Omega-3 Polyunsaturated Fatty Acid Supplementation Confers Long-Term Neuroprotection Against Neonatal Hypoxic–Ischemic Brain Injury Through Anti-Inflammatory Actions

Wenting Zhang, MD, PhD*; Xiaoming Hu, MD, PhD*; Wei Yang, MS; Yanqin Gao, MD; Jun Chen, MD

Background and Purpose—Current available therapies for neonatal hypoxia/ischemia (H/I) brain injury are rather limited. Here, we investigated the effect of omega-3 polyunsaturated fatty acids on brain damage and long-term neurological function after H/I in neonates.

Methods—Female rats were treated with or without an omega-3 polyunsaturated fatty acids-enriched diet from the second day of pregnancy until 14 days after parturition. Seven-day-old neonates were subjected to H/I and euthanized 5 weeks later for evaluation of tissue loss. Neurological impairment was assessed progressively for 5 weeks after H/I by grid walking, foot fault, and Morris water maze. Activation of microglia and production of inflammatory mediators were examined up to 7 days after H/I.

Results—Omega-3 polyunsaturated fatty acid supplementation significantly reduced brain damage and improved long-term neurological outcomes up to 5 weeks after neonatal H/I injury. Omega-3 polyunsaturated fatty acids exerted an anti-inflammatory effect in microglia both in an in vivo model of H/I and in in vitro microglial cultures subjected to inflammatory stimuli by inhibiting NF-κB activation and subsequent release of inflammatory mediators.

Conclusions—Our results suggest that omega-3 polyunsaturated fatty acids confer potent neuroprotection against neonatal H/I brain injury through, at least partially, suppressing a microglial-mediated inflammatory response. (Stroke. 2010;41:2341-2347.)

Key Words: hypoxia/ischemia • inflammation • polyunsaturated fatty acids

Neonatal hypoxic/ischemic (H/I) brain injury is a leading cause of perinatal mortality and morbidity. Unfortunately, all clinically available agents to treat asphyxiated infants have failed to show a consistent, significant benefit in reducing long-term neurological deficits. Thus, a new preventive and therapeutic strategy for perinatal H/I brain injury is imperative.

Abundant evidence points to inflammation as a major player in the progression of H/I-induced brain injury in both adults and neonates. Cerebral inflammatory response is characterized by the activation of microglia and subsequent production of inflammatory mediators and free radicals. Although prompt microglial activation after brain injury has been recognized to be beneficial to brain recovery, prolonged overstimulation of microglia is incriminated in causing secondary damage to neurons and ultimately exacerbating structural injury and neurological dysfunction. The immature brain is exceedingly susceptible to the consequences of inflammatory response compared with its adult counterpart. In fact, the extent of inflammatory response has been demonstrated to correlate with the severity of brain injury and neurological deficits in infants with H/I. Therefore, interventions aimed at attenuating postischemic inflammation are promising as therapeutic strategies to reduce brain damage and improve long-term recovery after H/I injury.

It has been shown that omega-3 polyunsaturated fatty acids (n-3 PUFAs) are able to modulate inflammatory responses. The beneficial impacts of n-3 PUFAs have been shown in many human peripheral diseases with an inflammatory component. The brain is enriched with long-chain n-3 PUFAs, especially docosahexaenoic acid (DHA). The effect of n-3 PUFAs on neurological disorders displaying neuroinflamma-
tion is also under investigation. Some research has shown that n-3 PUFAs protect against ischemic brain damage in adult stroke models. Our recent study, along with other reports, has suggested the beneficial effect of DHA pretreatment on brain damage and functional outcomes at 7 days after H/I in neonatal rats. However, the long-term effect of n-3 PUFAs and the mechanisms whereby n-3 PUFAs exert their protection on the immature brain with H/I are largely unknown.

In the current study, we found that dietary supplementation of n-3 PUFAs to pregnant female rats resulted in robust and prolonged neuroprotection in the neonatal brain after H/I. We further demonstrated that the protective effect of n-3 PUFAs against H/I is attributed, at least partially, to the attenuated activation of the microglial NF-κB signaling pathway and inflammatory responses.

