SPINAL INJECTION OF DOCOSAHEXAENOIC ACID ATTENUATES CARRAGEENAN-INDUCED INFLAMMATORY PAIN THROUGH INHIBITION OF MICROGLIA-MEDIATED NEUROINFLAMMATION IN THE SPINAL CORD

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Abstract—Neuroinflammation in the spinal cord plays a critical role in the processing of inflammatory pain. Docosahexaenoic acid (DHA), a predominant omega-3 polyunsaturated fatty acid in the central nervous system, is known to modulate inflammatory responses in various neurodegenerative disorders. In this study, we investigated whether DHA could reduce inflammatory pain and inhibit neuroinflammation in the spinal cord following carrageenan injection in mice. Intrathecal (i.t.) injection of DHA at 15 min before carrageenan injection blocked carrageenan-induced pain hypersensitivity for more than 6 h. In addition, i.t. injection of DHA at 3 h after carrageenan transiently reversed carrageenan-induced heat hyperalgesia and mechanical allodynia. Furthermore, DHA treatment reduced carrageenan-induced activation of microglia, phosphorylation of p38 mitogen-activated protein kinase (MAPK), and production of proinflammatory cytokines (tumor necrosis factor-α – TNF-α and interleukin-1β – IL-1β) in the L4–5 spinal cord. In cultured microglial cells, DHA dose-dependently reduced lipopolysaccharide (LPS)-induced phosphorylation of p38, production of proinflammatory cytokines (TNF-α, IL-1β, IL-6) and chemokines (CCL2, CCL3 and CXCL10). p38 inhibitor SB203580 inhibited LPS-induced expression of proinflammatory cytokines and chemokines in a dose-dependent manner. Taken together, these results provide evidence that DHA has antinociceptive effect in inflammatory pain, which may be attributed to, at least partially, suppressing a microglia-mediated inflammatory response through inhibition of p38 MAPK activation. © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: inflammatory pain, carrageenan, docosahexaenoic acid, microglia, cytokines, chemokines.

INTRODUCTION

Pain is one of the most common symptoms encountered in the clinic, and inflammatory pain represents an important type of pain (Woolf and Costigan, 1999; Kuner, 2010; Ji et al., 2011). Neuroinflammation in both peripheral and central nervous system plays an important role in the pathogenesis of chronic pain (White et al., 2005; Moalem and Tracey, 2006; Myers et al., 2006). In peripheral, multiple inflammatory mediators such as prostaglandin, nerve growth factor, and proinflammatory cytokines (e.g., tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)) are produced in inflamed or damaged tissues and sensitize the nerve endings (Abbadi et al., 2009; Stein and Lang, 2009). In the central nervous system, glial cells (e.g. microglia and astrocytes) release powerful neuromodulators such as cytokines and chemokines (e.g. CCL2, CCL3, CXCL10) and are involved in the modulation of the excitability of spinal cord sensory neurons (DeLeo and Yezierski, 2001; Abbadi et al., 2009; Milligan and Watkins, 2009; Gao and Ji, 2010a; Ji et al., 2011). Inhibition of the neuroinflammation that mediated by glial cells has been shown to attenuate inflammatory or neuropathic pain (Raghavendra et al., 2003; Ledeboer et al., 2005; Zhuang et al., 2006).

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are two major bioactive components of omega-3 polyunsaturated fatty acids (n-3 PUFAs), which were found in fatty fish and highly concentrated in the brain and retina in humans (Fliesler and Anderson, 1983; Lands, 1992). Compared to only 0.1% of EPA in total brain fatty acid, DHA comprises 10–20% and is the main n-3 PUFAs in the brain (McNamara and Carlson, 2006). Human studies have shown that an increased dietary intake of n-3 PUFAs is associated with reduced pain in rheumatoid arthritis (Ruggiero et al., 2009; Miles and Calder, 2012), inflammatory bowel disease (Bassaganya-Riera and Hontecillas, 2010), and dysmenorrhea (Harel et al., 1996). Rats treated with n-3 PUFAs have shown to markedly increase threshold for thermal pain (Yehuda and Carasso, 1993). However, the antinociceptive effect and the underlying mechanism of DHA in inflammatory pain still remain unclear.

