission tomography. (J Pediatr 2003;143:S26-S34)

The nutritionally essential, polyunsaturated fatty acids (PUFAs), arachidonic acid (AA, 20:4 n–6) and docosahexaenoic acid (DHA, 22:6 n–3), make up about 20% and 30% of fatty acids (FAs) in phospholipids of brain and retina, respectively,1,2 and modulate the structure and function of these organs.3-5 PUFAs cannot be synthesized de novo from 2-carbon fragments by mammalian tissue, nor do biochemical pathways exist for the interconversion of their n–3 and n–6 forms. Thus, they must be obtained from dietary sources. Linoleic acid (LA, 18:2 n–6) and α-linolenic acid (α-LNA, 18:3 n–3) in the diet can serve as precursors of AA and DHA, respectively.6

Controversy exists about the forms, pathways, and rates of long-chain FA transfer from blood into brain, and about their incorporation, subsequent metabolism, and interactions within brain.7-9 However, a consensus is forming that normal brain function and structure depend on a correct balance between n–3 and n–6 PUFAs and that if this balance is disturbed, cognitive and behavioral changes may result.10 Dietary insufficiency of n–3 when fed with high n–6 PUFAs throughout fetal and infant development has been shown to decrease retinal, visual, and brain development in monkeys.3,11 Studies in preterm infants also have shown that dietary PUFA supplementation increases visual function development and some aspects of neural development.12,13 However, the effects of dietary PUFAs on the development of term gestation infants are uncertain.14 Based on clinical studies in preterm infants, an expert panel recently recommended that formulas for preterm infants contain LA and α-LNA at a ratio of 16:1 to 6:1, and that if added, AA and DHA should be at a ratio of 1.5:1 to 2:1.15

A number of brain processes are thought to depend on the presence of adequate concentrations of AA and DHA as well as on balanced interactions between n–3 and n–6 PUFAs.16 These processes include apoptosis,17 gene transcription,18 neurite outgrowth,19 membrane excitability,20 prostaglandin formation,21,22 desaturation–elongation, membrane fluidity and elasticity,23-25 cerebral ischemia,26 and inflammatory and immunological events.27

| AA       | Arachidonic acid |
| COX      | Cyclooxygenase   |
| DHA      | Docosahexaenoic acid |
| FA       | Fatty acid       |
| HETE     | Hydroxyeicosatetraenoic acid |
| LA       | Linoleic acid    |
| α-LNA    | α-Linolenic acid |
| PET      | Positron emission tomography |
| PLA2     | Phospholipase A2 |
| cPLA2    | Cytosolic phospholipase A2 |
| PUFA     | Polyunsaturated fatty acid |
| sn       | Stereospecifically numbered |

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In addition to fetal and infant development, decreased n–3 compared with n–6 PUFA has been implicated as contributing to the clinical signs and complications of Alzheimer disease and age-related cognitive disturbances, depression and alcoholism. Peroxisomal disorders are characterized by a marked deficit in brain DHA. In addition, evidence that dietary n–3 PUFA supplementation may be beneficial in bipolar disorder patients and that lithium feeding of rats reduces AA but not DHA turnover in their brain phospholipids (see Table II below), has led to the hypothesis that bipolar disorder symptoms involve excessive n–6 compared with n–3 brain PUFA metabolism.

To test the hypothesis that imbalances in brain PUFA metabolism can lead to disturbed brain function and to examine how a normal PUFA balance maintains normal function, we need new methods to quantify and image brain PUFA metabolism in vivo. I present such a method and data obtained with it in this paper. The method has been used to examine brain AA and DHA metabolism in unanesthetized rats and is being extended for human studies with positron emission tomography (PET).

**Experimental Method**

In unanesthetized, loosely restrained rodents, the FA method involves injecting intravenously an albumin-bound unesterified radiolabeled FA, then measuring labeled and unlabeled unesterified FA concentrations in plasma at fixed times until the animal is killed and its brain is subjected to high-energy microwaving to instantaneously stop metabolism. Total lipids are extracted from the brain and analytical techniques are used to quantify the labeled and unlabeled FA in individual lipid pools (phospholipids, neutral lipids, unesterified fatty acids, acyl-CoAs). In parallel studies, after the animal is killed, the intact brain, without prior microwaving, is removed and frozen, then sliced into coronal sections that are subjected to quantitative autoradiography. Data derived from these studies have shown that intravenously injected FA

