**n-3 Polyunsaturated Fatty Acids Reduce Neonatal Hypoxic/Ischemic Brain Injury by Promoting Phosphatidylyserine Formation and Akt Signaling**

Wenting Zhang, MD, PhD; Jia Liu, MD; Xiaoming Hu, MD, PhD; Peiying Li, MD, PhD; Rehana K. Leak, PhD; Yanqin Gao, PhD; Jun Chen, MD

**Background and purpose**—Omega-3 polyunsaturated fatty acids (n-3 PUFAs) attenuate neonatal hypoxic/ischemic (H/I) brain damage, but the underlying mechanisms are not fully understood. This study tested the hypothesis that n-3 PUFAs enhance Akt-dependent prosurvival signaling by promoting the biosynthesis of phosphatidylyserine in neuronal cell membranes.

**Methods**—Dietary n-3 PUFA supplementation was initiated on the second day of pregnancy in dams. H/I was induced in 7-day-old rat pups by ipsilateral common carotid artery occlusion followed by hypoxia (8% oxygen for 2.5 hours). Neurological outcomes, brain tissue loss, cell death, and the activation of signaling events were assessed after H/I. The effects of n-3 PUFAs (docosahexaenoic acid and eicosapentaenoic acid) on oxygen-glucose deprivation–induced cell death and the underlying mechanism of protection were also examined in primary cortical neuron cultures.

**Results**—n-3 PUFAs reduced brain tissue loss at 7 days after H/I and improved neurological outcomes, whereas inhibition of PI3K/Akt signaling by LY294002 partially abrogated this neuroprotective effect. Docosahexaenoic acid/eicosapentaenoic acid also prevented ischemic neuronal death through the Akt prosurvival pathway in vitro. Furthermore, docosahexaenoic acid/eicosapentaenoic acid increased the production of phosphatidylyserine, the major membrane-bound phospholipids, after ischemia both in vitro and in vivo. A reduction in membrane phosphatidylyserine by shRNA-mediated knockdown of phosphatidylyserine synthetase-1 attenuated Akt activation and neuronal survival after docosahexaenoic acid/eicosapentaenoic acid treatment in the oxygen-glucose deprivation model.

**Conclusions**—n-3 PUFAs robustly protect against H/I-induced brain damage in neonates by activating Akt prosurvival pathway in compromised neurons. In addition, n-3 PUFAs promote the formation of membrane phosphatidylyserine, thereby promoting Akt activity and improving cellular survival. *(Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.115.010815.)*

**Key Words:** docosahexaenoic acids ■ eicosapentaenoic acid ■ neurons ■ omega-3 fatty acids ■ phosphatidylyserine synthase

Perinatal hypoxia/ischemia (H/I) is a common cause of death and long-term neurological disability in children. 1 Although advances in obstetric care have increased the number of infants surviving H/I, no current strategies effectively reduce or rescue post H/I neuronal damage and prevent the resulting encephalopathy. The extent of neuronal damage after H/I is at least, in part, determined by a balance between pro-death and prosurvival intracellular signals. The PI3K/Akt signaling cascade is one of the major survival pathways in cells and has been increasingly implicated in neuronal survival after H/I damage. Phosphorylation of Akt at Ser-473 and Thr-308, a consequence of PI3K activation, is observed early after cerebral ischemia, especially in the ischemic penumbra, in both adults 2 and neonates. 3 Intracerebroventricular injections of LY294002, a PI3K inhibitor, reduces phosphorylation of Akt and exacerbates neuronal damage after ischemia. 4 In contrast, activation of the PI3K/Akt signaling pathway provides marked neuroprotective effects against cerebral ischemia. 4 Therefore, Akt activity-enhancing drugs may represent a new class of therapeutics for the treatment of H/I in neonates.