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Abbreviations: COX-2, cyclooxygenase-2; DHA, docosahexaenoic acid; DMEM, Dulbecco’s Modified Eagle’s Medium; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; IL-1β, interleukin-1β; INOS, inducible NO synthase; i.t., intrathecal; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; n-3 PUFAs, omega-3 polyunsaturated fatty acids; PWT, paw withdrawal latency; PWT, paw withdrawal threshold; TNF-α, tumor necrosis factor-α.
DHA has long been considered neuroprotective and anti-inflammatory (De Smedt-Peyrusse et al., 2008; Ebert et al., 2009). DHA-rich n-3 fatty acid supplementation decreases the release of IL-6 and IL-1β from peripheral blood mononuclear cells after stimulation with lipopolysaccharide (LPS) (Vedin et al., 2008), and changes the inflammation regulation-related gene expression in the blood (Vedin et al., 2012). DHA supplementation also significantly prevented hypoxia/ischemia-induced upregulation of TNF-α, IL-1β, IL-6, cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) in the cortex (Zhang et al., 2010). In addition, DHA suppresses interferon-γ-induced inflammatory cytokine expression in BV2 microglial cultures (Lu et al., 2010). Whether DHA in the spinal cord level could regulate the production of pain-related inflammatory mediators is largely unknown.

In the present study, we compared the prevention and reversal effect of the spinal injection of DHA on carrageenan-induced inflammatory pain, and checked the effect of DHA on carrageenan-induced activation of microglia and astrocytes in the spinal cord. As p38 mitogen-activated protein kinase (MAPK) has been implicated to play an important role in mediating the expression of inflammatory mediators (Ji and Suter, 2007), we further examined whether the anti-inflammatory effect of DHA is mediated by inhibition of p38 MAPK in microglial cells in both spinal cord and cell cultures.

EXPERIMENTAL PROCEDURES

Animals

Male ICR mice (8-week-old), obtained from the Experimental Animal Center of Nantong University, were used in this study. The animals were maintained on a 12:12-light–dark cycle at a room temperature of 22 ± 1 °C with free access to food and water. Blinded experiments were performed according to the guidelines of the International Association for the Study of Pain. The experimental procedures were approved by the Animal Care and Use Committee of Jiangsu Province. Inflammatory pain was induced by intraplantar injection of 20-μl carrageenan (1%, Sigma, St. Louis, MO, USA) in sterile 0.9% saline. The control group was injected with the same volume of saline.

Drugs and administration

DHA salt (Sigma, St. Louis, MO, USA) was dissolved in saline. For intrathecal injection, a spinal cord puncture was made with a 30-gauge needle between the L4 and L5 level to deliver the reagents (10 μl) to the cerebral spinal fluid. Immediately after the needle entry into subarachnoid space, a brisk tail flick could be observed.

Behavioral analysis

Animals were habituated to the testing environment daily for at least 2 days before baseline testing. The room temperature and humidity remained stable for all experiments. For testing heat sensitivity, animals were put in plastic boxes placed on a glass plate, and the plantar surface was exposed to a beam of radiant heat through a transparent glass surface (Plantar Test Apparatus 390G, IITC Life Science, Woodland Hills, CA, USA). The glass temperature was set at 30 °C during all experiments. The baseline latencies were adjusted to 10–14 s with a maximum of 20 s as cutoff to prevent potential injury. The latencies were averaged over three trials, separated by a 5-min interval. For testing mechanical sensitivity, animals were put in boxes on an elevated metal mesh floor and allowed 30 min for habituation before examination. The plantar surface of the hindpaw was stimulated with a series of von Frey hairs with logarithmically increasing stiffness (0.02–2.56 g). Stooling, Kiel, WI, USA), presented perpendicular to the plantar surface (2–3 s for each hair). The 50% paw withdrawal threshold (PWT) was determined using Dixon’s up-down method (Dixon, 1980).

