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Docosahexaenoic Acid Suppresses Neuroinflammatory Responses and Induces Heme Oxygenase-1 Expression in BV-2 Microglia: Implications of Antidepressant Effects for Omega-3 Fatty Acids

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Accumulating evidence suggests that the pathophysiology of depression might be associated with neuroinflammation, which could be attenuated by pharmacological treatment for depression. Omega-3 polyunsaturated fatty acids (PUFAs) are anti-inflammatory and exert antidepressant effects. The aim of this study was to identify the molecular mechanisms through which docosahexaenoic acid (DHA), the main omega-3 PUFA in the brain, modulates oxidative reactions and inflammatory cytokine production in microglial and neuronal cells. The results of this study showed that DHA reduced expressions of tumor necrosis factor-α, interleukin-6, nitric oxide synthase, and cyclo-oxygenase-2, induced by interferon-γ, and induced upregulation of heme oxygenase-1 (HO-1) in BV-2 microglia. The inhibitory effect of DHA on nitric oxide production was abolished by HO-1 inhibitor zinc protoporphyrin IX. In addition, DHA caused AKT and ERK activation in a time-dependent manner, and the DHA-induced HO-1 upregulation could be attenuated by PI-3 kinase/AKT and MEK/ERK inhibitors. DHA also increased IKKα/β phosphorylation, IκBα phosphorylation, and IκBα degradation, whereas both nuclear factor-κB and IκB protease inhibitors could inhibit DHA-induced HO-1 expressions. The other major n-3 PUFA, eicosapentaenoic acid, showed similar effects of DHA on inflammation and HO-1 in repeated key experiments. In connecting with inflammation hypothesis of depression and clinical studies supporting the antidepressant effects of omega-3 PUFAs, this study provides a novel implication of the antidepressant mechanisms of DHA.
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INTRODUCTION

There is compelling evidence to suggest that neuroinflammation and inflammatory mediator production contribute to the pathophysiology of depression. For example, depressed patients have been found to have higher levels of proinflammatory cytokines, including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), as well as interferon-γ (IFN-γ; Maes *et al*, 2009; Maes, 2008). From prospective observations of animal and clinical studies, behavioral changes induced by proinflammatory cytokines, eg, IL-1β, IFN-γ, and IFN-α, resemble symptoms of depression, and are probably the most strikingly supportive evidence for the inflammation theory of depression (Dantzer *et al*, 2008; Miller *et al*, 2009; Su, 2009). In fact, inflammatory cytokines have been found to interact with many pathophysiological domains, including neurotransmitter metabolisms, neuroendocrine functions, synaptic plasticity, and oxidative status, which might characterize depression (Raison *et al*, 2006). The neuro-inflammatory activation contributes to the overproduction of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) and the loss of glial elements (Ida *et al*, 2008; Li *et al*, 2008b, 1999), which are consistent with neuropathological findings characterizing depression (Miller *et al*, 2009). Interestingly, antidepressant medications, including noradrenaline reuptake inhibitors (O'Sullivan *et al*, 2009) and tricyclic antidepressants (TCAs; Tai *et al*, 2006), have been found to attenuate the oxidative stress and inflammatory reaction by suppressing the expression of iNOS and several proinflammatory cytokines.

The mechanism of antioxidative and anti-inflammatory reactions from antidepressant medications has been found

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to be associated with an enhancement of heme oxygenase-1 (HO-1) expression (Kim *et al*, 2008; Shin *et al*, 2009; Tai *et al*, 2009). HO is a rate-limiting enzyme that catalyzes the conversion of heme to equimolar amounts of CO, iron, and biliverdin (Abraham and Kappas, 2008). Two main HO isoforms, HO-1 and HO-2, can be found in human brain. Unlike the constitutively expressed HO-2, HO-1 (also referred to as heat-shock protein 32, HSP32) can be strongly inducible in response to diverse stress-related cellular stimuli, including its substrate heme, heavy metals, UV radiation, endotoxin, inflammatory cytokines, and prostaglandins (Abraham and Kappas, 2008). The induction of HO-1 in brain neuronal and nonneuronal cells is important in neuroprotection and neuroplasticity (Choi and Kim, 2008; Le *et al*, 1999), which are also characteristics of antidepressant mechanisms (Charney and Manji, 2004; Krishnan and Nestler, 2008).

N-3 (or omega-3) polyunsaturated fatty acids (PUFAs) have an important role in neurobiological mechanisms of depression (Horrobin and Bennett, 1999; Su *et al*, 2000) and cytokine-induced sickness and depressive behaviors (Song *et al*, 2004a; Su *et al*, 2010; Su, 2008). Specifically, societies consuming a large amount of omega-3 PUFAs seem to have a lower prevalence of major depressive disorder (Hibbeln, 1998). In addition, major depression is associated with low levels of n-3 PUFAs in peripheral blood tissues (Lin *et al*, 2010; Maes *et al*, 1996; Peet *et al*, 1998) and in postmortem brains (McNamara *et al*, 2007). Consistent with the observational studies, several clinical trials, if not all (Marangell *et al*, 2003; Silvers *et al*, 2005), have shown that omega-3 PUFAs were effective in treating patients with major depression. (Freeman *et al*, 2006; Lin and Su, 2007; Su *et al*, 2008). Moreover, n-3 PUFAs have a protective effect against the development of depression-like behaviors induced by stress (Carlezon *et al*, 2005; Huang *et al*, 2008) or proinflammatory cytokines (Song *et al*, 2004a, 2007, 2008) in animal models of depression.