Epidemiological studies have shown an inverse association between the intake of n-3 polyunsaturated fatty acids...
(n-3 PUFAs) and the risk of ischemic stroke.\textsuperscript{5,6} n-3 PUFAs are a family of unsaturated fatty acids that play an essential role in the anatomic and biomedic-}


tical integrity of the brain. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the main n-3 PUFAs in the central nervous system.\textsuperscript{7} Almost all DHA and EPA in the normal brain are located in the cell membrane bilayer.\textsuperscript{8} The high electron density of DHA and EPA impart fluidity to the cell membrane, a feature that is essential for the survival, renewal, and other biochemical functions of cells. Beyond conferring membrane fluidity, DHA and EPA are used continuously for the biogenesis and maintenance of neuronal membranes, and associated with many physiological functions, such as learning and memory.\textsuperscript{8–10} Both DHA and EPA are structurally intermingled with larger phospholipid molecules in the neuronal membrane, in particular aminophospholipids, such as phosphatidylserine and phosphatidyethanolamine. Our group and others have demonstrated that supplementation with n-3 PUFAs, especially DHA, robustly reduces the extent of neuronal damage and neurological deficits after neonatal H/I\textsuperscript{11} and adult cerebral ischemia.\textsuperscript{12–14} However, the cellular and molecular mechanisms of n-3 PUFAs-afforded neuroprotection after neonatal H/I brain injury are not fully understood.

In this study, we used primary neuronal cultures and a rat model of neonatal H/I to elucidate the mechanisms underlying n-3 PUFA-afforded protection against ischemic neuronal injury. We demonstrated that Akt activation is critical in mediating n-3 PUFA-afforded neuronal protection. Furthermore, we found that DHA and EPA increase the neuronal membrane content of phosphatidylserine, which in turn enhances Akt activity and promotes neuronal survival after ischemia.

Materials and Methods

\textbf{n-3 PUFA Treatment and Model of Neonatal H/I Brain Injury}

Pregnant Sprague–Dawley rats (Charles River Laboratories) were maintained on a regular diet (Prolab 5P76 Isopro 3000, PMI Nutrition, n-3 PUFA low, N3L) or an n-3 PUFA-enriched diet (n-3 PUFA high, N3H) starting the second day of pregnancy until 14 days after parturition. Daily chow consumption of dams and weight gain of offspring were monitored throughout the experiment and showed no significant difference between N3L and N3H groups (not shown). Seven-day-old neonates were subjected to H/I brain injury as previously described.\textsuperscript{15} Briefly, the left common carotid artery was ligated under anesthesia with isoflurane. After 1.5 hours of recovery, rat pups were subjected to hypoxia in a chamber balanced with a humidified atmosphere of 8% oxygen/92% nitrogen for 2.5 hours and submerged in a 37°C water bath to maintain normothermia.

In \textit{vitro} Model of Neuronal H/I

Primary cortical neurons were prepared from 17-day-old Sprague–Dawley rat embryos and incubated in a humidified atmosphere containing 95% air and 5% \textit{CO}_2. Transient oxygen-glucose deprivation (OGD) for 60 minutes was applied to 12-day-old cultures to model ischemia-like conditions in vitro.\textsuperscript{15} DHA or EPA was added to the cultures 24 hours before OGD. Cell death was evaluated after OGD using live/dead staining and the lactate dehydrogenase assay (Sigma) as described.\textsuperscript{15}

\textbf{Statistical Analysis}

All data are reported as mean±SEM. Significant differences between mean were assessed by ANOVA and post hoc least significant difference tests for multiple comparisons. \textit{P}≤0.05 was considered statistically significant. All behavioral and histological observations were made by blinded investigators.

Full description of all the experimental procedures are available in the online-only Data Supplement.

\section*{Results}

\textbf{n-3 PUFAs Protect Against Neonatal H/I Brain Damage}

Infarct volume and cell morphology were visualized using Cresyl Violet staining of Nissl substance at 7 days after H/I. There was a large area of tissue loss after H/I in the ipsilateral cerebral cortex, striatum, and hippocampus of N3L animals. As expected, n-3 PUFA supplementation significantly attenuated H/I-induced tissue loss in all these H/I sensitive regions, resulting in a reduction of total tissue loss (Figure 1A). As shown in Figure 1B, numerous dying cells characterized by shrunken profiles and pyknotic nuclei were present on the ipsilateral side of N3L animals and perinatal n-3 PUFA supplementation reduced H/I-induced cell damage. No significant difference was observed on the contralateral side between N3L and N3H animals.