Cell culture

The microglial BV2 cells were seeded in a 6-well plate at a density of 3 × 10^5 cells/cm² and cultured in a medium containing 10% FBS (fetal bovine serum) in high glucose DMEM (Dulbecco’s Modified Eagle’s Medium). For primary microglial culture, the cerebral hemispheres (neonatal mice, P2) were isolated and transferred to ice-cold Hank’s buffer and the meninges were carefully removed. Tissues were then minced into 1-mm pieces, triturated, filtered through a 10-μm screen and then plated into 75-cm² flasks. The medium was replaced twice a week. After 12–14 days, the flasks were shaken on a rotary shaker at 220 rpm for 4 h. The resulting cell suspension, rich in microglia, was placed in a 6-well plate at a density of 5 × 10^5 cells/cm². Prior to stimulation with LPS, OPTI-MEM was replaced. The cells were incubated with LPS for either 1 or 6 h. The treatment of the DHA (3, 10, 30 μM) or SB203580, (5, 50 μM) was started 30 min prior to LPS treatment. After the treatments, the cells were collected for Real-time PCR or Western blot.

Tissue collection

Animals were rapidly killed after deep anesthesia with isoflurane, and the L4–5 spinal segments were quickly removed and frozen at −80 °C for later Real-time PCR or Western blot analysis.

Real-time PCR

The total RNA of the spinal cord or cultured cells was extracted using Trizol reagent (Invitrogen). RNA quantity and purity were determined using Eppendorf Biophotometer. One microgram of total RNA was reverse transcribed using a mixture of random primers according to the manufacturer’s protocol (TaKaRa, Japan). Real-time quantitative PCR analysis was performed in the Real-time Detection System (Rotor-Gene 6000) by SYBR green I dye detection. The mRNA expressions of IBA-1 (ionized calcium binding adaptor molecule 1), GFAP (glial fibrillary acidic protein), TNF-α, IL-1β, IL-6, CCL2, CCL4, CXCL10 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were checked. The detailed primer sequences for each gene are listed in Table 1. The PCR reaction process for cells was first incubated at 95 °C for 30 s, followed by 40 cycles of thermal cycling at 95 °C for 5 s and 60 °C for 45 s. Data were collected after each cycle and displayed graphically (Rotor-Gene 6000 Series Software 1.7). Melt curves were performed on completion of the cycles to ensure that nonspecific products were absent. Quantification was performed by normalizing Ct (cycle threshold) values with GAPDH Ct and analyzed with the 2^-ΔΔCt method.
Immunohistochemistry

Animals were terminally anesthetized with isoflurane and perfused through the ascending aorta with saline followed by 4% paraformaldehyde. After the perfusion, the spinal cord segments (L4–5) were removed and postfixed in the same fixative overnight, then replaced with 15% sucrose overnight. Spinal sections (transverse, free-floating, 30 μm) were cut in a cryostat and blocked with 2% goat serum in 0.3% Triton for 1 h at RT and incubated overnight at 4 °C with p-p38 or IBA-1 primary antibodies (anti-p-p38, 1:300; mouse, Cell Signaling, Beverly, MA, USA; anti-IBA-1, 1:3000, rabbit, Wako, Tokyo, Japan). The sections were then incubated for 1 h at RT with Cy3- or FITC-conjugated secondary antibodies (1:1000, Jackson ImmunoResearch, West Grove, PA, USA). The stained sections were examined with a Leica fluorescence microscope, and images were captured with a CCD Spot camera.

Western blot

The spinal cord or cultured cells were homogenized in a SDS sample buffer containing a mixture of proteinase and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL, USA). Protein samples (30 μg) were separated on SDS–PAGE gel and transferred to nitrocellulose blots. The blots were blocked with 5% milk and incubated overnight at 4 °C with antibody against p-p38 (rabbit, 1:500; Cell Signaling, Beverly, MA, USA; anti-IBA-1, 1:500, mouse, Cell Signaling, Beverly, MA, USA), and β-actin (mouse, 1:20,000; Sigma, St. Louis, MO, USA). These blots were further incubated with HRP-conjugated secondary antibody, developed in enhanced chemiluminescence (ECL) solution, and exposed onto Hyperfilm (Amersham Biosciences) for 1–10 min. Specific bands were evaluated by apparent molecular size. The intensity of the selected bands was captured and analyzed using Image J software (NIH).