The function of n-3 PUFAs in the brain is diverse, but the mechanisms of antidepressant effects of n-3 PUFAs have yet to be elucidated. Although n-3 PUFAs can regulate monoamine neurotransmitters that interact with central pathophysiological domains in depression, accumulating evidence implies that anti-inflammation might also be involved (Su, 2009; Venna *et al*, 2009). The two major bioactive components of n-3 PUFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), particularly found in fatty fish, are not synthesized in the human body and should be obtained directly from the diet (Lands, 1992). EPA has an important role in balancing inflammation by antagonizing membrane arachidonic acid (an n-6 PUFA), reducing prostaglandin E2 synthesis (Farooqui *et al*, 2006), and reversing inflammatory responses induced by cytokines and LPS in microglia (Kawashima *et al*, 2009; Lynch *et al*, 2007; Moon and Pestka, 2003), and has also been found to have beneficial effects on cytokine-induced sickness and depressive behaviors (Song *et al*, 2004a, b, 2008), as well as antidepressant effects in patients with depression (Nemets *et al*, 2006; Peet and Horrobin, 2002; Su *et al*, 2003). Compared with only 0.1% of EPA in total brain fatty acid, DHA comprises 10–20% and is the main n-3 fatty acid in the brain (McNamara and Carlson, 2006) and has long been considered neuroprotective (Bazan, 2006) and anti-inflammatory (De Smedt-Peyrusse *et al*, 2008; Ebert *et al*, 2009). However, the antidepressant mechanisms through neuroinflammation pathways for DHA still remain unclear.

Recently, Brand *et al* (2010) have found that membrane lipid modification by PUFAs, especially DHA, promoted upregulation of HO-1, but sensitized oligodendroglial cells to oxidative stress. Their study, however, applied stimuli from heat and H_2O_2 oxidative stresses, rather than inflammatory stimuli. To our knowledge, the role of HO-1 in anti-inflammatory effects of DHA has not yet been reported. In this study, we applied an *in vitro* cell model to show DHA's effects on inflammatory stimulation in immortalized microglial cells, BV-2. Microglial cells reside in the parenchyma and have a critical role in host defense, tissue repair, and neuroprotection/neurotoxicity in the brain (Hanisch and Kettenmann, 2007). Microglial activation involves changes in cell phenotype and the expression of new proteins such as iNOS and cyclo-oxygenase-2 (COX-2), which in turn might result in neuronal damages through the release of nitric oxide (NO) and proinflammatory cytokines (Bal-Price and Brown, 2001; Block *et al*, 2007). Active microglial cells can be immortalized into the BV-2 cell line, which retains most of the morphological, phenotypical, and functional properties of freshly isolated microglial cells (Blasi *et al*, 1990). The goal of this study was to identify the molecular mechanism through which DHA modulates oxidative reaction and proinflammatory cytokine production in microglial cells.

MATERIALS AND METHODS

Materials

DHA and EPA were purchased from Sigma-Aldrich (St Louis, MO). Fetal bovine serum (FBS), DMEM, and OPTI-MEM were purchased from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA). Goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated IgG, primary antibodies against IκBα, IKKα/β, p65, p50, PI-3 kinase (p85), β-actin, AKT, ERK2, phospho-ERK1/2, and phospho-AKT (Ser^{473}), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against IKKα/β, phosphorylated at $Ser^{180/181}$, and p65, phos phorylated at Ser^{573}, were purchased from Cell Signaling and Neuroscience (Danvers, MA). Wortmannin, TPCK, and PDTC were purchased from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

Cell Culture

All protocols for animal experiments were approved by Animal Care Committees of China Medical University. Sprague–Dawley rats were obtained from the National Laboratory Animal Center in Taiwan. The detailed procedures of primary microglia cultures have been described elsewhere (Lu *et al*, 2009, 2007). Briefly, glial cells were cultured for 12–14 days in DMEM/F12 (Gibco, Grand Island, NY) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. To separate microglia, flasks were shaken for 5 h at 180 r.p.m. in a rotary shaker at 37°C. Detached cells were passed through a 70 μm nylon mesh filter (BD Falcon, Franklin Lakes, NJ) and then plated

into 24-well plates at a density of 5×10^5 cells/well. The purity of microglia cultures was assessed using CD11b antibody, and more than 95% of cells were stained positively. Cells were cultured in DMEM with 2% FBS for 2 days before drug treatment.

BV-2, the murine cell line, has been generated by infecting primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus (J2). Phenotypically, BV-2 cells tested positive for MAC1 and MAC2 antigens. As BV-2 cells retain most of the morphological, phenotypical, and functional properties described for freshly isolated microglial cells, they can be considered as immortalized active microglial cells (Blasi *et al*, 1990). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with 10% FBS at 37°C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. Confluent cultures were passaged by trypsinization.

MES23.5 is a dopaminergic cell line hybridized from murine neuroblastoma-glioma N18TG2 cells with rat mesencephalic neurons, which show several properties similar to those of primary neurons originated in the substantia nigra (Crawford *et al*, 1992). Cells were cultured in DMEM/F12 containing Sato's components growth medium supplemented with 5% FBS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin at 37°C, in a humid 5% CO_2, 95% air environment. For experiments, cells were seeded at a density of $1 \times 10^5/cm^2$ in plastic plates.

