Accumulation of Dietary Docosahexaenoic Acid in the Brain Attenuates Acute Immune Response and Development of Postischemic Neuronal Damage

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Background and Purpose—Consumption of fish has been shown to reduce risk of coronary heart disease and, possibly, of ischemic stroke. Because docosahexaenoic acid (DHA) is the most likely neuroactive component within fish oil, we hypothesized that exposing mice to a DHA-enriched diet may reduce inflammation and protect neurons against ischemic injury.

Methods—To visualize the effects of DHA on neuroinflammation after stroke, TLR2-fluc-GFP transgenic mice were exposed to either a control diet, a diet depleted in n-3 polyunsaturated fatty acid, or a diet enriched in DHA during 3 months. Real-time biophotonic/bioluminescence imaging of the TLR2 response was performed before and after middle cerebral artery occlusion, whereas cytokines concentrations and stroke area analyses were performed at 3 and 7 days after middle cerebral artery occlusion, respectively.

Results—We show that a 3-month DHA treatment prevented microglial activation after ischemic injury, reduced the ischemic lesion size, and increased levels of the antiapoptotic molecule Bcl-2 in the brain. Additional analysis revealed a significant decrease in the levels of COX2 and IL-1β, but not in other proinflammatory cytokines. Importantly, long-term DHA supplementation significantly changed the n-3:n-6 polyunsaturated fatty acid ratio in the brain.

Conclusions—Collectively, these data indicate that diet-induced accumulation of DHA in the brain protects against postischemic inflammation and injury. Because DHA is widely available at low cost and has an excellent safety profile, our data suggest that increased DHA intake may provide protection against acute immune response/brain damage in ischemic stroke. (Stroke. 2011;42:00-00.)

Key Words: stroke □ nutraceutical □ biophotonic/bioluminescence imaging □ neuroinflammation □ TLR2-reporter mouse

Cardiovascular diseases, which include coronary heart disease and stroke, are leading causes of death and disability in industrialized countries and, therefore, identifying primary prevention strategy represents a public health priority.1 Large prospective cohort studies and randomized trials have shown that fish oil intake decreases the risk of death from coronary heart disease. Several lines of epidemiological evidence also indicate that consumption of fish is associated with reduced risk of ischemic stroke,2 although this association is not consistently detected.3 Because fish is a major source of docosahexaenoic acid (DHA, 22:6n-3), it can be hypothesized that DHA is a potential nutraceutical candidate for treatment of ischemic stroke.

Accumulation of DHA in the brain has been associated with reduction of neuroinflammation4–7 and activation of antiapoptotic pathways,8 2 mechanisms of action implicated in ischemic stroke. Given that restraining acute inflammatory processes may be beneficial in ischemic stroke,1,9 we hypothesized that long-term intake of DHA-enriched diet may change n-3:n-6 PUFA ratio in cerebral tissue, thereby protecting brain cells from an uncontrolled inflammation response and consequent ischemic injury.

By using mouse models for live biophotonic/bioluminescence imaging of microglial activation, we report here that diet-induced accumulation of DHA in the brain modulates postischemic inflammation/innate immune response and protects against ischemic injury.

Materials and Methods

Experimental Animals

All experiments were performed on TLR2-fluc-GFP transgenic mice (C57BL/6 background), in which luciferase and GFP reporters are...
driven under the transcriptional control of the murine Toll-like receptor 2 gene promoter.\textsuperscript{10} Males (n=9–12 per group, age 2–3 months) were put on 1 of the customized diets (Supplemental Table 1, http://stroke.ahajournals.org) for 3 months (Figure 1). The formulas of these isocaloric purified diets were precisely determined to avoid batch-to-batch disparity (Supplemental Table 1). DHA-treated mice received approximately 0.7 g/kg per day of DHA, protected from oxidation through gelatin microencapsulation (MEG-3, Ocean Nutrition Inc). All experimental procedures were approved by the Laval University Animal Care Ethics Committee and are in accordance with the Canadian Council on Animal Care.