Quantification and statistics

For behavioral studies, one-way ANOVA followed by Newman–Keuls post hoc test was used for comparison of temporal variations within the same group and student t-test was used for comparisons between groups. For the quantification of Western blot, the density of specific bands for p-p38, β-actin was measured with imaging analysis software (Image J, NIH). The size of rectangle was fixed for each band and the background near that band was subtracted. p-p38 levels was normalized to β-actin. The quantification of Real time-PCR was normalized to GAPDH. The Western blot and Real-time PCR data were analyzed with student t-test. All the data were presented as mean ± SEM, and P < 0.05 was considered statistically significant in all cases.

RESULTS

Intrathecal injection of DHA inhibits carrageenan-induced heat hyperalgesia and mechanical allodynia

We tested the paw withdrawal latency (PWL) to radiant heat stimulation and the PWT to von Feyfilaments stimulation both before and 1, 3, 6, 24, and 48 h after unilateral injection of carrageenan or saline. As shown in Fig. 1A, carrageenan injection induced a significant decrease of PWL (P < 0.001). The PWL dropped from 13.0 ± 0.3 s to 8.4 ± 0.3 s at 1 h (P < 0.001 vs. baseline), 7.1 ± 0.2 s at 3 h (P < 0.001 vs. baseline), 8.2 ± 0.6 s at 24 h (P < 0.001 vs. baseline), and recovered at 48 h. Saline injection did not change the PWLs at all time points (P > 0.05). In addition, carrageenan also induced mechanical allodynia. The PWT dropped from 1.91 ± 0.2 g before carrageenan (baseline) to 0.42 ± 0.09 g at 1 h after carrageenan injection. The mechanical allodynia remained at the peak level for 6 h, and fully recovered at 48 h. The animals in saline group did not show significant changes in PWL (Fig. 1B). These results indicate that intraplantar carrageenan induces significant heat hyperalgesia and mechanical allodynia.

To examine whether pretreatment of DHA could prevent the development of carrageenan-evoked pain hypersensitivity, DHA at the doses of 1, 3, and 10 μg were administered intrathecally 15 min before the injection of carrageenan. As shown in Fig. 1C, DHA at the dose of 10 μg blocked carrageenan-induced heat hyperalgesia. The effect appeared at 2 h after carrageenan injection (10.74 ± 0.47 s for DHA and 7.67 ± 0.23 s for vehicle, P < 0.001) and maintained for more than 24 h (11.28 ± 0.40 s for DHA and 8.63 ± 0.41 s for vehicle, P < 0.001). However, intrathecal injection 1 or 3-μg DHA had no significant effect on PWL (Fig. 1C). We also tested mechanical allodynia for animals injected with vehicle, 3-μg DHA, and 10-μg DHA. The results showed that pretreatment with 10-μg DHA attenuated carrageenan-induced mechanical allodynia from 0.5 h to 6 h. DHA at the dose of 3 μg only increased PWT at 3 h after carrageenan injection (Fig. 1D).

To test whether DHA could reverse the established pain hypersensitivity, we intrathecally administered DHA at 3 h after carrageenan injection when heat hyperalgesia and mechanical allodynia were fully