Western Blot Analysis

BV-2 cells were treated with DHA for indicated time periods and then washed with cold PBS that had been lysed for 30 min on ice with radioimmunoprecipitation assay buffer (50 mM HEPES (PH 7.4), 150 mM NaCl, 4 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% Triton X-100, 0.25% sodium deoxycholate, 50 mM 4-(2-aminoethyl) benzene sulfonylfluoride, 50 μg/ml leupeptin, and 20 μg/ml aprotinin). Protein samples were separated using sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk in PBS for 1 h at room temperature and then probed with primary antibodies. After undergoing three PBS washes, the membranes were incubated with secondary antibodies. Blots were visualized by enhanced chemiluminescence using a Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Reverse Transcription and Quantitative Real-Time PCR

Total RNA was extracted from BV-2 cells using a TRIzol kit (MDBio, Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA.

Quantitative real-time PCR was performed using SYBR-green detection of PCR products in real time using the 48-well StepOne Real-Time System (Applied Biosystems, Foster City, CA). The following sequence-specific oligonucleotide primers were used:

iNOS: 5′-CCCAGAGTTCCAGCTTCTGG-3′ and 5′-CCAAGCCCCTCACCATTATCT-3′;
COX-2: 5′-TGGGGTGATGAGCAACTATT-3′ and 5′-AAG GAGCTCTGGGTCAAACT-3′;
IL-1β: 5′-TGGGGGAGATTCTCACTTTG-3′ and 5′-CCATC AGCGTTCCCATACTT-3′;
IL-6: 5′-CCAGTTGCCTTCTTGGGACTG-3′ and 5′-CAGGTCTGTTGGGAGTGGTATCC-3′;
TNF-α: 5′-AAAATTCGAGTGACAAGCCTGTAG-3′ and 5′-CCCTTGAAGAGAACCTGGGAGTAG-3′;
GAPDH: 5′-CTCAACTACATGGTCTACATGTTCCA-3′ and 5′-CTTCCCATTCTCAGCCTTGACT-3′.

Immunocytofluorescent Staining

Cells were seeded onto glass coverslips and exposed to DHA for 24 h, then washed with PBS and fixed with 4% paraformaldehyde for 15 min, after which they were permeabilized with Triton X-100 for 30 min. After blocking with 5% nonfat milk in PBS buffer, cells were incubated with rabbit anti-p50 or anti-p65 antibodies for 1 h at room temperature. After a brief wash, cells were incubated with a goat anti-rabbit FITC-conjugated secondary antibody (1 : 100; Leinco Tec., St Louis, MO). Finally, the cells were washed again, mounted, and visualized with a fluorescence microscope.

Statistics

The values given are means ± SEM. The significance of difference between the experimental and control groups was assessed by Student's *t*-test. The difference was significant if the *p*-value was <0.05.

RESULTS

DHA Suppresses IFN-γ-Induced Inflammatory Cytokine Expression in Bv-2 Microglia

We used the BV-2 microglia cell line to study the antineuroinflammatory mechanism of DHA. To determine the effect of DHA on iNOS and COX-2 protein levels, BV-2 cells were treated with LPS or IFN-γ plus DHA, and protein levels were detected using western blotting (Figure 1a and b). BV-2 cells were pretreated with various concentrations of DHA for 30 min and then stimulated with IFN-γ for another 24 h. As shown in Figure 1c and d, DHA effectively inhibited IFN-γ-induced iNOS and COX-2 expression in a concentration-dependent manner. The cell culture medium was then harvested to determine nitrite content by Griess reaction. Results show that IFN-γ increased the production of NO up to 5.5-fold. DHA effectively antagonized IFN-γ-induced NO production (Figure 1e). To determine whether the mechanisms are similar across n-3 PUFAs, we repeated the experiments with EPA and found that EPA inhibited IFN-γ-induced iNOS and COX-2 expression (Supplementary Figure S1A), as well as NO production (Supplementary Figure S1B), in BV-2 microglia. DHA and EPA at concentrations ranging from 3 to 30 μM did not affect cell viability using the MTT assay (data not shown).

With real-time PCR analysis, we examined the effects of DHA and IFN-γ on BV-2 microglial expressions in mRNA levels of iNOS, COX-2, IL-6, and TNF-α. Not shown in the figures, IFN-γ induced iNOS, COX-2, IL-6, and TNF-α