Surgical Procedures
As described,\textsuperscript{10–13} the surgery was performed on 2-to-3-month old male mice (transgenics in C57BL/6 background; 20–25 g). The animals were anesthetized (2% isoflurane) and unilateral transient focal cerebral ischemia was induced by intraluminal filament occlusion (12 mm long 6–0 silicon-coated monofilament) of the left middle cerebral artery occlusion (MCAO) during 1 hour followed by 72 hours, body temperature was regularly checked and maintained at 37°C with a heating pad. As previously described,\textsuperscript{12} to confirm 72 hours, body temperature was regularly checked and maintained at 37°C with a heating pad. To avoid cooling for the first 72 hours, body temperature was regularly checked and maintained at 37°C with a heating pad. As previously described,\textsuperscript{12} to confirm successful MCAO at 6 and 24 hours following surgery, the animals were examined for early neurological deficits. Only the mice expressing positive phenotypes including circulating behavior, slight motor deficits of contralateral front paw, and reduced spontaneous activity were selected for the study.

Histological Evaluation of the Size of Infarction
The size of infarction was evaluated at 48 hours and at 7 days after MCAO. The samples were postfixed overnight in 4% paraformaldehyde and equilibrated in phosphate-buffered 20% sucrose for 48 hours. Brains were embedded in Tissue-Tek (O.C.T. compound) and cut into coronal section with a Cryostat. Tissue sections (all the stroke area region n=7/diet) were stained with cresyl-violet and were digitized. The area of infarction, direct stroke area, was quantified with ImageJ program (version 1.42q for Windows, National Institutes of Health), and infarct area was calculated as percentage of control, nonstroke area in the contralateral hemisphere with appropriate corrections for brain edema.\textsuperscript{12}

Immunofluorescence Analysis
As previously described,\textsuperscript{11} the sections were then incubated overnight at room temperature using primary antibodies: 1:750 Rabbit polyclonal anti-Iba1 (Wako), 1:250 mouse anti-TLR2 (eBioscience), and 1:500 mouse monoclonal anti-green fluorescent protein (GFP; Invitrogen). After wash, the sections were incubated in corresponding fluorescent goat secondary antiserum (Invitrogen).

Cytokine Arrays
As described,\textsuperscript{11} the protein expression analysis of inflammatory cytokine was performed using mouse antibody array (Raybio\textsuperscript{®}Mouse Inflammation Antibody Array 1.1, Ray Biotech, #AAM-INF-1L). Protein lysate was obtained by homogenization of brain of transgenic mice in 1X Cell Lysis Buffer (included in the Ray Biotech kit) with Protease inhibitor cocktail (Sigma #P8340). Samples for each group (3 mice/group) were pooled and incubated with the array membrane overnight at 4°C. The membranes were then processed according to Raybiotech protocol. Membrane were exposed to X-ray film (Kodak film Biomax MR1, #8701302) and analyzed by Agfa Arcus II system and ImageJ software.

Western Blotting
Total protein extracts were obtained from the ipsilateral hemisphere of the brain 48 hours after cerebral ischemia by homogenization in a 1X Cell Lysis Buffer (included in the Ray Biotech kit) with Protease inhibitor cocktail (Sigma #P8340). The proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and detected using monoclonal primary antibodies against anti-COX2 (1:2,000; Sigma), and anti-Bcl-2, and rabbit polyclonal antibody (1:750; New England Biolabs) and antitax (1:750; New England Biolabs). The Western blots were revealed using the western lightning chemiluminescence kit (Perkin Elmer).

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Lipid Extraction and Gas Chromatography
The determination of fatty acid content was performed in frozen extracts from the contralateral striatum, using previously published procedures.\textsuperscript{14}

Statistical Analysis
All data are expressed as mean±SEM. Statistical difference was assessed by 1-way analysis of variance (ANOVA) followed by post hoc comparison (Bonferroni’s) test (P≤0.05). When appropriate, nonparametric analysis was conducted using a Kruskal-Wallis test.