### Table 1. Summary of the real-time polymerase chain reaction (PCR) primers sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequences (5′→3′)</th>
<th>Reverse primer sequences (5′→3′)</th>
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<tr>
<td>IBA-1</td>
<td>ATGAGCCCAAAGCAGGGATT</td>
<td>CTTCAAGTTTGGACGGCAG</td>
</tr>
<tr>
<td>GFAP</td>
<td>CCAAGTAAACAACACCTGGA</td>
<td>TCCAGCCATTTCAACCTTTTC</td>
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<tr>
<td>TNF-α</td>
<td>GTTCATGCGCCAGACCTCAGC</td>
<td>GGCACCACTAAGTTGGTGTCTTGG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TCCAGGTAGGAGCATGACGAC</td>
<td>GAACGCAGCCAGCACTGTTA</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCACTTCACAAATGCGAGGTTT</td>
<td>CCAGTTTTGGTACCCATCATTTTC</td>
</tr>
<tr>
<td>CCL2</td>
<td>GCATCACCAGTTGTTGCAA</td>
<td>CTCAGGCTACTCATTTGGAGGATA</td>
</tr>
<tr>
<td>CCL3</td>
<td>CATGACACTCTGCAACCAAGGTCTT</td>
<td>GAGCAAGAGGCTGTCGTTTTCA</td>
</tr>
<tr>
<td>CXCL10</td>
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<td>AGGACCTAGCACCATCCACTGGAAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAATGGTGAAGGTCGCTGTGAAC</td>
<td>CAAACATTCTCCTTGTCCACAG</td>
</tr>
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</table>
developed. As shown in Fig. 1E, intrathecal 10-μg DHA attenuated ongoing hyperalgesia (10.97 ± 0.39 s for DHA and 7.74 ± 0.23 s for vehicle, \( P < 0.01 \)) and lasted for more than 1 h (9.73 ± 0.78 s for DHA and 7.47 ± 0.34 s for vehicle, \( P < 0.05 \)). DHA at 3 μg also showed mild but significant analgesic effect, whereas DHA at 1 μg had no effect (Fig. 1E). DHA at the dose of 10 μg, but not 3 μg partially reversed mechanical allodynia at 30 min after DHA injection (1.01 ± 0.06 g for DHA and 0.30 ± 0.05 g for vehicle, \( P < 0.001 \), Fig. 1F). These data suggest the analgesic effect of DHA by pretreatment persists longer than that by posttreatment.
Intrathecal DHA suppresses carrageenan-induced activation of microglia

To investigate whether the effect of DHA on carrageenan-induced inflammatory pain is mediated through inhibition of glial activation, we checked the mRNA expression of microglial marker, IBA-1 and astrocytic marker, GFAP in the spinal cord after carrageenan injection by Real-time PCR. Carrageenan induced rapid IBA-1 mRNA increase, which began from 1 h, peaked at 3 h and maintained for more than 24 h. However, GFAP mRNA was only increased at 6 and 24 h after carrageenan injection (Fig. 2A).

We then tested the effect of DHA on IBA-1 and GFAP mRNA expression. The mice were intrathecally injected 10⁻¹g DHA 15 min prior to carrageenan injection. The mRNA expression of IBA-1 and GFAP in the spinal cord was checked at 3 h after carrageenan injection. As shown in Fig. 2B, intrathecal 10⁻¹g DHA decreased carrageenan-induced upregulation of IBA-1 mRNA. But GFAP mRNA was not affected. These results indicate that microglial activation precedes astrocytic activation following carrageenan injection and pretreatment DHA reduces the activation of microglia.

Intrathecal DHA suppresses carrageenan-induced activation of p38 MAPK in the spinal cord

Studies have shown that spinal p38 MAPK is an important intracellular kinase in microglia and plays a crucial role in the pathogenesis of chronic pain (Ji and Suter, 2007). We then examined whether DHA could inhibit carrageenan-induced p38 activation in the spinal cord. The immunohistochemistry showed a marked increase in the number of p-p38 immunoreactivity (p-p38-IR) cells in the dorsal horn of carrageenan mice compared with naïve mice (Fig. 3A, B). IBA-1 immunostaining was also increased at 3 h after carrageenan injection (Fig. 3C, D). Double immunofluorescence indicated that p-p38 was co-localized with IBA-1 (Fig. 3E–G).

We then checked p-p38 expression by Western blot. As shown in Fig. 3H, I, carrageenan increased p-p38 expression in the spinal cord at 3 h after injection (P < 0.05). Pretreatment with DHA blocked carrageenan-induced upregulation of p-p38 protein (vehicle vs. DHA, P < 0.05).