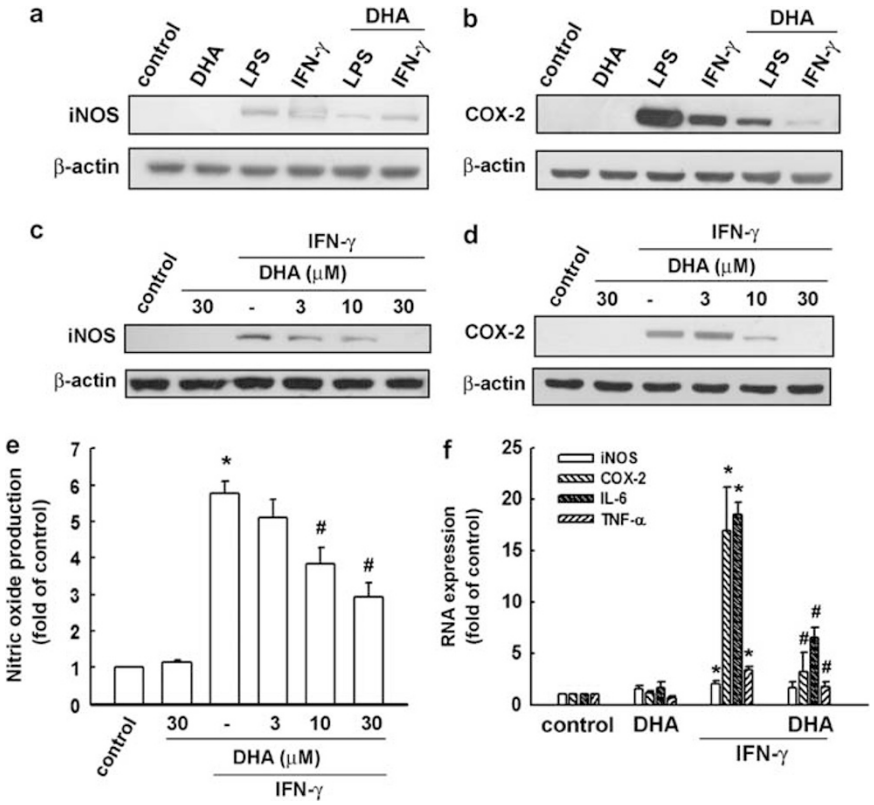


Figure 1 DHA suppresses IFN-γ-induced iNOS and COX-2 expression. BV-2 cells were pretreated with DHA (30 μM) for 30 min before application of LPS (100 ng/ml) or IFN-γ (30 μg/ml). Whole-cell lysis protein was extracted and subjected to western blot analysis for iNOS (a) and COX-2 (b) expression after 24 h of incubation with LPS or IFN-γ. Cells were pretreated with various concentrations of DHA (0, 3, 10, or 30 μM) for 30 min before IFN-γ (30 μg/ml) application. Whole-cell lysis protein was extracted and subjected to western blot analysis for iNOS (c) and COX-2 (d) expression after 24 h of incubation with IFN-γ. (e) The culture mediums were collected and analyzed using Griess reaction. (f) BV-2 cells were pretreated with various concentrations of DHA (0, 3, 10, or 30 μM) for 30 min before being stimulated with IFN-γ (30 μg/ml) for 6 h. Relative mRNA levels of iNOS, COX-2, TNF-α, and IL-6 were analyzed using real-time PCR and normalized with GAPDH. Each bar represents means ± SEM. $^{*}P<0.05$ compared with the control group. $^{\#}P<0.05$ compared with the IFN-γ treatment group. Similar results were obtained from at least four independent experiments.

expressions in a time- and concentration-dependent manner. We then analyzed levels of total mRNA extracted from BV-2 microglia after a 6-h IFN-γ stimulation. As shown in Figure 1f, pretreatment with DHA significantly inhibited IFN-γ-induced iNOS, COX-2, IL-6, and TNF-α mRNA expressions.

DHA Induces HO-1 Upregulation in BV-2 Microglia Cells

As HO-1 induction participates in the negative regulation of inflammatory cytokine expression, we investigated whether DHA leads to HO-1 induction. When BV-2 microglia were treated with DHA for 24 h, HO-1 levels were increased in a concentration-dependent manner (Figure 2a). Similarly, EPA also increased HO-1 protein expression in BV-2 microglia (Supplementary Figure S2A). In Figure 2b and c, we further analyzed HO-1 mRNA expressions induced by DHA in various concentration- and time-dependent conditions in BV-2. We also analyzed HO-1 mRNA expressions induced by DHA (Figure 2d) and EPA (Supplementary Figure S2B) in primary cultured microglia. We then used ZnPPIX, an HO-1 inhibitor, to examine whether HO-1 expression is required in the anti-inflammatory pathways exerted by DHA. As shown in Figure 2d, the presence of ZnPPIX abrogated the inhibitory effects of DHA on IFN-γ-induced NO production. Similarly, the inhibitory effects of EPA on IFN-γ-induced NO production could also be abrogated by ZnPPIX treatment (Supplementary Figure S2C).

DHA Stimulates HO-1 Upregulation Through PI-3 Kinase/AKT And ERK Signaling Pathways in BV-2 Microglia

We investigated the signaling pathway involved in the HO-1 expression induced by DHA. As shown in Figure 3a, DHA-induced HO-1 expression was markedly antagonized by pretreatment with the PI-3 kinase/AKT inhibitor LY294002 or the MEK inhibitor PD98059. These inhibitors did not affect cell viability as assessed using MTT assay (data not shown). Furthermore, DHA activated AKT and ERK1/2 phosphorylation in a time-dependent manner (Figure 3b and c). These data suggest that activation of PI-3 kinase/AKT and ERK pathways is required for the upregulation of HO-1 induced by DHA in BV-2 microglia.