\textbf{n-3 PUFAs Improve Neurological Outcomes After Neonatal H/I}

Sensorimotor dysfunction was evaluated by measuring gait and the righting reflex at 2 to 7 days after H/I or sham operation (Figure 1C). No significant difference was detected in neurological function between the 2 sham groups exposed to different diets. n-3 PUFA supplementation significantly ameliorated H/I-induced acute sensorimotor dysfunctions, as revealed by improved performance in the gait test at 2 to 3 days after H/I and an improved righting reflex at 2 to 6 days after H/I. No significant difference was observed between the 2 diet groups at 7 days post H/I, suggesting potential behavioral compensation over time (Figure 1C).

The Morris water maze was administered at 8 weeks after H/I to evaluate long-term cognitive deficits. No significant differences were observed in water maze performances between the 2 sham-operated groups with different diets (Figure 1D). Strikingly, n-3 PUFA supplementation significantly attenuated H/I-induced cognitive disabilities, as manifested by reduced time to locate the hidden platform in cued trials (improved learning) and increased time spent in the target quadrant when the platform was removed in the probe test (improved memory). There was no difference in swimming velocity among groups. These findings demonstrate that n-3 PUFAs improve spatial learning and memory without any confounding effect on swimming speed.

\textbf{n-3 PUFAs Attenuate H/I-Induced Cell Death}

The effects of n-3 PUFAs on H/I-induced cell death was determined by detecting activated caspase-3 and caspase-9, DNA fragmentation, and Fluoro-Jade B staining of degenerating neurons. Elevations in cleaved caspase-3 and caspase-9, which were detected at 16 and 24 hours post injury, were significantly blocked by n-3 PUFA treatment as expected (Figure 2A and 2B). Consistently, immunostaining revealed
a dramatically reduced number of caspase-3-positive cells in N3H brains when compared with N3L brains at 24 hours after H/I insult (Figure 2C). Neuronal DNA damage, as visualized by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) staining in NeuN-positive cells, was significantly increased in the ipsilateral cortex at 24 hours after H/I in N3L animals, whereas n-3 PUFAs almost completely suppressed H/I-induced DNA damage (Figure 2D). In addition, degenerating neurons characterized by bright Fluoro-Jade B fluorescence throughout the nucleus and cell body were diminished by n-3 PUFAs in the ipsilateral cortex (Figure 2D). Taken together, these results suggest that n-3 PUFAs have a robust protective effect against H/I-induced cell death.

**n-3 PUFAs-Afforded Neuroprotection is Akt Dependent**

Activation of Akt is important for neuronal survival in the ischemic brain. Consistent with our previous report, phosphorylated Akt (p-Akt) in cortical extracts was initially reduced 30 minutes after H/I, elevated transiently 3 hours after H/I, and then significantly reduced below control levels from 9 hours onwards. In contrast, n-3 PUFAs supplementation promoted the phosphorylation of Akt at 0.5 and 16 to 24 hours after H/I (Figure 3A). Immunofluorescent staining was then performed at 24 hours after H/I to determine alterations in neuronal expression of p-Akt. Declined expression of p-Akt and widespread condensed nuclei were observed in NeuN-positive neurons in the ipsilateral cortex of N3L animals. As expected, n-3 PUFAs supplementation markedly inhibited the decline of neuronal p-Akt after H/I (Figure 3B).

The prosurvival role of Akt is associated with its downstream substrate, glycogen synthase kinase 3β (GSK-3β). GSK-3β serves as a proapoptotic enzyme by negatively regulating various transcriptional factors, including AP-1 and CREB. On activation, Akt phosphorylates and inactivates GSK-3β at its N-terminus (Ser9). Here, we found that the elevated levels of p-Akt after H/I in the N3H animals were accompanied by increased phosphorylation of GSK-3β (Figure 3A), further confirming that Akt activity is indeed enhanced by n-3 PUFAs.

To further substantiate the involvement of Akt in the neuroprotective mechanism of n-3 PUFAs, the PI3K inhibitor...
LY294002 was applied intracerebroventricularly 1 hour before H/I. The activation of p-Akt and p-GSK-3β in N3H pups at 24 hours after H/I was inhibited by LY294002 as expected (Figure 3C). Furthermore, LY294002 significantly, albeit partially, abolished the protective effects of n-3 PUFAs at 7 days after H/I (Figure 3D).