Intrathecal DHA reduces carrageenan-induced upregulation of proinflammatory cytokines in the spinal cord

It has been reported that the activated astrocytes and microglia can release a number of signaling molecules including the classic immune signals: cytokines (e.g. TNF-α, IL-1β and IL-6) and chemokines (e.g. CCL2, CCL3, and CXCL10), which play important roles in inflammatory pain (Ji and Suter, 2007; Gao and Ji, 2010a). Therefore, the mRNA expression of these proinflammatory cytokines and chemokines in the spinal cord was further examined by Real-time PCR. As shown in Fig. 4, high levels of TNF-α, IL-1β, IL-6 and CCL2 mRNA were observed at 3 h after carrageenan injection. Intrathecial injection of DHA (10 μg) attenuated upregulation of TNF-α and IL-1β induced by carrageenan, whereas the mRNA expression of other cytokines or chemokines did not change (Fig. 4).

DHA inhibits LPS-induced expression of proinflammatory cytokines and chemokines through p38 MAPK in cultured microglial cells

To further check whether the inhibition effect of DHA on inflammatory mediators’ production is through p38 MAPK pathway in microglia, we cultured BV2 cells. BV-2 is an immortalized murine microglial cell line which replicate readily and is easy to maintain in culture (Blasi et al., 1990). Based on others’ work (Lu et al., 2010; Zhang et al., 2010), the DHA doses of 3, 10, and 30 μM were chosen. Cells were pretreated with various concentrations of DHA for 30 min and then stimulated with LPS (1 μg/ml) for another 6 h. The Real-time PCR results showed that exposure to LPS induced a significant upregulation of TNF-α, IL-1β, IL-6, CCL2, CCL3 and CXCL10 compared with control. DHA at the dose of 30 μM effectively decreased LPS-induced production of these cytokines and chemokines.
compared with LPS treatment. DHA at the concentration of 3 and 10 μM had no significant effect (Fig. 5A).

As Horvath et al. (2008) reported that BV2 cells partially model primary microglia, we then primarily cultured microglia and compared the effect of DHA. As shown in Fig. 5B, LPS induced similar mRNA increase of TNF-α, IL-1β, IL-6 and CCL2 at 3 h. Intrathecally administered DHA 15 min before carrageenan decreases upregulation of TNF-α and IL-1β mRNA expression, whereas has no effect on mRNA expression of IL-6, CCL2, CCL3, or CXCL10. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. naive. #P < 0.05 and ##P < 0.01 vs. vehicle, n = 5.

We then checked activation of p38 by Western blot in BV2. Cells were pretreated with 30 μM DHA for 30 min and then stimulated with LPS (1 μg/ml) for another 1 h. The Western blot results showed that LPS induced a rapid activation (phosphorylation) of p38. Pretreatment of cells with 30 μM DHA decreased p38 activation (Fig. 6A, B). These results suggest that DHA suppresses p38 activation and inflammatory mediators’ production in cultured microglia.

To assess whether p38 is involved in LPS-induced expression of proinflammatory mediators, the effect of p38 inhibitor SB203580 was tested. According to our previous experiment (Gao et al., 2009), the doses of 5 and 50 μM were chosen. Cells were pretreated with SB203580 for 30 min and then stimulated with LPS (1 μg/ml) for another 6 h. The Real-time PCR results showed pretreatment of 5 μM SB203580 had no effect on the expression of these mediators. But SB203580 at a dose of 50 μM effectively inhibited mRNA expression of TNF-α, IL-1β, IL-6, CCL2, CCL3, and CXCL10. These results suggest that p38 plays an important role in mediating the effects of LPS on production of inflammatory mediators (Fig. 6C).
Fig. 5. DHA dose-dependently reduces LPS-induced expression of proinflammatory cytokines and chemokines in BV2 microglial cells (A) and primary cultured microglia (B). DHA at the dose of 30 μM suppressed LPS-induced mRNA upregulation of TNF-α, IL-1β, IL-6, CCL2, CCL3 and CXCL10. Cells were pretreated with DHA (3, 10, and 30 μM) for 30 min and then exposed to LPS (1 μg/ml) for 6 h. *P < 0.05, **P < 0.01, and ***P < 0.001, vs. control. #P < 0.05, ##P < 0.01, and ###P < 0.001, vs. LPS treatment (positive control), n = 3.