Results
DHA-Enriched Diet Attenuates Acute Microglial Activation/TLR2 Response and Decreases Ischemic Lesion
We investigated whether long-term intake of DHA may change n-3:n-6 PUFA ratio in the brain and modulate postischemic inflammation. As shown in Figure 1, TLR2 reporter mice were put on either control, n-3-PUFA-depleted, or DHA-enriched diets (Supplemental Table 1) for a period of 3 months and then were subjected to transient MCAO (Figure 1). After stroke, the microglial activation/innate immune responses were longitudinally imaged from the brains of live TLR2-luc-GFP mice (Figure 2A). As shown in Figure 2C, D and as demonstrated in our previous study, after stroke the TLR2-driven GFP transgene was strongly induced in microglial cells.\textsuperscript{10} The quantitative analysis of photon emissions revealed a strong anti-inflammatory effect of DHA treatment at 6, 24, and 48 hours after cerebral ischemia as compared with control or low n-3 PUFA diet (Figure 2B). Overall, chronic DHA treatment almost completely blunted induction of TLR2 promoter, suggesting profound preventive effects of DHA against postischemic inflammation. Next, we investi-
gated whether DHA treatment affected early monocyte recruitment after stroke. To analyze whether the robust anti-inflammatory effect of DHA-enriched diet was associated with neuroprotection, we measured stroke area at 48 hours and at 7 days after MCAO. As shown in Figure 3A,B, the analysis of cresyl-violet-stained brain sections at 48 hours and at 7 days after MCAO revealed a significant decrease in the size of ischemic lesions in mice subjected to DHA-enriched diet compared with control and low n-3 PUFA diets (*P<0.05).

Previous work suggests that DHA may inhibit neuronal apoptosis through activation of cell survival pathways. Therefore, we analyzed the levels of anti- and proapoptotic proteins in ischemic brains of mice subjected to different PUFA-enriched diets. Western blot analysis revealed that DHA-enriched diet increased levels of the antiapoptotic protein Bcl-2 after stroke (Figure 4A) although left levels of proapoptotic protein Bax unchanged (Figure 4B). Hence, the DHA-mediated decrease in size of ischemic lesion was associated with increased levels of Bcl-2.

Long-Term Dietary DHA Intake Changes the Content of a Total Brain Fatty Acid Levels and Decreases Proinflammatory Cytokines After Stroke

Next, we investigated whether observed anti-inflammatory/neuroprotective effects were indeed caused by change in n-3:n-6 PUFA ratio in the brain. As shown in Figure 5, the DHA-supplemented diet significantly increased concentrations of DHA in the striatum, a value 10% higher than in controls (P<0.05) and 28% higher than in animals fed low n-3 PUFA diet (P<0.001; Figure 5A). Moreover, DHA treatment decreased the levels of arachidonic acid (ARA), an n-6 PUFA with inflammatory properties, in the striatum...
Figure 3, DHA-enriched diet reduces size of ischemic lesion. Analysis of cresyl-violet-stained brain sections at day 2 A and day 7 B after MCAO revealed a significant decrease in the size of infarcted area in the ipsilateral hemisphere of mice subject to DHA-enriched diet compared with control and low n-3 PUFA diets. Infarct volume is expressed as percentage of control, nonstroked area in the contralateral hemisphere. Values indicate mean±SEM (*P<0.05). MCAO indicates middle cerebral artery occlusion; DHA, docosahexaenoic acid; SEM, standard error of the mean.