<table>
<thead>
<tr>
<th>PUFA</th>
<th>Plasma unesterified concentration, $c_{\text{plasma}}$ (nmol/mL)</th>
<th>Incorporation coefficient, $k^*$ (1/s)</th>
<th>Incorporation rate, $J_{\text{in}}$ (µmol/g/d)</th>
<th>Brain esterified concentration, $c_{\text{brain}}$ (µmol/g)</th>
<th>Turnover $J_{\text{in}}/c_{\text{brain}}$ (%/d)</th>
<th>Half-life 0.693/turnover (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonate</td>
<td>9–16</td>
<td>$4.5 \times 10^{-7}$</td>
<td>0.3–0.6</td>
<td>13.0</td>
<td>3–5%</td>
<td>12–23</td>
</tr>
<tr>
<td>Docosahexaenoate</td>
<td>7–25</td>
<td>$5.5 \times 10^{-7}$</td>
<td>0.4–1.2</td>
<td>15.3</td>
<td>2–8%</td>
<td>7–34</td>
</tr>
</tbody>
</table>

Table I. Rates of incorporation and turnover of arachidonic and docosahexaenoic acids due to replacement and metabolic loss in brain lipids of unanesthetized rats

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>$\lambda^\dagger$</th>
<th>Inositol</th>
<th>Choline</th>
<th>Ethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.04 ± 0.00</td>
<td>15.3 ± 0.4</td>
<td>18.3 ± 0.6</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.18 ± 0.02</td>
<td>2.6 ± 0.1</td>
<td>5.0 ± 0.3</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>Docosahexaenoate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.03 ± 0.00</td>
<td>17.7 ± 1.7</td>
<td>3.1 ± 0.4</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.03 ± 0.00</td>
<td>31.0 ± 9.0</td>
<td>4.5 ± 1.2</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Palmitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.02 ± 0.00</td>
<td>29.1 ± 2.6</td>
<td>7.0 ± 0.4</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.02 ± 0.00</td>
<td>26.0 ± 1.2</td>
<td>5.1 ± 0.3</td>
<td>3.5 ± 0.1</td>
</tr>
</tbody>
</table>

Table II. Chronic lithium administration selectively reduces arachidonate turnover due to deesterification-reesterification in rat brain phospholipids

Steady-state ratio of acyl-CoA to plasma specific activity.
Turnover equals $J_{\text{in}}/c_{\text{brain}}$.
From Chang, 1996; Chang, 1999.

In addition to fetal and infant development, decreased n–3 compared with n–6 PUFA has been implicated as contributing to the clinical signs and complications of Alzheimer disease and age-related cognitive disturbances, depression and alcoholism. Peroxisomal disorders are characterized by a marked deficit in brain DHA. In addition, evidence that dietary n–3 PUFA supplementation may be beneficial in bipolar disorder patients and that lithium feeding of rats reduces AA but not DHA turnover in their brain phospholipids (see Table II below), has led to the hypothesis that bipolar disorder symptoms involve excessive n–6 compared with n–3 brain PUFA metabolism.

To test the hypothesis that imbalances in brain PUFA metabolism can lead to disturbed brain function and to examine how a normal PUFA balance maintains normal function, we need new methods to quantify and image brain PUFA metabolism in vivo. I present such a method and data obtained with it in this paper. The method has been used to examine brain AA and DHA metabolism in unanesthetized rats and is being extended for human studies with positron emission tomography (PET).
Tracers disappear from blood with a half-life of less than 1 minute and that 1% of the injected dose is rapidly incorporated into stable brain lipids, particularly phospholipids. In awake human beings or anesthetized monkeys, the method involves administration of a positron-labeled PUFA such as \([1-11C]AA\) and quantitatively imaging “net” brain incorporation of tracer using PET.

Compartmental Representation of Brain Arachidonic Acid Metabolism

Figure 1 illustrates the compartments and pathways of brain AA metabolism and of plasma-brain AA exchange that contribute to the “arachidonic acid cascade.” This cascade includes a continuous cycle in which AA is released from phospholipids and then is reincorporated, or lost by conversion to eicosanoids or other bioactive metabolites, by oxidation or by backdiffusion to plasma (not shown). AA and its eicosanoid products in this cascade are important second messengers that affect many brain processes. Comparable cycles may be constructed for the metabolism of DHA (where conversion to “docosanoids” has been demonstrated to be catalyzed by cyclooxygenase [COX]-2 in brain parenchyma and lipoxygenase in the pineal gland) and of palmitic acid (in this case, de novo synthesis from 2-carbon fragments within brain is included and conversion to “eicosanoid like” molecules doesn’t occur).