Fig. 6. DHA inhibits LPS-induced p38 activation in BV2 microglial cells. (A) Western blot shows protein levels of p38 phosphorylation after stimulation with LPS or pretreated with DHA. Cells were pretreated with DHA (30 μM) for 30 min and then exposed to LPS (1 μg/ml) for 1 h. *P < 0.05, vs. control. #P < 0.05, vs. LPS treatment (positive control), n = 3. (C) p38 inhibitor SB203580 inhibits LPS-induced production of proinflammatory cytokines and chemokines in BV2 microglial cells in a dose-dependent manner. *P < 0.05 and ***P < 0.001, vs. control. ##P < 0.01 and ###P < 0.001, vs. LPS treatment (positive control), n = 3.
DISCUSSION

In this study, we investigated the antinociceptive effect of DHA on carrageenan-induced inflammatory pain and explored possible mechanisms. We demonstrated that DHA exhibited a significant antinociceptive effect in a dose-dependent manner. In particular, the effect of pretreatment is more persistent than that of posttreatment. We further illustrated that pretreatment with DHA blocked carrageenan-induced microglial activation, p38 MAPK phosphorylation and TNF-α and IL-1β mRNA expression in the spinal cord. DHA also inhibited LPS-induced inflammatory mediators’ production through activation of p38 in cultured microglia.

DHA prevents carrageenan-induced inflammatory pain

A meta-analysis demonstrates that dietary omega-3 fatty acids alleviate inflammatory pain in patients (Goldberg and Katz, 2007). Rats treated with a high n-3/n-6 PUFAs ratio diet exhibit a markedly increased threshold for thermal pain and neuropathic pain (Yehuda and Carasso, 1993; Perez et al., 2005). In addition, recent studies showed that oral administration of DHA dose-dependently reduced pain-related behaviors induced by formalin (Nakamoto et al., 2010). Our study for the first time examined the prevention and reversal effect of spinal DHA on carrageenan-induced inflammatory pain. Intrathecal injection DHA (10 µg) 15 min before carrageenan effectively prevented the development of heat hyperalgesia and mechanical allodynia, and the effect persists for 24 h for heat hyperalgesia and 6 h for mechanical allodynia. However, the reversal effect of DHA is only 1 h for heat hyperalgesia and 30 min for mechanical allodynia. Xu et al. (2010) showed that intrathecal injection of DHA attenuated complete Freund’s adjuvant (CFA)-induced heat hyperalgesia for 30 min. These results suggest that dietary supplement DHA may play an important role in the prevention of inflammatory pain, whereas the treatment effect of DHA is relatively weak. In addition, the effect of DHA on heat hyperalgesia persists longer than on mechanical allodynia.

DHA inhibits microglia activation and P38 activation

In recent years, non-neuronal cells such as immune cells and glial cells (microglia and astrocytes) have been implicated to play a critical role in the pathogenesis of chronic pain (Milligan et al., 2003; Tsuda et al., 2004; Marchand et al., 2005; Chiang et al., 2007; Romero-Sandoval et al., 2008). Although both microglia and astrocytes were activated in the spinal cord following peripheral nerve injury or tissue injury, microglial activation precedes astrocytic reaction in most cases (Ji and Suter, 2007; Gao and Ji, 2010b). Behavioral studies have shown that inhibition of microglial activation by minocycline prevents/delays neuropathic pain development (Raghavendra et al., 2003; Ledeboer et al., 2005), whereas inhibition of astrocytes’ function reverses nerve-injury- or nerve inflammation-induced mechanical allodynia (Milligan et al., 2003; Zhuang et al., 2006), suggesting the respective role of microglia and astocytes in the development and maintenance of neuropathic pain. In this study, carrageenan injection induced early microglial activation and late astrocytic activation. Pretreatment with DHA in the spinal cord blocked carrageenan-induced pain and decreased IBA-1 mRNA upregulation. It was reported that intrathecal administration of microglial inhibitor minocycline attenuates carrageenan-induced hyperalgesia (Wirkner et al., 2007). These data support the pivotal role of microglia in the initiation of carrageenan-induced inflammatory pain.