Figure 4. Dietary intake of DHA increases the levels of Bcl-2 after stroke. A, Western blot analysis showed no difference in the level of the proapoptotic protein Bax in the ischemic brains of the mice fed with the different diet. B, Significant increase in level of antiapoptotic protein Bcl-2 was detected in the brains of mice exposed to long-term DHA-enriched diet as compared with control or low n-3 PUFA diet 48 hours after cerebral ischemia (*P<0.05). DHA indicates docosahexaenoic acid.

Differential Effects of n-3 and n-6 PUFA on Proinflammatory Cytokines Levels
There is growing evidence that postischemic inflammation plays an important role in the evolution of ischemic brain injury. One of the characteristics of the brain inflammatory response 24 to 72 hours after transient MCAO is a marked increase in the levels of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6. Here, we found that chronic n-3 PUFA depletion potentiated the postischemic increase in IL-1β and IL-6 compared with controls (Figure 6A and C). In contrast, supplementation with high-DHA selectively prevented the increase of IL-1β in ischemic brains compared with animals fed n-3-PUFA-depleted and/or control diets (Figure 6A). Interestingly, we did not observe any PUFA-induced effects on the expression levels of TNF-α (Figure 6B).

Competition between n-6 and n-3 PUFA occurs at the level of prostaglandin formation at the cyclooxygenase (COX) and lipoxygenase levels. Previous studies suggest that n-3 PUFAs such as DHA can suppress induction of COX-2 by competing with ARA for enzymatic metabolism, inducing the production of less inflammatory derivatives. Therefore, we hypothesized that shifting upward the n-3:n-6 PUFA ratio in the brain of mice would reduce COX-2 induction after stroke. Analysis of the brains after MCAO confirmed that levels of COX2 were significantly decreased following the high-DHA diet compared with controls (32.18%; P<0.05) and low n-3 PUFA diet (27.63%; P<0.05; Figure 6D).
MCAO revealed a strong neuroprotective effect of the DHA-enriched diet. Interestingly, the decrease in size of ischemic lesion was accompanied by an increase in levels of the antiapoptotic molecule Bcl-2, a decrease in levels of both COX2 and the proinflammatory cytokine IL1-

Indeed, accumulation of DHA in the brains of mice almost completely blunted the robust activation of TLR2 normally associated with microglial activation following cerebral ischemia.10,19 While recent studies suggest that activated microglial cells in certain conditions may confer neuroprotection,11,20 a current view is that uncontrolled acute postischemic inflammation response is generally neurotoxic,21,22 and modulation of the early inflammatory response is associated with marked neuroprotection.12,23,24 Our data are supporting this later view as the anti-inflammatory effect of DHA was strongly neuroprotective as well. Namely, the long-term dietary DHA intake was associated with smaller ischemic lesions and significant increase in levels of the antiapoptotic protein Bcl2. Observed neuroprotection is consistent with previous work on rodent models of cerebral ischemia, where DHA or fish oil preparation administered days before global cerebral ischemia improved functional recovery and reduced brain injury.25,26 Moreover, recent work by Belayev et al demonstrated that DHA27 infused intravenously after the induction of cerebral ischemia in mice exerts a robust neuroprotection. In addition to DHA, some novel docosanoids have been shown to inhibit poststroke infiltration neutrofils and exert anti-inflammatory effects.28 Finally, DHA treatment has been shown to exert neuroprotective action in other central nervous system diseases where a strong neuroinflammatory component has been described, including Alzheimer’s and Parkinson’s diseases.29,30

However, 1 of the most striking observations reported here is the specificity of the DHA-mediated effects. Namely, DHA-enriched diet exerted significant anti-inflammatory and neuroprotective effect, whereas α-linoleic acid from the control diet did not. This is despite the fact that dietary α-linoleic acid was partly converted into brain DHA. A likely explanation is that only DHA supplementation was effective at significantly reducing the concentration of ARA in the brain, as shown in Figure 5. The ensuing increase in DHA:ARA ratio would favor DHA competing with ARA for metabolism on membrane release, resulting in a reduction of ARA proinflammatory derivatives such as COX-produced prostaglandins.5,6,17,18 The DHA-induced reduction of COX-2 reported here and by others16,18 would thus potentiate the reduction of proinflammatory metabolites.