**Figure 2.** n-3 Polyunsaturated fatty acids attenuate hypoxia/ischemia (H/I)-induced cell death. A, Representative Western blot images of cleaved caspase-3 and cleaved caspase-9. B, Quantification of data shown in (A). Cortical extracts were obtained from animals on N3L and N3H diets after sham operation or H/I (n=5 per condition). #P≤0.05 vs sham, *P≤0.05 vs N3L H/I at the same time point. C and D, Tissue sections from cerebral cortex were stained for cleaved caspase-3 (C) NeuN, TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) (D, top), or Fluoro-Jade B (D, bottom) at 24 hours after H/I (n=4 per group). Scale bar, 25 μm.

**Figure 3.** Activation of Akt in neurons is critical for the neuroprotective effects of n-3 polyunsaturated fatty acids against hypoxia/ischemia (H/I). A, Western blot analyses of phosphorylated Akt (p-Akt), Akt, p-GSK-3β, and GSK-3β in cortical extracts. Cortical extracts were obtained from animals on N3L and N3H diets after sham operation or H/I (n=6 per condition). #P≤0.05 vs sham, *P≤0.05 vs N3L H/I at the same time point. B, Representative coronal tissue sections immunostained for NeuN and p-Akt or labeled with Hoechst 33258 (n=4 per group). Scale bar, 20 μm. C and D, Five millilitre LY294002 (50 mmol/L) were injected during the course of 15 minutes at 1 hour before H/I. C, top, Western blots of p-Akt, total Akt, p-GSK-3β, total GSK-3β, and β-actin in cortical extracts at 24 hours after sham operation or H/I. D, Tissue loss at 7 days after H/I in N3L and N3H groups with or without LY294002 injection (n=14 per group). #P<0.05 vs sham, *P<0.05, **P<0.01. GSK indicates glycogen synthase kinase.
n-3 PUFAs Prevent OGD-Induced Neuronal Death

To confirm the direct neuroprotective effects of n-3 PUFAs on neurons and verify the underlying mechanisms, we used a well-established in vitro OGD model of H/I. Primary cortical neurons were pretreated with different concentrations of DHA, EPA, or vehicle for 24 hours. Lactate dehydrogenase release and cell viability, as determined by quantification of live/dead staining, were measured at 24 hours after OGD. DHA and EPA remarkably attenuated lactate dehydrogenase release (Figure 4A) and preserved cell viability (Figure 4B) post-OGD, with maximal protection observed at 20 \( \mu \)mol/L of DHA or 10 \( \mu \)mol/L of EPA. The OGD-induced decrease of p-Akt was inhibited by DHA and EPA in primary cortical neurons (Figure 4C). Moreover, the Akt inhibitor mitigated the neuroprotection afforded by DHA and EPA (Figure 4D).

Thus, the in vitro experiments replicated the in vivo results and confirmed that n-3 PUFAs prevent ischemic neuronal death by enhancing the Akt cell survival pathway.

Phosphatidylserine is Required for the Neuroprotection by n-3 PUFAs

Because the PI3K inhibitor only partially inhibited n-3 PUFA- afforded neuroprotection against H/I, additional mechanisms of protection upstream of PI3K-Akt or independent of PI3K may be engaged by n-3 PUFAs. Interestingly, we found that the brain content of phosphatidylserine was significantly increased in the N3H P7 rat (Table). Further analyses of PUFA content in phosphatidylserine revealed an increase in the amount of n-3 PUFAs and a concomitant decrease in n-6 PUFAs in N3H rats. As a result, the ratio of n-3 PUFAs/n-6 PUFAs in phosphatidylserine significantly increased in n-3 PUFAs-supplemented P7 rats. This phenomenon is also evident in vitro in that pretreatment with DHA or EPA dramatically enhanced the neuronal membrane content of phosphatidylserine in a concentration-dependent manner (Figure 5A).

Lentivirus-mediated knockdown of phosphatidylserine synthase-1 (PSS1), an enzyme that catalyzes the biosynthesis of phosphatidylserine from phosphatidylcholine or phosphatidylethanolamine, completely abrogated the accumulation of membrane phosphatidylserine induced by fatty acids (Figure I in the online-only Data Supplement), demonstrating the importance of PSS1 in the actions of DHA and EPA.