DHA Induces HO-1 Expression Through The NF-κB Signaling Pathway

It has been reported that nuclear factor-κB (NF-κB) activation is important for HO-1 induction in many cells

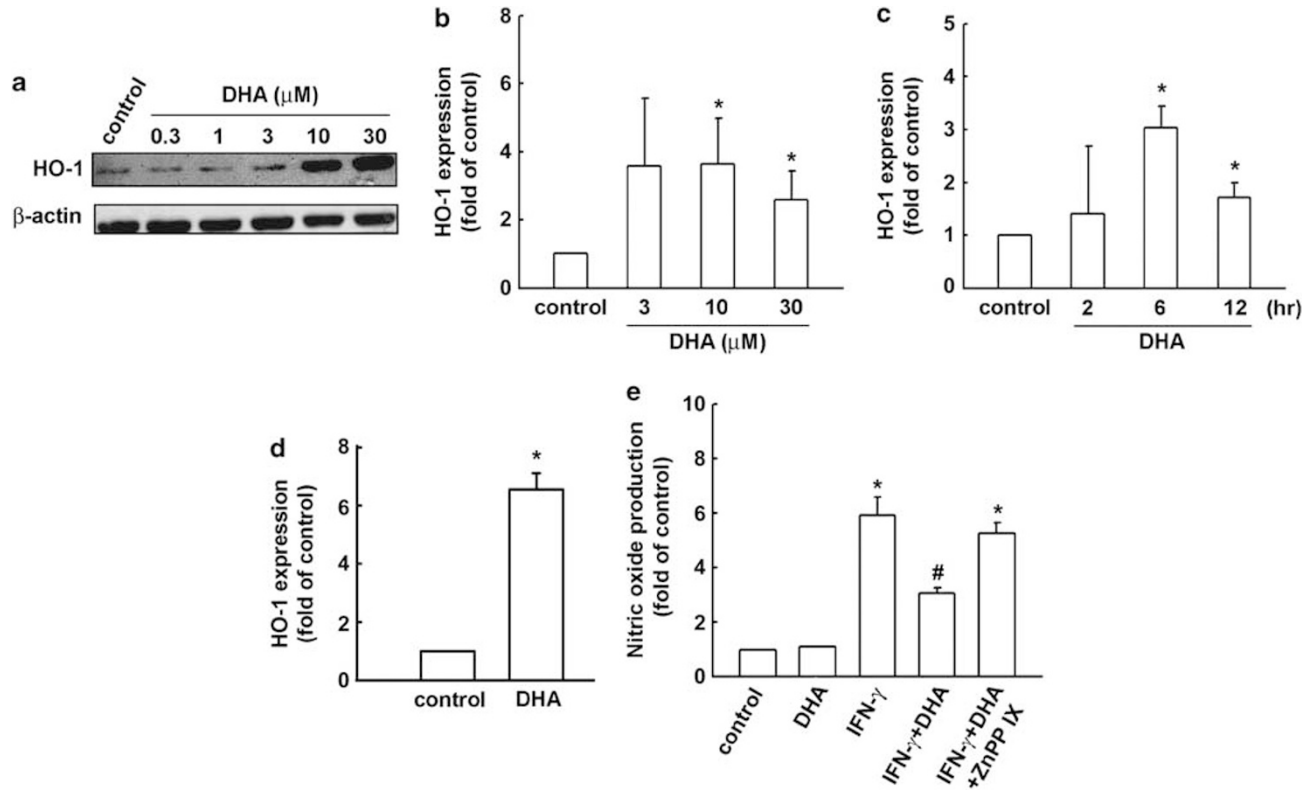
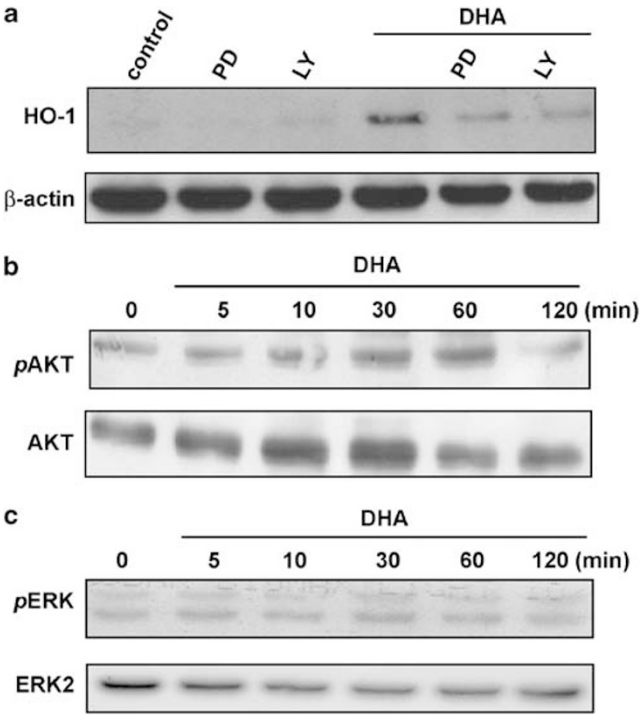


Figure 2 DHA induces HO-1 upregulation in BV-2 microglia cells. (a) BV-2 cells were stimulated with various concentrations of DHA for 24 h. Whole-cell lysates were subjected to western blot analysis using an antibody against HO-1. Cells were stimulated with various concentrations of DHA for 6 h (b) or stimulated with DHA (30 μM) for indicated time periods (c). Rat primary cultured microglia were stimulated with DHA (30 μM) for 6 h (d). The mRNA level of HO-1 was analyzed using real-time PCR. Results are expressed as the mean ± SEM of four independent experiments. (e) Cells were pretreated with ZnPPIX (10 μM) for 30 min and treated with DHA for another 30 min before IFN-γ (30 μg/ml) application. NO production was analyzed using Griess reaction. Note that ZnPPIX effectively antagonized IFN-γ-induced NO production. Each bar represents means ± SEM from at least four independent experiments. $^{*}p<0.05$ compared with the control group; $^{\#}p<0.05$ compared with the IFN-γ treatment group.