Materials and Methods
n-3 PUFA Treatment and Model of Neonatal H/I Brain Injury
The experimental procedure was approved by the Institutional Animal Use and Care Committee at the University of Pittsburgh. Timed pregnant female Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) were fed a regular laboratory rodent diet (Prolab 5P76 Isopro 3000; PMI Nutrition) with a low n-3 PUFA concentration (0.5%) or a regular diet supplemented with n-3 PUFAs (DHA/EPA, 15 mg in every gram of regular diet) from the second day of pregnancy to 14 days after parturition. Seven-day-old rat pups were subjected to H/I brain injury as previously described. Briefly, the left common carotid artery was ligated under anesthesia with 3% isoflurane. After a 1.5-hour recovery period, the pups were placed in a chamber containing a humidified atmosphere of 8% O₂/92% N₂ for 2.5 hours and then returned to their dams.

Assessment of Brain Damage
Brain sections were stained with cresyl violet as previously described for tissue loss assessment. Volume loss in the ipsilateral versus contralateral hemisphere was determined by calculating the amount of surviving tissue per section using the MCID image analysis system (St Catharine’s, Ontario, Canada). The percentage of volume loss is eventually calculated using the following equation: (volume of contralateral hemisphere / volume of ipsilateral hemisphere) / volume of contralateral hemisphere.

Neurological Evaluation
Animals were subjected to behavioral assessment at 2 to 5 weeks after H/I. Rats were placed on a stainless steel grid floor (20 cm × 40 cm with a mesh size of 4 cm²) elevated 1.5 m above the floor to evaluate locomotor activity and coordination as previously described. Cognitive deficits were examined using the Morris water maze at 5 weeks after H/I as detailed previously.

Real-Time Polymerase Chain Reaction Analysis
Real-time polymerase chain reaction was performed as described. Quantitative polymerase chain reaction was performed with the Opticon 2 Real-Time Polymerase Chain Reaction Detection System (Bio-Rad) using corresponding primers and the SYBR green Polymerase Chain Reaction Master Mix (Applied Biosystems). The cycle time values of the genes of interest were first normalized with

Figure 1. Cortical lipid profile of neonates. A, Timeline showing the induction of H/I and the time points for various assays. B, Concentration of DHA and EPA in the cortex from the N3L and N3H diet groups. C, The cortical concentration of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) of 14-day-old neonates from the regular diet group (N3L, n=6) and the n-3 PUFAs-supplemented group (N3H, n=7). Data are expressed as percentage of total fatty acids. D, The ratio of n-3/n-6 PUFAs. N=7 per group. ##P<0.01 versus N3L sham; *P<0.05, **P<0.01, ***P<0.001 versus N3L H/I.

Figure 2. Long-term neuroprotection of n-3 PUFAs against neonatal H/I brain injury. A, Representative photographs of cresyl violet-stained brain coronal sections at 5 weeks after H/I brain damage. B, Quantification of tissue loss at 5 weeks after H/I (n=17 per group). *P<0.05, **P<0.01 versus N3L H/I. C, Micrographs show the histology changes in the cortex (CTX), striatum (ST), and hippocampus CA1 (CA1) from lesioned (ipsilateral) and unlesioned (contralateral) sides. Scale bar=25 μm.
glyceraldehyde-3-phosphate dehydrogenase of the same sample and then the gene expression levels were calculated and expressed as fold changes versus sham.

**Lipid Extraction and Fatty Acid Analysis**
Cortex samples were fast frozen and dried into powder in a vacuum freeze dryer until subjected to analysis. The fatty acids were extracted as described previously. Tissue fatty acid methyl ester peak identification was performed by comparison to the peak retention times of a 30-component methyl ester standard (Sigma). The concentration of each fatty acid was determined by calculating the areas of peaks.

**Microglia Cell Line**
The microglial BV2 cells were cultured at 3 × 10^5/mL in Dulbecco modified Eagle medium media with 10% fetal bovine serum. Cultures were treated with DHA or EPA salt (Sigma) dissolved in media for 48 hours followed by inflammmagen stimulation. Nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production were measured as described.

**Statistical Analysis**
All data are reported as mean±SEM. Significant differences between means were assessed by analysis of variance and post hoc Scheffe tests for multiple comparisons. \( P < 0.05 \) was considered statistically significant.