To further investigate the role of phosphatidylserine in Akt activation and neuronal survival after OGD, neurons were transfected with PSS1 shRNA using lentivirus vector 3 days before OGD, and then treated with DHA and EPA 2 days later. PSS1 shRNA inhibited DHA/EPA-enhanced Akt phosphorylation (Figure 5B) and neuronal survival (Figure 5C) at 2 and 6 hours after OGD. Taken together, these data suggest that PSS1-dependent phosphatidylserine formation is essential for DHA/EPA-mediated Akt activation and ischemic protection (Figure 5D).

Discussion

H/I brain damage remains a serious problem for neonates because of the lack of effective preventive or therapeutic strategies. Our previous research has shown that perinatal...
supplementation with an n-3 PUFA-enriched diet increases the cortical content of n-3 PUFAs in neonates, and confers long-term neuroprotection against H/I brain injury through suppression of the nuclear factor-κB–mediated inflammatory response. Here, our in vivo and in vitro studies suggest that n-3 PUFAs may also directly protect neurons from H/I injury in immature brains. This is consistent with previous work showing that n-3 PUFAs prevent neuronal death induced by multiple stimuli, including serum deprivation, amyloid-β oligomers, and traumatic brain damage. However, there is some controversy in that treatment with DHA or EPA has also been reported to increase neuronal death in response to prion protein. Thus, it is possible that the effects of n-3 PUFAs on neuronal survival depend on the nature of the stimulus or insult.

Our results suggest that n-3 PUFAs promote Akt signaling, a cardinal prosurvival factor that helps mitigate ischemic brain damage. The procedure of Akt activation is separated into 3 steps: membrane translocation, conformational change, and phosphorylation. As the first and the most fundamental step, cytosolic Akt moves toward the plasma membrane by interacting with PIP3. PI3K is a protein well known to regulate PIP3 production from PIP2 on the intracellular leaflet of cell membranes and to promote Akt phosphorylation and activation. We found that n-3 PUFAs increased phosphorylation and activation of the PI3K/p85 subunit (Figure IIA and IIB in the online-only Data Supplement), which could result in the activation of downstream Akt. However, the data showing that the PI3K inhibitor only partially inhibited n-3 PUFA-afforded

<p>| Table. Phosphatidylserine Content and PUFA Composition in Phosphatidylserine of P7 Rat Brains After 2 Different Diets |</p>
<table>
<thead>
<tr>
<th>N3L</th>
<th>N3H</th>
</tr>
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<tbody>
<tr>
<td>Phosphatidylserine, nmol/mg protein</td>
<td>88.53 (81.78–95.20)</td>
</tr>
<tr>
<td>DHA (n-3) percentage</td>
<td>37.40% (35.10–39.70)</td>
</tr>
<tr>
<td>EPA (n-3) percentage</td>
<td>0.67% (0.62–0.72)</td>
</tr>
<tr>
<td>DPA (n-3) percentage</td>
<td>2.06% (1.99–2.13)</td>
</tr>
<tr>
<td>ALA (n-3) percentage</td>
<td>3.38% (3.17–3.59)</td>
</tr>
<tr>
<td>AA (n-6) percentage</td>
<td>26.80% (25.06–28.54)</td>
</tr>
<tr>
<td>LA (n-6) percentage</td>
<td>6.97% (6.48–7.46)</td>
</tr>
<tr>
<td>DTA (n-6) percentage</td>
<td>15.2% (14.05–16.35)</td>
</tr>
<tr>
<td>DPA (n-6) percentage</td>
<td>3.34% (2.79–3.89)</td>
</tr>
<tr>
<td>N-3/N-6 ratio</td>
<td>0.8318</td>
</tr>
</tbody>
</table>

Brain lipid extracts were examined for PUFA content in phosphatidylserine using GC analysis; n=8 in each group. AA indicates arachidonic acid; ALA, alpha-Linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DTA, docosatetraenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; and PUFA indicates polyunsaturated fatty acid.

*P<0.05, **P<0.01.
neuroprotection against H/I suggest that there might be additional mechanisms of protection upstream of PI3K-Akt or independent of PI3K.