(Chen *et al*, 2004; Hill-Kapturczak *et al*, 2001; Juan *et al*, 2005; Lin *et al*, 2007; Nakao *et al*, 2008). We therefore examined whether the NF-κB pathway is involved in DHA-induced HO-1 expression. NF-κB activation was assessed with the translocation of NF-κB from cytosol to nucleus. As shown in Figure 4a, DHA treatment resulted in a marked translocation of NF-κB p50 and p65 from the cytosol to the nucleus in microglia. Furthermore, NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC, 30 μM) and IκBα phosphorylation inhibitor (Bay 117082, 3 μM) effectively antagonized the enhancement of HO-1 expression induced by DHA (Figure 4b). Again, treatment with those inhibitors did not affect cell viability assessed using MTT assay (data not shown). The immunocytochemical examination of NF-κB localization has also been examined. As shown in Figure 4c, NF-κB p50 and p65 were primarily located in the cytosol

Figure 3 DHA stimulates HO-1 upregulation through PI-3 kinase/AKT and ERK signaling pathways in BV-2 microglia. (a) BV-2 cells were pretreated with the PI-3 kinase inhibitor LY294002 (10 μM) or MEK inhibitor PD98059 (30 μM) for 30 min before DHA treatment. Whole-cell lysates were subjected to western blot analysis using an antibody against HO-1. (b and c) Cells were incubated with DHA for indicated time periods (0–120 min). Whole-cell lysates were subjected to western blot analysis using antibodies against the phosphorylation of AKT and ERK1/2. Similar results were obtained from at least three independent experiments.

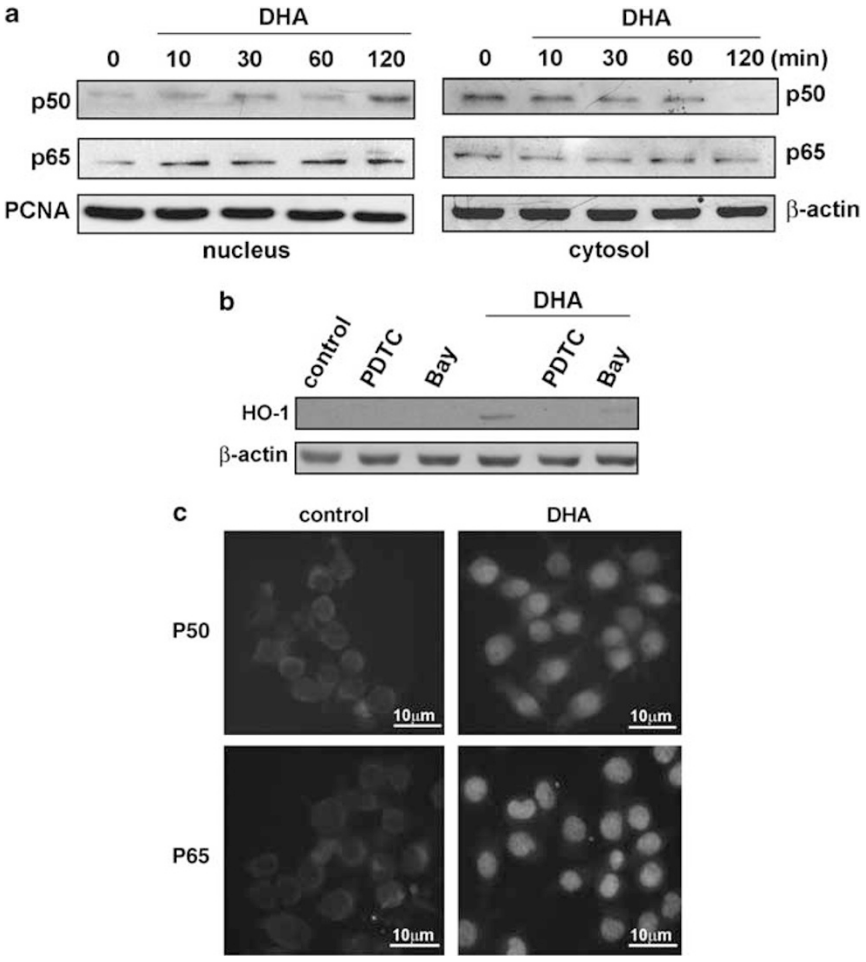


Figure 4 DHA induces HO-1 expression through the NF-κB signaling pathway. (a) BV-2 cells were treated with DHA for indicated time intervals, and the levels of cytosolic and nuclear p50 or p65 were determined by immunoblotting with a p65- or p50-specific antibody. PCNA and β-actin were used as loading controls for nuclear and cytosolic fractions, respectively. (b) Cells were pretreated with PDTC (30 μM) or Bay 11-7082 (3 μM) for 30 min, followed by stimulation for 24 h with DHA (30 μg/ml). Whole-cell lysis protein was subjected to western blot analysis for HO-1. (c) The nuclear translocation of p50 or p65 after 1 h of DHA treatment is shown using immunofluorescence assay. Scale bar, 10 μm.

during the resting state (control panel), and were translocated into nuclei in response to DHA treatment for 1 h.