Plasma-Brain Exchange

Figure 1 illustrates how an intravenously injected radiolabeled AA can be used to quantify AA incorporation and turnover in brain phospholipids, and AA loss from brain by metabolism. The unesterified AA in blood is >99% bound to circulating plasma albumin but can rapidly dissociate from the albumin as blood passes through the brain. It can be calculated that virtually all albumin-bound AA in plasma will dissociate within the 1 to 2 seconds that blood passes through the brain, and that 95% will be extracted by brain. Because the rate of extraction by brain is independent of changes in cerebral blood flow, this rate reflects only the plasma unesterified AA concentration and brain metabolic demand.
of circulating lipoproteins do not measurably enter brain, at least in adult mammals. The quantity incorporated in brain phospholipids from plasma is determined by the rate of esterification via acyl-CoA (Fig 1). The unidirectional rate of incorporation can be determined from measured plasma radioactivity and concentration of the FA and the amount of radioactivity esterified into brain lipids over the period of time from administration of the radiolabeled FA.

The rate of incorporation, equals the product of the unesterified unlabeled plasma concentration $c_{\text{plasma}}$ and of the incorporation coefficient $k^*$,

$$J_{\text{inc}} = k^* c_{\text{plasma}}$$

$k^*$ is measured incorporated brain radioactivity at time of death $T$ after tracer injection, divided by integrated plasma radioactivity (plasma input function) between the start of injection at time $t = 0$ and $t = T$,

$$k^* = \frac{c_{\text{brain}}(T)}{\int_0^T c_{\text{plasma}} \, dt}$$

and $c_{\text{plasma}}$ and $c_{\text{brain}}(T)$ are plasma and brain radioactivity (at $T$), respectively.

### Incorporation into Brain Phospholipids

AA in plasma can cross the blood-brain barrier and enter an "exchangeable" brain unesterified AA pool at the endoplasmic reticulum (Fig 1). From this pool, it can be rapidly activated as a thioester to arachidonoyl-CoA, then be transferred as an ester to stereospecifically numbered ($sn$)-2 sites of available lysophospholipids. Studies have indicated that AA is esterified mainly into the $sn$-2 position of phosphatidylinositol or phosphatidylcholine, whereas DHA is esterified mainly into the $sn$-2 position of brain phosphatidylethanolamine and phosphatidylcholine. Less than 10% of either PUFA that enters brain is lost through $\beta$-oxidation in the process. In contrast, 50% of labeled palmitic acid that enters brain undergoes $\beta$-oxidation, whereas the remainder is esterified mainly into the $sn$-1 position of phosphatidylcholine.

### Evidence for Two Unesterified Brain AA Compartments and Coordination Between Phospholipase A2 and Cyclooxygenase-2 at Synapses

At a steady-state brain AA concentration, the rate of AA esterification from brain arachidonoyl-CoA into the $sn$-2 position of phospholipids must equal its rate of release, which is catalyzed by phospholipase A2 (PLA2). There are three major PLA2 isoforms in brain: (1) an AA-selective cytosolic cPLA2 (85 kDa, Type IV), which requires Ca$^{2+}$ for translocation to the membrane followed by phosphorylation for activation; (2) an AA-selective secretory sPLA2 (14 kDa, Types IIA and V), also Ca$^{2+}$ dependent; (3) a nonselective Ca$^{2+}$-independent intracellular iPLA2 (101 kDa, Type VI).

After AA is released by PLA2, Figure 1 illustrates that it enters an unesterified brain AA pool that does not directly exchange with AA in plasma and is largely located at the synapse. This is the precursor pool for conversion to eicosanoids. It is not yet known how much AA released from phospholipids is converted to eicosanoids either at rest or in response to PLA2 activation, or how much of the released AA is reesterified into phospholipids. In this regard, in a PUFA-deficient cell line, about 15% of released AA was converted to eicosanoids at a high media AA concentration.

Two unesterified brain AA pools, one that can exchange with plasma AA and one that cannot, are consistent with evidence from cultured cells that the AA that is released from phospholipids by PLA2 is preferentially converted to eicosanoids, whereas exogenous unesterified AA is not converted. The presence of two distinct AA pools is also suggested from studies with labeled AA infusion in awake rats, that show that unlabeled AA, when released from brain phospholipids during neuroinflammation, reduces the brain unesterified AA specific activity (radioactivity/concentration) without changing the specific activity of brain arachidonoyl-CoA. In normal brain, the specific activity of arachidonoyl-CoA approximates the specific activity of total unesterified brain AA.