Another potentially important finding of our study is that n-3 PUFAs increase phosphatidylserine accumulation in neuronal membranes. This is in line with existing evidence in different cell types that DHA induces alterations in the phospholipid composition of membranes and increases phosphatidyserine biosynthesis.29-31 Phosphatidylserine is important for the structural and physical properties of biological membranes and has been shown to modulate protein localization.32 Compared with phosphatidylcholine, the most abundant phospholipid in eukaryotic cell membranes, phosphatidylserine in neurons comprises only ≈10% of total phospholipids. Phosphatidylserine is conventionally recognized as a signal for the removal of apoptotic cells. In normal cells, phosphatidylserine is preferentially concentrated in the inner leaflet of the plasma membrane in an energy-dependent manner.33,34 Apoptosis disturbs the asymmetrical distribution of phosphatidylserine and results in the appearance of phosphatidylserine on the outside of cells. Phosphatidylserine exposure at the outer surface of cells triggers their uptake and removal by macrophages.35 The distribution of phosphatidylserine is highly distinct among different tissues and different cell types. It is mainly enriched in the retina and brain, especially in neuronal plasma membranes, a finding that implicates phosphatidylserine in neuronal development, function, or survival.7

In this study, we discovered that phosphatidylserine may be involved in additional functions in neurons beyond cell removal. Our results suggest that phosphatidylserine biosynthesis is critical for DHA- or EPA-affected Akt activation and neuroprotection, as inhibition of phosphatidylserine synthesis by lentivirus-mediated knockdown of PSS1 reduced phosphatidylserine content in the cell membrane, inhibited the phosphorylation of Akt, and abolished the observed protective effects of both fatty acids. Therefore, the alterations in neuronal phosphatidylserine synthesis may profoundly influence cell survival by modulating the Akt survival signaling pathway.

The exact mechanisms by which phosphatidylserine content in the membrane modulates Akt activity are not clear. It is possible that elevated membrane phosphatidylserine after n-3 PUFAs treatment modulates activity of multiple membrane proteins along a signaling pathway by regulating their membrane targeting. Consistent with this assumption, membrane-concentrated phosphatidylserine has been proven to facilitate PI3K-dependent membrane translocation of Akt, a prerequisite step for its full activation.28,29,36 Moreover, there are interactions between Akt and phosphatidylinerse that enable Akt phosphorylation at Ser-473 even in the absence of PI3P in vitro.36 This may explain the partial inhibition of n-3 PUFAs in neuronal membranes and the Akt inhibitor.

Although the neuroprotective effect of n-3 PUFAs against H/I is critically dependent on Akt activity, other mechanisms may also be contributing factors. For example, phosphatidylinerse may participate in the membrane translocation and activation of other prosurvival signaling molecules, such as PKC and Raf-1 kinases.30,31,37 It has also been shown that ERK 1/2 activation contributes to cell survival after H/I.38 We observed that n-3 PUFAs enhanced phosphorylation of ERK 1/2 in the cortex after H/I (Figure IIB and IIC in the online-only Data Supplement), suggesting that Akt may act in concert with other neuroprotective pathways to promote neuronal survival. This possibility is consistent with evidence of cross talk between the Akt and ERK signaling cascades.39 In addition, emerging evidence suggests that n-3 PUFAs may achieve neuroprotection through indirect mechanisms by regulating the function of non-neuronal glial cells, including microglia40 and astrocytes.41 It is therefore possible that n-3 PUFAs may interfere with destructive glial responses, which are important for the progression of neuronal damage after H/I.

In conclusion, our research suggests that perinatal supplementation with n-3 PUFAs robustly protects against brain damage with improved neurological outcomes after H/I. We have further illustrated that n-3 PUFAs inhibit neuronal cell death in a phosphatidylserine- and Akt-dependent manner. Specifically, n-3 PUFAs enhance the biosynthesis of phosphatidylserine in neurons. On the one hand, sufficient phosphatidylserine on the neuronal membrane may facilitate the membrane translocation of Akt in a PI3K-dependent manner, thereby enhancing the subsequent phosphorylation and activation of Akt. On the other hand, phosphatidylserine may also directly interact with Akt and enhance its phosphorylation/activation. Activated Akt may then activate the proapoptotic protein GSK-3β and, perhaps, other substrates, resulting in suppression of neuronal cell death after H/I (Figure 5D). These studies support the view that n-3 PUFAs supplementation during pregnancy may help prevent ischemic damage to the developing brain and preserve neurological function in infancy and that phosphatidylserine and Akt are promising targets for improving neonatal brain repair.