**Results**

**The Cerebral Concentration of n-3 PUFAs Is Elevated and Maintained at a High Level After H/I in n-3 PUFA-Treated Pups**
Female gestant rats were given a regular diet (n-3 PUFAs low, N3L) or an n-3 PUFA-enriched fish oil supplementation diet (n-3 PUFAs high, N3H) from the second day of pregnancy until 14 days after parturition (Figure 1A). There was no significant difference in maternal consumption of chow and weight gain of neonates between N3L and N3H groups (Supplemental Figure I; available at http://stroke.ahajournals.org). The offspring were subjected to H/I or sham operation at postnatal Day 7. The cortical content of n-3 PUFAs was measured in 14-day-old neonates (7 days after H/I). There was a significant increase in the content of 2 major n-3 fatty acids, DHA and EPA (Figure 1B) leading to the elevation of n-3 PUFA fraction (Figure 1C) and overall n-3/n-6 PUFA ratio (Figure 1D) in pups of the N3H group. Of note, the ratio of n-3/n-6 in the N3H group was maintained at a high level (approximately 3) 7 days after H/I brain damage, whereas it significantly decreased (from 2.5 to 1.9) in the N3L group after H/I (Figure 1D). No difference was detected between the N3H and N3L groups in the proportions of saturated fatty acids, monounsaturated fatty acids, and n-6 polyunsaturated fatty acids (Figure 1C).

**n-3 PUFAs Confer Long-Term Neuroprotection Against Neonatal H/I**
Cresyl violet staining was performed to examine the infarct area and cell morphology per brain region at 5 weeks after insult. As shown in Figure 2A–B, n-3 PUFAs significantly attenuated H/I-induced brain tissue loss in the cortex, striatum, and hippocampus, resulting in a reduction in overall tissue loss from 56.4% to 28.7%. Microscopic images show a large area of tissue loss surrounded by shrunken, dying cells in the ipsilateral brain of the N3L group (Figure 2C, middle panel). Perinatal n-3 PUFA supplementation preserved cresyl violet-stained neurons in penumbra at 5 weeks after H/I (Figure 2C, right panel). Taken together, these results reveal a potent neuroprotective effect of n-3 PUFAs against neonatal H/I injury.
Grid walking and foot fault tests were performed between 2 and 5 weeks after H/I. Impairment of sensorimotor behavior was observed in rats on the N3L diet after H/I, manifested by significantly increased right forelimb (Figure 3A) and hind limb (Figure 3B) fault rates compared with corresponding sham-operated controls. Supplementation of n-3 PUFAs nearly prevented the H/I-induced foot fault (Figure 3A–B), indicating the beneficial effect of n-3 PUFAs on attenuating sensorimotor dysfunction after H/I. Locomotor activity, assessed by total steps in the grid-walking test, did not change significantly between groups at 3 to 5 weeks after H/I. The brief decrease in locomotor activity at 2 weeks after H/I in the N3L group reflected a transient disability in overall motor activity after acute brain injury (Figure 3C). The Morris water maze was further performed at 5 weeks after H/I. Without any influence on swimming velocity (Figure 3F), supplementation of n-3 PUFAs ameliorated cognitive disability (Figure 3D) and memory deficit (Figure 3E) induced by H/I. There was no significant difference among all behavior performances between the 2 sham-operated groups (Supplemental Figure II).