Our study also provides key in vivo information on how DHA may modulate innate immune response and neuroinflammation. As shown previously, postischemic cell damage results in massive phospholipase-mediated release of free fatty acids, including ARA and DHA from the membrane phospholipids.4,27 Among those agents, DHA derivatives like neuroprotectin 1 and resolvins have been attributed strong anti-inflammatory properties.15,31,32 For example, neuroprotectin 1 may have been involved in the DHA-induced effects on Bcl-2 and COX-2.15,31 Here, we found that animals treated with DHA had lower brain levels of IL-1β and COX-2, increased Bcl-2, and yet TNF-α or IL-6 remained unchanged. Overall, our findings suggest that DHA had profound and specific effects against the neuroinflammatory and apoptotic ischemic cascade.

Conclusions

The results of our study clearly demonstrated that chronic DHA intake blunted inflammatory response induced by cerebral ischemia, thus preventing brain injury in an animal model. This further suggests that chronic dietary exposure of
DHA shifts upward the n-3:n-6 PUFA ratio, thereby generating an anti-inflammatory and neuroprotective environment. Our data bring into consideration the possibility that increased DHA consumption may represent an affordable prophylactic intervention to decrease detrimental consequences of ischemic stroke.

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Disclosures
None.

References
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ONLINE SUPPLEMENT

SUPPLEMENTAL TABLE 1

**TABLE S1.** Dietary formulae and fatty acid content determined by gas chromatography
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<table>
<thead>
<tr>
<th></th>
<th>Diet A Control</th>
<th>Diet B Low n-3 PUFA</th>
<th>Diet C High DHA</th>
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<tr>
<td>Proteins (% w/w)</td>
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<td>Carbohydrates (% w/w)</td>
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#### Ingredients (g/kg)

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<th>Diet A Control</th>
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<th>Diet C High DHA</th>
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<td>500</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
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<td>50</td>
</tr>
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<td>Safflower</td>
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#### Fatty acids (g/kg)

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<th>Fatty acid</th>
<th>Diet A Control</th>
<th>Diet B Low n-3 PUFA</th>
<th>Diet C High DHA</th>
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<tr>
<td>Total n-3 PUFA</td>
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<tr>
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<td>Docosahexaenoic acid (DHA)</td>
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<tr>
<td>Total n-6 PUFA</td>
<td>13.45</td>
<td>32.38</td>
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<tr>
<td>Linoleic acid (LA)</td>
<td>13.45</td>
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<tr>
<td>Arachidonic acid (ARA)</td>
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<td>0.00</td>
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<tr>
<td>n-6 PUFA:n-3 PUFA ratio</td>
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<td>~95.0</td>
<td>~2.2</td>
</tr>
</tbody>
</table>

* Determined by gas chromatography.

Abbreviations: DHA, docosahexaenoic acid; MEG-3, High-DHA microencapsulated powder; PUFA, polyunsaturated fatty acids; USP, United States Pharmacopeia.

Notes: The formulas of these isocaloric purified diets were precisely determined to avoid batch-to-batch disparity or the presence of phytoestrogens or other common contaminants. They were the same in terms of total calories (~5 kcal/g), fibers, vitamins, minerals, and antioxidants. The control diet contained canola and soybean oil, thus providing alpha-linolenic acid, a 18 carbon n-3 PUFA. The low n-3 PUFA diet was based on safflower oil. Finally, the high DHA diet contained ~5 g of DHA and ~1.2 g of eicosapentaenoic acid (EPA, 20:5n-3) per kg of pelleted diet equivalent to a daily dose of approximately 0.7 g kg⁻¹ day⁻¹ of long-chain n-3 PUFA (assuming a mouse of 25 g eats 3 g of food per day).