We then examined the upstream molecules involved in DHA-induced NF-κB activation. Stimulation of cells with DHA induced IKKα/β phosphorylation, IκBα phosphorylation, and IκBα degradation in a time-dependent manner (Figure 5a and b). Pretreatment of cells with PI-3 kinase inhibitors (LY294002 or wortmannin) or MEK inhibitors (PD98059 or U0126) attenuated DHA-induced IKKα/β phosphorylation and IκBα phosphorylation (Figure 5d and e), suggesting that IKKα/β activation is regulated by PI-3 kinase/AKT or ERK signaling pathways. Treatment of BV-2 microglia cells with DHA also increased p65 phosphorylation at Ser^{573} in a time-dependent manner (Figure 5c). These results showed that DHA effectively inhibited IFN-γ-induced inflammatory mediator expression. In addition, DHA also induced HO-1 upregulation through the activation of PI-3 kinase/AKT and ERK signaling pathways, leading to IKKα/β phosphorylation, p65 phosphorylation, and activation of NF-κB in the microglia.

DISCUSSION

The main finding of this study is that DHA potently inhibits oxidative reactions and proinflammatory responses in microglia, probably by promoting the upregulation of HO-1 expression. To our knowledge, this is the first report that shows the role of HO-1 in the anti-inflammatory effects of DHA and identifies the molecular mechanism through which DHA modulates oxidative reaction and proinflammatory cytokine production in microglial cells.

First, the inflammatory stimuli of IFN-γ increase the upregulation of oxidative and proinflammatory mediators, including iNOS, COX-2, IL-6, and TNF-α, which can be attenuated by DHA. The results of our current study are consistent with the findings from previous studies showing that DHA is antioxidative and anti-inflammatory (De Smedt-Peyrusse *et al*, 2008; Ebert *et al*, 2009; Komatsu *et al*, 2003; Saw *et al*, 2010). However, there are some studies showing that DHA is pro-oxidative in higher doses (Brand *et al*, 2010; Hirafuji *et al*, 2002). In the nervous system, DHA

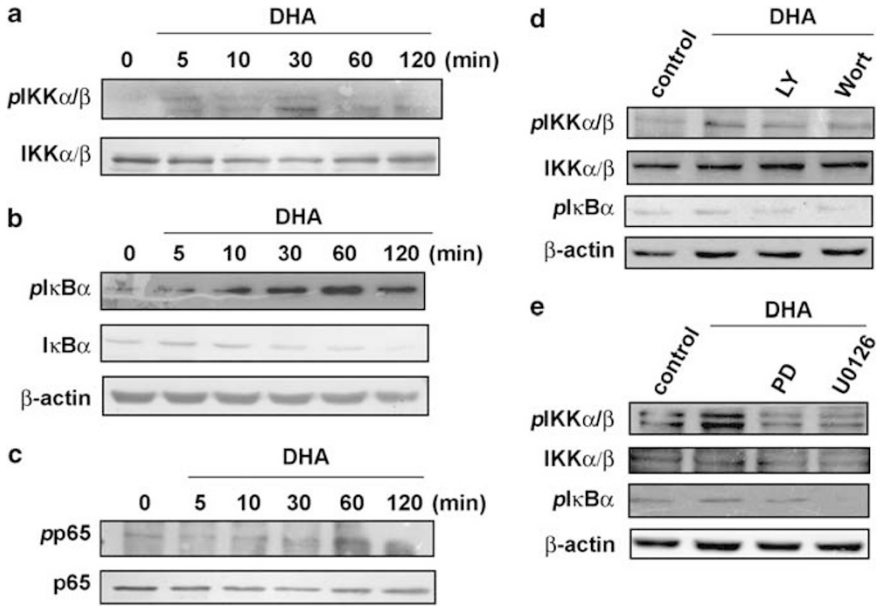


Figure 5 DHA induces IKKα/β activation, IκBα phosphorylation, IκBα degradation, and p65 Ser^{573} phosphorylation in BV-2 microglia. BV-2 cells were incubated with DHA for indicated time periods, and phospho-IKKα/β (a), phospho- IκBα and IκBα (b), or phosphor-p65 (c) expression levels were determined using western blot analysis. Note that DHA induced IKKα/β phosphorylation, IκBα phosphorylation and IκBα degradation, and p65 phosphorylation in a time-dependent manner. Cells were pretreated with LY294002 (10 μM) or wortmannin (100 nM) (d), or with PD98059 (30 μM) or U0126 (1 μM) (e), for 30 min, followed by stimulation with DHA for 60 min. Cell lysates were then evaluated using immunoblotting. Results are representative of four independent experiments.

has diverse functions. For example, DHA can regulate membrane receptors and influence membrane fluidity at the molecular level, and promote neurite growth and neuronal survival at the cellular level (Su, 2009). Although antioxidant and anti-inflammatory effects of DHA may be prominent, its pro-oxidant potential and risk of an increase in lipid peroxide formation have to be taken into consideration (Brand *et al*, 2010; Yavin *et al*, 2002). Future studies investigating the paradoxical oxidant effects of DHA are warranted.