**n-3 PUFAs Suppress Cerebral Inflammation After H/I**

As resident macrophages in the brain, microglia are intricately involved in the initiation and propagation of inflammatory response and cytokine production in neonatal H/I brain injury. Microglial activation was measured using a microglial marker, Iba1. There was no obvious microglial activation at 3 hours after H/I in N3L animals. At 24 hours after H/I, activated microglia characterized by an amoeboid shape and stout processes were extensively distributed at the center of the lesion sites in cortex from N3L animals. These activated microglia were still accumulated in the perilesion area at 7 days after insult. n-3 PUFA supplementation robustly ameliorated the activation of microglia after H/I (Figure 4A). We further measured several inflammatory mediators in the cortex 24 hours after H/I insult, when inflammatory response can be reliably detected (Figure 4B) and before irreversible brain damage has occurred. Consistent with our previous report, the mRNA levels of TNF-α, interleukin-1α (IL-1α), IL-1β, IL-6, cyclo-oxygenase-2 (COX-2), and inducible NO synthase were significantly upregulated in the ipsilateral cortex of N3L animals after H/I. n-3 PUFA supplementation significantly prevented H/I-induced increases in all 6 factors tested (Figure 4C). The time course of inducible NO synthase and COX-2 protein expression after H/I brain damage was further examined (Figure 4D–E). Expression of both inducible NO synthase and COX-2 was increased as early as 3 hours after H/I and remained elevated up to 24 hours postinjury in animals on the N3L diet. n-3 PUFAs suppressed the expression of inducible NO synthase and COX-2 from 16 hours onward. A prominent inflammatory response was also observed in striatal and hippocampal areas after H/I, which was also inhibited by n-3 PUFAs (data not shown). Taken together, these data suggest that n-3 PUFAs inhibited a sustained inflammatory response post-H/I.

**DHA and EPA Attenuate the Release of Proinflammatory Mediators From Microglia on Inflammatory Stimuli**

To verify the direct anti-inflammatory effect of n-3 PUFAs on microglia and to further investigate the underlying mechanisms, BV2 microglial cells were cultured in 96-well plates
and treated with DHA or EPA for 48 hours followed by lipopolysaccharide (100 ng/mL) stimulation for another 24 hours. Pretreatment with DHA significantly attenuated lipopolysaccharide-induced NO (Figure 5A) and TNF-α (Figure 5B) release in a dose-dependent manner starting at 20 μmol/L. The effect of EPA was qualitatively similar to, albeit weaker than, the effect of DHA (Figure 5C–5D). The lowest concentrations of DHA (40 μmol/L) and EPA (80 μmol/L) that give optimal inhibition of NO and TNF-α release were used for subsequent experiments. DHA and EPA also inhibited TNF-α release from microglia in the presence of other inflammatory stimuli, including IL-1 and 4-phorbol 12-myristate 13-acetate (PMA; 40 nmol/L) or IL-1 (20 ng/mL). Production of TNF-α was measured 24 hours later. #P<0.01 versus control; +P<0.05, *P<0.01, **P<0.001, ***P<0.0001 versus inflammmagen-treated.

**n-3 PUFAs Inhibit the NF-κB Signaling Pathway in Microglia**

NF-κB is an important transcription factor that modulates a diverse array of genes related to inflammatory response, including TNF-α, IL-1β, COX-2, and inducible NO synthase. Thus, we further examined the effect of DHA and EPA on NF-κB activity in microglia. As shown in Figure 6A, DHA and EPA treatment significantly inhibited lipopolysaccharide-induced NF-κB DNA binding activity. Western blot analyses showed a significant decrease in p65 levels in cytosolic fraction from BV2 cells at 1 hour postlipopolysaccharide (Figure 6B). At the same time, nuclear p65 expression levels increased significantly, consistent with increased translocation of p65 from cytosol into nucleus after lipopolysaccharide. Pretreatment with DHA or EPA prevented the p65 nuclear translocation.

The inhibitory effect of n-3 PUFAs on NF-κB signaling was further confirmed in the in vivo neonatal H/I model. Animals on the N3L diet showed significantly increased accumulation of nuclear p65 at 3 to 24 hours after H/I compared with sham-treated animals. Supplements of n-3 PUFAs completely abolished this increase beginning 3 hours after H/I (Figure 6C–D). Enhanced phosphorylation of IκBα, another indicator of NF-κB activation, can be detected as early as 3 hours after insult in N3L animals, when the increased nuclear accumulation of p65 was also detected. n-3 PUFAs coincidentally inhibited the H/I-induced phosphorylation of IκBα.

**Discussion**

In this study, we demonstrated that n-3 PUFA supplementation significantly elevated cerebral n-3 PUFA levels, reduced brain damage, and improved long-term neurological outcomes up to 5 weeks after neonatal H/I injury. We further illustrated that n-3 PUFAs exert a protective effect against H/I through, at least partially, suppressing the microglial-mediated inflammatory response.