Localizing the released AA at the synapse is consistent with immunohistochemical evidence that cPLA2 and the COX-2 that converts AA to prostaglandins are both localized postsynaptically. cPLA2 and COX-2 may act in a coordinated fashion within the AA cascade, perhaps by forming an active complex with each other on the phospholipid membrane. Additionally, cPLA2 and COX-2 are likely to have co-evolved, as their genes are adjacent to each other on human chromosome 1.

### Arachidonic Acid Recycling in Phospholipids

Figure 1 illustrates that the released AA that is not converted to eicosanoids will diffuse, when bound to a fatty acid binding protein (FABP), to the AA pool at the endoplasmic reticulum (Fig 1). From there, it can exchange with AA in plasma or be activated to arachidonoyl-CoA by an acyl-CoA synthetase with the consumption of 2 ATP molecules. A small fraction of AA in arachidonoyl-CoA will undergo $\beta$-oxidation, whereas the remainder will be esterified into the $sn$-2 position of available lysophospholipids by an acyltransferase. AA-selective acyl-CoA synthetases and acyltransferases have been described and are colocalized at endoplasmic reticulum membranes.

Two AA "cycles" can be identified in Figure 1. The first, AA deesterification-reesterification within phospholipids, involves the serial actions of PLA2, acyl-CoA synthetase, and acyltransferase. This cycle is considered to regulate downstream brain AA metabolism, particularly its conversion to bioactive eicosanoids. The second cycle involves loss of AA within brain to eicosanoids, by $\beta$-oxidation, and by back diffusion to plasma (which may be quite low because of AA retention within brain by fatty acid binding proteins), followed by replacement by plasma AA. Two comparable brain cycles exist for DHA.
Kinetics of Metabolism-Replacement Cycle

At a steady state, it can be seen from Figure 1 that the rate at which unesterified unlabeled AA is incorporated into brain phospholipids from plasma approximates the net rate of AA release from brain phospholipids followed by loss to eicosanoids, by β-oxidation, and by back-diffusion to plasma.68 The rate of incorporation of unesterified plasma AA and DHA into rat brain phospholipids has been estimated as 0.3 to 0.6 and 0.4 to 1.2 μmol/g per day, respectively (Table I). When divided by the concentration of the respective esterified FA in brain, these rates provide estimates of the rate of turnover due to loss followed by replacement, equal to 3% to 5% per day for AA and 2% to 8% per day for DHA. Corresponding half-lives (0.693/turnover) equal 12 to 23 and 7 to 34 days (Table I). Such long half-lives may explain why it takes many weeks to recover normal brain DHA concentrations in animals fed an n–3 adequate diet after prolonged n–3 dietary deprivation.77 A slow recovery from n–3 PUFA deprivation can also reflect slow removal from brain phospholipids of accumulated docosapentaenoic acid (22:5 n–6).1

It is possible that plasma LA can contribute to the brain arachidonoyl-CoA pool, after it is elongated and desaturated in brain. Comparable lipid solubilities and molecular weights suggest that the unesterified LA and α-LNA precursors in plasma can enter brain as fast as their AA and DHA.68,78 Indeed, LA and α-LNA have been shown to be taken up by the brain of the immature functionally hepatectomized rat, after their intravenous injection,79 and LA has been reported to be converted to AA in isolated microsomes from the immature pig brain.80 To the extent that LA and α-LNA are converted to AA and DHA within brain (which remains to be determined), daily turnover rates of brain AA and DHA, calculated from the incorporation rates of these PUFAs, would be greater than the estimates in Table I.68

Kinetics and Independence of Deesterification-Reesterification Cycles of Arachidonic and Docosahexaenoic Acids

The ratio of AA turnover due to its loss from brain and replacement from plasma (Table I), to AA turnover due to deesterification-reesterification in phospholipids, can be experimentally determined during continuous tracer infusion as the specific activity of the acyl-CoA pool relative to the specific activity of plasma.9,38 This ratio (λ) normally approximates 0.04 (Table II), which implies that unesterified AA in plasma contributes about 4% to brain acyl-CoA, compared with a much higher contribution by unesterified AA released from phospholipids. The relative contributions are roughly illustrated by the relative thicknesses of arrows in Figure 1.