The MAPKs -JNK (c-Jun N-terminal kinase), ERK (extracellular signal-regulated kinase), and p38 are important molecules in chronic pain sensitization and are differentially activated in spinal cord gial cells (Ji et al., 2009). In particular, p38 MAPK has been shown to be activated in spinal microglia after nerve injury or tissue injury (Jin et al., 2003; Tsuda et al., 2004). Behavioral studies showed that i.t. injection of p38 inhibitor SB203580, FR167653 or CNI-1493 prevents the development of neuropathic pain (Jin et al., 2003; Tsuda et al., 2004; Hains and Waxman, 2006; Wen et al., 2007). Our study showed that carrageenan induced p38 activation in the spinal cord microglia at 3 h after injection, which was reduced by pretreatment with DHA. Our in vitro data also showed DHA (30 µM) diminished LPS-induced p38 activation in BV2 microglia. Other studies using different cells showed that DHA reduced the basal expression of p-p38 in CD4 (+) T-cells (Attakpa et al., 2009) and lung cancer cells (Serini et al., 2008). DHA also suppresses the activity of p38 MAPK in LPS-stimulated dendritic cells (Wang et al., 2007) and TNF-α-stimulated endothelial cells (Xue et al., 2006). These data indicate that the analgesic effect of DHA may be mediated by inhibition of p38 activation in microglia.

DHA reduces inflammatory mediators’ production

It has been demonstrated that activated microglia and astrocytes release neuromodulators, such as growth factors, proinflammatory cytokines, and chemokines, which contribute to pain facilitation (Watkins et al., 2001; Gao and Ji, 2010a; Gwak et al., 2012; Yang et al., 2012). Our results showed that carrageenan induced pain hypersensitivity that associated with increased mRNA expression of TNF-α, IL-1β, IL-6 and CCL2 in the spinal cord at 3 h after injection. However, CCL3 and CXCL10 mRNA expression was not changed, suggesting they, in the spinal cord level, may not be related with carrageenan-induced inflammatory pain at this time point. Electrophysiological studies have shown that TNF-α, IL-1β, IL-6, and CCL2 could directly regulate synaptic transmission in dorsal horn neurons (Kawasaki et al., 2008; Gao et al., 2009). Milligan et al. (2003) showed that inhibition of TNF-α and IL-1β prevent or reverse inflammatory neuropathy in rats. Here DHA intrathecal administration decreased TNF-α and IL-1β expression, suggesting the antinociceptive
effect of DHA may be through inhibition of the proinflammatory cytokines TNF-α and IL-1β.

Although intrathecal DHA only reduced the expression of TNF-α and IL-1β mRNA in the spinal cord, our in vitro data showed that DHA reduced LPS-induced mRNA expression of more mediator, including IL-6, CCL2, CCL3, and CXCL10. The discrepancy may be due to the low expression of these chemokines in the spinal cord and the relative low dose of DHA given by intrathecal. It was reported that DHA supplementation significantly prevented hypoxia/ischemia-induced upregulation of TNF-α, IL-1β, IL-6, COX-2 and iNOS in the ipsilateral cortex (Zhang et al., 2010). DHA also reduced expressions of TNF-α, IL-6, iNOS induced by IFN-γ in BV2 microglial cells (Lu et al., 2010). Given that DHA blocked LPS-induced p38 activation and p38 inhibitor decreased LPS-induced expression of inflammatory mediators, the inhibition effect of DHA on inflammatory mediators’ production is through the p38 MAPK pathway in microglia.

CONCLUSIONS

Inflammatory pain, such as arthritis pain, is a growing health problem. The commonly used opioids and COX inhibitors for treating inflammatory pain are limited by side effects. Our results showed that intrathecal given DHA mainly targeting at the spinal cord level could effectively block carrageenan-induced pain hypersensitivity, suggesting dietary supplementation of DHA or n-3 PUFA may have preventive effect on inflammatory pain. Furthermore, our in vivo and in vitro data indicate that the analgesic effect of DHA in the spinal cord may be through p38 MAPK-mediated microglia activation and subsequent inflammatory mediators’ production. Collectively, our results provide a novel implication of antinociceptive mechanism of DHA.

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