The neuroprotective effect of n-3 PUFAs has been observed in the acute phase of ischemia assessed from 24 hours to 7 days postinjury in an adult stroke model. However, the effect of n-3 PUFAs on long-term brain damage and neurological functional recovery, particularly in immature brains, is unknown. Our current study demonstrated a robust protective effect of n-3 PUFAs against H/I-induced brain damage and functional deficits lasting at least 5 weeks after H/I insult. This is the first report demonstrating the prolonged neuroprotection of n-3 PUFAs on H/I brain injury. This information is critical for translating the results to human clinical trials, in which most subjects have chronic impairments.
Although the importance of n-3 PUFAs in neonatal H/I has begun to be appreciated, no information is available regarding the change of endogenous n-3 PUFAs in the developing brain after H/I. It has been found in an adult stroke model that DHA is released from cell membrane after ischemic stroke and that some of the DHA is metabolized, which may potentially result in the decrease of n-3 PUFAs in the brain after ischemia. Although some of these DHA metabolites, protectin and resolvin, have been shown to be neuroprotective, excessive degradation of n-3 PUFAs will influence the fluidity of neuronal membrane and compromise the related physiological functions, which may ultimately exacerbate the brain damage. In this study, we have shown that H/I induced a prolonged (7 days after H/I) decrease of n-3 PUFAs in the neonatal brain, suggesting that the immature brain lacks the capability to quickly compensate for the loss of n-3 PUFAs. Dietary supplementation significantly increased cerebral n-3 PUFAs in normal neonates and maintained them at a high level through H/I, which could ensure neuroprotection against brain damage.

Prominent microglial activation has been observed within the immature brain early after H/I and up to 42 days after injury. Prolonged overactivation of microglia has widely been recognized to deteriorate neonatal H/I brain injury by producing and releasing neurotoxic substances that include inflammatory cytokines and reactive oxygen species. In this study, we showed, both in vitro in a microglial cell line and in vivo in the H/I-affected neonatal brain, that n-3 PUFAs suppressed microglial activation and the production of proinflammatory mediators through inhibiting the activation of NF-κB. These data suggest that the neuroprotective effect of n-3 PUFAs in the H/I brain is ascribed to its interference with the signaling processes governing the inflammatory response in microglia. We have also found that DHA and EPA markedly enhanced microglial peroxisome proliferator-activated receptor-γ activity (data not shown), which is a recently identified prosurvival factor that protects against ischemic brain injury, at least in part, by anti-inflammatory mechanisms. Our ongoing studies will determine whether peroxisome proliferator-activated receptor-γ activation plays a critical role in mediating the neuroprotective effect of n-3 PUFAs after H/I.

Taken together, our results suggest that n-3 PUFAs exert potent and prolonged neuroprotection in neonatal H/I brain injury. The enrichment of n-3 PUFAs in the brain robustly suppressed microglia-mediated post-H/I inflammatory responses through an NF-κB-dependent mechanism. It is possible that other mechanisms contribute to the neuroprotective effect of n-3 PUFAs. For example, we have observed that n-3 PUFA treatment enhanced revascularization after H/I, which may improve intraischemic cerebral perfusion and contribute to n-3 PUFA-afforded neuroprotection against H/I. Additionally, n-3 PUFA supplementation has been shown to improve neuronal/cognitive development in neonates. Thus, further investigation into n-3 PUFAs as a prophylactic or therapeutic agent for neonatal H/I brain injury is warranted.

Acknowledgments
We thank Carol Culver for editorial assistance.

Sources of Funding
This project was supported by National Institutes of Health grants NS45048, NS056118, and NS036736 (to J.C.); Chinese Natural Science Foundation grant (30870794) and International Collaboration grant (08410703000) from Shanghai Science & Technology Bureau (to Y.G. and J.C.); and Special Research Funds from Chinese Ministry of Science & Technology to State Key laboratories. X.H. is supported by a fellowship award from the American Heart Association (10POST4150028).

Disclosures
None.
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Stroke. 2010;41:2341-2347; originally published online August 12, 2010;
doi: 10.1161/STROKEAHA.110.586081

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