In rats, AA and DHA turnover rates due to deesterification-reesterification in brain phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol can be of the order of 15% to 20% per hour (Table II), corresponding
to half-lives of just a few hours. This evidence for rapid PUFA recycling within some phospholipids is consistent with the recognized participation of PUFAs in signal transduction, neuroplasticity, and other important cell processes. Net FA recycling within brain phospholipids consumes about 5% of the ATP produced by the brain as a whole.

Two recent studies in awake rats suggest that the deesterification-reesterification cycles of AA and DHA in brain phospholipids are independent of each other. For example, Table II illustrates that feeding rats lithium chloride for 6 weeks to produce a “therapeutically equivalent” brain lithium concentration of 0.7 mmol40,83,84 reduced AA turnover in brain phospholipids by 80% while not affecting turnover of DHA or palmitic acid. Concurrently, during tracer infusion the specific activity of arachidonoyl-CoA was increased, suggesting a reduced contribution of AA released from brain phospholipids compared with control. In another study, three generations of rats were deprived of n–3 PUFAs in their diet. The DHA concentration and DHA turnover rate in brain phospholipids were markedly reduced, but there was no change in AA concentration or turnover rate.

Together, the lithium and n–3 PUFA deprivation studies suggest that independent sets of coordinately regulated enzymes—belonging to PLA2, acyl-CoA synthetase and acyltransferase classes—determine AA and DHA turnover rates within brain phospholipids. While we do not yet know which of these enzymes was targeted by n–3 PUFA deprivation, we have shown that lithium chloride feeding downregulated the transcription, and thus the activity, of the AA-selective cPLA2, which explained lithium’s reduction of AA turnover. The AA-selective iPLA2 was unaffected. Lithium’s effects on cPLA2 and AA turnover were accompanied by a posttranscriptional reduction in COX-2 activity and reduced formation of prostaglandin E2, an AA metabolite produced through COX.

Imaging Signaling Transduction Involving Phospholipase A2

PLA2 may be coupled through a G protein to a number of neuronal or astrocytic receptors, including cholinergic muscarinic receptors, dopaminergic D2 receptors, serotonergic 5-HT2A/C receptors, or β2-adrenergic receptors. Because of this coupling, it has been possible to use quantitative autoradiography in awake rats to image AA incorporation into specific brain regions, after PLA2 activation by cholinergic, dopaminergic, or serotoninergic drugs acting at these receptors. Figure 2 illustrates increased incorporation of radiolabeled AA into brain regions containing muscarinic M1 receptors, in response to the muscarinic agonist arecoline. The increased incorporation could be blocked by the muscarinic antagonist, atropine, or by the PLA2 inhibitor mannoalide. It was shown to represent selectively increased esterification of label into synaptic membrane phosphatidylcholine and phosphatidylinositol. Increased labeled DHA incorporation into brain synaptic membrane phosphatidylethanolamine and phosphatidylcholine has been noted in response to arecoline.

PET Scanning of Brain PUFA Metabolism

Our studies in awake rodents provide a conceptual and experimental basis for using intravenously injected radiolabeled AA or DHA to quantify baseline and activated brain processes involving PLA2. We are extending these
studies to image brain AA and DHA metabolism in human subjects by means of PET, when using the positron-emitting radiotracers, [1-13C]AA or [1-11C]DHA. PET is a non-invasive way to localize such radiotracers in brain because it can quantify and localize the gamma rays that are released at 180° from each other from emitted positrons, with a resolution of < 10 mm. Magnetic resonance imaging can be used in addition to PET to construct a 3-dimensional volumetric image of the brain, on which the “functional” PET images are superimposed for quantifying regional radioactivity. Mathematical models are used to calculate relevant regional biochemical or functional parameters from the data.

[1-13C]AA incorporation into brain has been successfully imaged with PET in healthy young human volunteers, after a preclinical study in anesthetized macaques first established uptake and dose parameters for the tracer, and confirmed that uptake was independent of cerebral blood flow. Figure 3 illustrates mean horizontal [1-11C]AA images of incorporation coefficients \( k^* \) (Equation 1) in the human brain, and compares them with images in the same subjects of regional cerebral blood flow measured with \([15O]H_2O\). I thank Drs James C. Demar and Thad A. Rosenberger for their very helpful comments and suggestions about this manuscript.

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