Second, our results support previous reports (Tsoyi *et al*, 2008; Vareille *et al*, 2008) showing that HO-1 is a critical regulator of NO production in numerous cells. Specifically, we found that DHA inhibition of NO production was effectively abolished by ZnPPIX, an HO-1 inhibitor. In addition, previous reports have shown that PI-3 kinase/AKT and ERK pathways regulate HO-1 expression in various cells (Choi and Kim, 2008; Wu *et al*, 2006). Again, our results from the primary cultured microglia and BV-2 microglial cells support the role of these pathways in DHA-induced HO-1 expression. Specifically, DHA activated AKT and ERK1/2 phosphorylation, and blockade of PI-3 kinase/AKT and MEK/ERK pathways inhibited DHA-induced HO-1 expression. Furthermore, the HO-1 upregulation induced by both DHA and EPA could be observed in MES 23.5 dopaminergic cells (Supplementary Figure S3). Further experiments would be needed to specify the effects of HO-1 expression in neuronal cells.

Interestingly, the activation of AKT, ERK1/2 phosphorylation, and NF-κB seems to be associated with mechanisms of antidepressant effects of several traditional antidepressants (eg, specific serotonin reuptake inhibitors and TCAs; Charney and Manji, 2004; Duman *et al*, 1999, 2000; Li *et al*, 2008a; Manji *et al*, 2001; Mercier *et al*, 2004) and off-label agents used for depression treatment (eg, lithium, valproate, omega-3 PUFAs, and atypical antipsychotics; Chen *et al*, 1999; Lu *et al*, 2004; Lu and Dwyer, 2005; Manji *et al*, 2001; Seti *et al*, 2009; Zhang *et al*, 2009). A recent report has shown that a TCA, amitriptyline, induces HO-1 expression in chronic morphine-infused rats (Tai *et al*, 2009). Interestingly, our additional experiment also showed that another TCA, desipramine, could increase HO-1 expression in BV-2 microglia. Furthermore, a desipramine cotreatment with DHA potentiates this HO-1 expression (Supplementary Figure S4, not shown in the Results section). In the regulatory level, the HO-1 gene promoter contains multiple potential regulatory transcription factor binding sites, including NF-κB, ARE, AP-1, and IL-6-responsive elements (Choi and Alam, 1996; Lavrovsky *et al*, 1994), suggesting a potential role for these transcription factors in modulating HO-1 expression. The NF-κB signaling pathway regulates several physiological processes and has been found to have a dual function in response to microenvironmental stimuli (Pasparakis, 2009). For example, NF-κB promotes the expression of proinflammatory genes, including genes encoding cytokines, chemokines, and adhesion molecules (Lu *et al*, 2009, 2007). On the other hand, NF-κB protects cells by inhibiting the accumulation of reactive oxygen species by activating transcription of antioxidant proteins (Sakon *et al*, 2003), and upregulates the transcription factor of an anti-inflammatory cytokine IL-10 by upregulating HO-1 expression (Lee and Chau, 2002; Saraiva and O'Garra, 2010; Tai *et al*, 2009). NF-κB has been found to be important for the induction of HO-1 expression in response to diverse stimuli, such as TGF-β-1, TNF-α, and LPS (Malaguarnera *et al*, 2005; Ning *et al*, 2002). The results of this study also show that NF-κB activation is essential for DHA-stimulated HO-1 expression, based on the fact that

NF-κB inhibitors antagonized DHA-induced HO-1 expression. Furthermore, DHA induced increases in IKKα/β phosphorylation, IκBα phosphorylation, IκBα degradation, and p65 Ser^{536} phosphorylation. These findings suggest that NF-κB signaling serves a multifaceted role to control the maintenance of tissue inflammation homeostasis (Pasparakis, 2009).

DHA is a major structural component of phospholipids in neuronal cell membranes, whereas EPA is present in neuronal cell membranes in a very small amount (McNamara and Carlson, 2006). Therefore, it has been proposed that DHA is more important in brain functioning than EPA (Peet and Stokes, 2005). However, EPA, rather than DHA, seems to be the effective component when treating clinical depression in published studies (Freeman *et al*, 2006; Lin and Su, 2007). The contradiction between theoretical mechanisms and clinical studies, however, is not supported by our current findings from cellular models. Specifically, our study has shown that DHA and EPA had similar effects on the regulation of inflammation and HO-1 expression. As the designs of previous clinical trials have focused on the augmentation effects of n-3 PUFAs by enrolling only depressed patients receiving antidepressant medications simultaneously (Freeman *et al*, 2006; Lin and Su, 2007), the antidepressant effect of DHA might be hidden if the effects of DHA and antidepressant medications are overlapped. According to the results of lower DHA levels in depression in our previous meta-analytic review (Lin *et al*, 2010), it is too early to exclude the antidepressant effects of DHA. Nevertheless, our findings should still be interpreted with caution. As the model of IFN-induced cellular changes is based on the neural inflammation theory of depression, the antidepressant effects of n-3 PUFAs found from this particular cellular model might not be generalized to clinical heterogeneity of depression.

In conclusion, our study elucidates the antioxidative and anti-inflammatory effects of DHA and the manner in which the regulatory molecular mechanisms of DHA-induced HO-1 expression operate through the PI-3 kinase/AKT and ERK signaling pathways by increasing IKKα/β phosphorylation, p65 Ser^{536} phosphorylation, NF-κB activation, and HO-1 protein expression in microglia. By connecting with increasing evidence pointing to inflammation dysfunction in depression and clinical studies supporting the antidepressant effects of omega-3 PUFAs, our results provide a novel implication of antidepressant mechanisms of omega-3 fatty acids.

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DISCLOSURE

The authors report no biomedical financial interests or potential conflict of interest.

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