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Disclosures

None.

References

2. Noshita N, Lewin A, Sugawara T, Chan PH. Evidence of phosphorylation of Akt and neuronal survival after transient focal cerebral


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Supplemental Methods:
Assessment of brain damage
Coronal brain sections extending rostrocaudally from +1.1 to -2.5 mm from bregma were obtained at 7 days after H/I and stained with Cresyl Violet for tissue loss assessment as previously described\(^1\). Tissue loss in the ipsilateral versus contralateral hemisphere was determined by calculating the area of surviving tissue in each section using the MCID image analysis system (St. Catharine’s). The percentage of volume loss is calculated as described. All measurements were made by a blinded investigator.

Immunohistochemistry, TUNEL staining and Fluoro-Jade B staining
Immunofluorescent staining was performed on 4% paraformaldehyde-perfused coronal sections from pups euthanized 24 hours after H/I as described previously\(^1\). The primary antibodies were applied to the tissue: anti-p-Akt (Ser473) (1:50; Cell Signaling), anti-cleaved-caspase-3 (1:200; Cell Signaling), or anti-NeuN (1:500; Chemicon International, Inc.). Terminal transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using the *in situ* Cell Death Detection Kit (Roche). Fluoro-Jade B staining was carried out according to the manufacturer’s instructions (Chemicon International, Inc.).

Neurological evaluation
Righting reflex and gait testing were performed within 7 days after H/I to evaluate sensorimotor neurological functions, as reported previously\(^2\). Briefly, the righting reflex was determined by placing the animals in a supine position and measuring the time needed for the animals to change to prone position with all four paws in contact with the floor. Gait testing measured the average time for animals to successfully move out of a 13-cm diameter circle on each observation day. Cognitive deficits were examined using the Morris water maze at 8 weeks after H/I as detailed previously\(^3\).

Intracerebroventricular injection
Intracerebroventricular injections were made with a 33-gauge needle and a 10 μl Hamilton syringe at the following coordinate: 2.0 mm rostral, 1.5 mm lateral, and 2.0 mm deep in relation to the lambda. Five μl LY294002 (50 mmol/L; Calbiochem) were injected over the course of 15 min at one hour before H/I.

Gene transfection of primary neurons
The primary neuronal cultures were infected for 3 days with Lenti-shPSS1, or the control vectors (empty vector and Lenti-EGFP). A scrambled sequence containing
the same nucleotide composition in a randomized order was used as control.

**Analysis of plasma membrane phosphotidylserine**
Isolation of the plasma membrane was performed using differential centrifugation as described previously. Analyses of the phospholipids were performed on an Agilent 1100 MSD coupled with an Agilent 1100 HPLC system using a Prodigy C18 column (150 × 2.0 mm, 5 mm; Phenomenex) as described previously. PS was separated and PUFA composition was analyzed by gas chromatography (GC) as described.

**Supplemental Figures:**

**Figure I.** Primary neuronal cultures were transfected with PSS1 shRNA or scrambled vehicle using lentivirus vector for three days. **A.** Representative images of MAP-2 staining (red) and transfected EGFP (lentivirus vector, green). **B.** Western blots of PSS1 showing PSS1 knockdown. **C.** Knockdown of PSS1 inhibits PS formation following DHA or EPA (n=4 per group). *p<0.05, **p<0.01 vs. PSS1 sc group.
Figure II. n-3 PUFAs activate PI3K/p85 and ERK1/2 after neonatal H/I. Cortical extracts were obtained from animals on N3L and N3H diets after sham operation or H/I. A. Western blot analyses of phosphorylated p85 (p-p85), total p85 (t-p85), and β-actin in cortical extracts. B. Quantification of the expression of p-p85 and p-Erk. C. Western blot analyses of phosphorylated Erk (p-Erk), total Erk (t-Erk), and β-actin in cortical extracts. n=6 per condition. #p≤0.05 vs. sham, *p≤0.05 vs. N3L H/I at the same time.

Supplemental References: