

NLRX1 ligand, docosahexaenoic acid, ameliorates LPS-induced inflammatory hyperalgesia by decreasing TRAF6/IKK/I κ B- α /NF- κ B signaling pathway activity

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ABSTRACT

The nucleotide-binding oligomerization domain-like receptor X1 (NLRX1) has been associated with various anti-inflammatory mechanisms. We investigated whether the NLRX1 ligand docosahexaenoic acid (DHA) ameliorates lipopolysaccharide (LPS)-induced inflammatory hyperalgesia by interacting with tumor necrosis factor receptor-associated factor 6 (TRAF6)/inhibitor of κ B (I κ B) kinase (IKK)/I κ B- α /nuclear factor- κ B (NF- κ B) signaling pathway in the central nervous system. Reaction time to thermal stimuli within 30 seconds was measured in male mice injected with saline, lipopolysaccharide (LPS), and/or DHA after 6 hours using the hot plate test. Co-immunoprecipitation and immunoblotting studies were performed to determine the activation of the TRAF6/IKK/I κ B- α /NF- κ B pathway in the brains and spinal cords of animals. Latency to the thermal stimulus was reduced by 30% in LPS-injected endotoxemic mice compared with saline-injected mice. Treatment with DHA significantly improved latency compared with endotoxemic mice. In the brain and spinal cord of LPS-injected mice, treatment with DHA also prevented the increase in the expression and/or activity of (1) IKK α /IKK β , IKK γ , and K63 U in the NLRX1-immunoprecipitated tissues, (2) IKK α /IKK β , K63 U, and K48 U in the IKK γ -immunoprecipitated tissues, and (3) I κ B- α , NF- κ B p65, and interleukin-1 β associated with decreased I κ B- α expression. These findings suggest that inhibition of IKK/I κ B- α /NF- κ B signaling by dissociation of NLRX1 from TRAF6 in response to LPS treatment contributes to the protective effect of DHA against inflammatory hyperalgesia.

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Introduction

Pain is a main cause of suffering in patients where systemic inflammation-related diseases like infectious conditions, neurological disorders, autoimmune diseases, or trauma are frequently seen (1,2). Inflammation can lead to not only hyperalgesia, which is defined by increased pain sensitivity and alleviated pain threshold but also sympathetic maintained pain and allodynia (3). Hyperalgesia seen in humans can be examined with reflexive pain tests in rodents that measure pain behavior given through nociceptors using thermal (hot plate, tail flick, Hargreaves, and cold plate tests), mechanical (such as pressure or tension) (von Frey, Randall-Selitto, and strain gauge tests), electrical, or chemical (such as acetone vapor) stimuli (2-5). Besides its limitations, the test is one of the traditional pain-evoked tests and is frequently used to measure the changes in nociceptive responses when determining the effectiveness of drug candidates having analgesic and/or anti-inflammatory effects in pre-clinical studies (6). Moreover, the administration of lipopolysaccharide (LPS) is a well-proven experimental approach to investigate the effects of acute and temporary immune stimulation on physiological, emotional, and behavioral impacts of sickness behavior which includes hyperalgesia in animals and healthy humans (4). The lipid A component of LPS, endotoxin, has also been shown to enhance pain sensation

in response to thermal stimuli at supraspinal and spinal levels, as demonstrated by the hot plate test, which is considered an integration of supraspinal pathways and a supraspinal controlled acute pain test (5).

Nucleotide-binding oligomerization domain-like receptor (NLR) X1 (NLRX1), a mitochondrial nucleotide-binding oligomerization domain-like receptor (NLR) has been implicated in various inflammatory conditions (7-9). In the central nervous system (CNS) of animals and humans, NLRX1 was shown to be expressed ubiquitously in the whole brain, cortical, and hippocampal tissues in addition to a variety of cells such as neurons, astrocytes, and microglia (10-13). It has been reported that NLRX1 can activate or suppress toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/tumor necrosis factor receptor-associated factor 6 (TRAF6)/inhibitor of κ B kinase (IKK)/nuclear factor- κ B (NF- κ B) pathway depending on experimental conditions (14-17). For example, NLRX1 activates NF- κ B as a result of increased reactive oxygen species production in cells stimulated by tumor necrosis factor- α (TNF- α) (12). NLRX1 can also inhibit activation of the classical (canonical) NF- κ B pathway via TLR4 by interacting with TRAF6 (14). In unstimulated cells, NLRX1 interacts with TRAF6. Upon stimulation by LPS, however, NLRX1 dissociates from TRAF6 and undergoes K63-linked polyubiquitination (17). Subsequently, it interacts with the K48 U (facilitates NF- κ B activa-

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tion via inhibitor of κ B [I κ B]) and K63 U (controls the binding of TRAF6 to IKK γ to activate NF- κ B) bonds of lysine residues of ubiquitin in the regulatory IKK γ subunit of the IKK complex leading to its polyubiquitination. This interaction leads to the inhibition of phosphorylation of the IKK α /IKK β catalytic subunits of the IKK complex and NF- κ B activation, which is associated with proinflammatory cytokine formation and mortality. Overall, the results suggest that NLRX1 also serves to prevent excessive host responses to a variety of inflammatory stimuli *in vitro* and *in vivo*. In addition, the results of several studies suggest that ω -3 polyunsaturated fatty acids that have the ability to enter the CNS (18), such as docosahexaenoic acid (DHA), NLRX1 ligand, have anti-inflammatory effects by inhibiting NF- κ B activity in various neuroinflammation models with NLRX1-dependent mechanism (19-21). There is only one study in the literature showing that intrathecal injection of DHA prevents chronic inflammatory pain in mice as tested by punctate mechanical allodynia perceived at the supraspinal level after intrathecal LPS or intraplantar complete Freund's adjuvant (CFA) injection (22). However, no studies are focusing on either the role of NLRX1 or the effect of NLRX1 ligands in perceived LPS-induced inflammatory hyperalgesia at the spinal and/or supraspinal level.

We have previously shown that LPS-induced inflammatory hyperalgesia is associated with decreased expression and/or activity of inducible nitric oxide (NO) synthase, neuronal NO synthase, soluble epoxide hydrolase, NLRC3, and peroxisome proliferator-activated receptors $\alpha/\beta/\gamma$ in addition to the anti-apoptotic phosphoinositide 3-kinase p85 α /Akt1/cyclic adenosine monophosphate-response element-binding protein 1/B-cell lymphoma-2 signalling pathway activity in the various tissues of mice including brain and spinal cord (23-28). Increased activity of nucleotide-binding domain and leucine-rich repeat protein (NLRP3)/apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC)/pro-caspase-1, NLRC4/ASC/pro-caspase-1, and caspase-11 inflammasomes, TLR4/MyD88/transforming growth factor-activated kinase/NF- κ B/cyclooxygenase (COX)-2 pathway, and nicotinamide adenine dinucleotide phosphate oxidase, which are associated with the production of pro-inflammatory cytokines, were also observed in the brain and spinal cord of LPS-treated mice (23-28). Given the beneficial role of NLRX1 in inflammation, we tested the hypothesis that anti-inflammatory NLRX1 ligands such as DHA prevent the LPS-induced inflammatory hyperalgesia by inhibiting the TRAF6/IKK/I κ B- α /NF- κ B signaling pathway in the CNS.

Materials and Methods

Animals

Balb/c mice (male; 20 to 40 g; n=58) (Research Center of Experimental Animals, Mersin University, Mersin, Turkey) were used in the experiments. The mice were housed under a 12-hour light/dark cycle and fed on standard chow. The procedures on animals were approved by the Mersin University Experimental Animals Local Ethics Committee (Approval date: May 13, 2019; Protocol number: 2019/16) and performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Inflammatory hyperalgesia model

The inflammatory hyperalgesia model was induced by intraperitoneal (i.p.) injection of LPS, and the latency of pain to nociceptive response was measured by the hot plate test as previously reported (25-29). To observe the pain behavior, mice were randomly divided into 7 groups, and the dose-response relationship was investigated with different DHA doses (30): (1) saline (10 ml/kg) (control group), (2) LPS (10 mg/kg; 10 ml/kg) (inflammatory hyperalgesia group), (3) LPS+DHA (1 mg/kg; 10 ml/kg), (4) LPS+DHA (2 mg/kg; 10 ml/kg), (5) saline+DHA (3 mg/kg; 10 ml/kg), (6) LPS+DHA (3 mg/kg; 10 ml/kg), and (7) LPS+DHA (10 mg/kg; 10 ml/kg). DHA (dissolved in saline; D8768; Sigma Chemical Co., St. Louis, MO, USA) was injected into the mice simultaneously with saline or LPS (dissolved in saline; L4130; *Escherichia coli* LPS, O111:B4; Sigma). Mice were placed individually on a plate pre-heated to $55 \pm 0.2^\circ\text{C}$ (AHP 9601, Commat Ltd., Ankara, Turkey). The latency to paw withdrawal within 30 seconds was recorded using the hot plate test after the mice showed the first sign of paw licking 6 hours after injection of saline, LPS, and/or DHA. The time point of 6 hours was preferred for the assessment of hyperalgesia according to our previous time-course studies (24-28). Mice were killed after the test by cervical dislocation, and the brains and spinal cords of the animals were harvested.

Co-Immunoprecipitation (IP) and immunoblotting (IB) studies

Co-immunoprecipitation studies were performed to determine the changes in the association of NLRX1 or IKK with TRAF6, IKK α , IKK β , phosphorylated IKK α /IKK β (p-IKK α /IKK β), IKK γ , phosphorylated IKK γ (p-IKK γ), K63 U, or K48 U proteins according to previously described methods (7,17,31,32). In addition, the expression of I κ B- α , phosphorylated I κ B- α (p-I κ B- α), NF- κ B p65, phosphorylated NF- κ B p65 (p-NF- κ B p65), and interleukin-1 β (IL-1 β) proteins using the IB method. Protein A/G PLUS-Agarose Immunoprecipitation Reagent (sc-2003; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to prepare immunoprecipitated samples as described by the manufacturer. Briefly, 500 μ g of protein for each sample was precleared with 20 μ l of protein A/G-agarose beads for 1 hour at 4°C , the beads were pelleted (1.000 x g for 5 minutes at 4°C), and the supernatants were incubated for 1 hour at 4°C with 2 μ g of antibodies specific for NLRX1 (sc-374514; Santa Cruz) or IKK (sc-71331; Santa Cruz). 20 μ l of protein A/G-agarose beads were added and incubated for 12 hours at 4°C in a rotating apparatus, centrifuged at 1.000 x g for 5 minutes at 4°C , washed four times with 1 ml of HEPES buffer, resuspended in 60 μ l of HEPES and 40 μ l of Laemmli sample buffer. The immunoprecipitated samples were kept at -80°C for measurement of protein expression of β -tubulin, TRAF6, IKK α , IKK β , p-IKK α /IKK β , IKK γ , p-IKK γ , K63 U, or K48 U. The total amount of protein in the immunoprecipitated samples was determined by the Coomassie blue method (33). Samples (10 μ g protein) were subjected to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). Nonfat dry milk in Tris-buffered saline (5%) was used to block the nitrocellulose membranes containing the transferred proteins. The membranes were then incubated with the primary antibodies in bovine serum albumin (BSA) (1:500 in 5% BSA) overnight at 4°C : (1)

NLRX1 antibody (sc-374514; Santa Cruz); (2) TRAF6 (sc-8409; Santa Cruz); (3) IKK α antibody (2682; Cell Signaling, Danvers, MA, USA); (4) IKK β antibody (8943; Cell Signaling); (5) p-IKK α /IKK β antibody (2078; Cell Signaling); (6) IKK γ antibody (sc-71331; Santa Cruz); (7) p-IKK γ antibody (sc-293135; Santa Cruz); (8) K63 U antibody (recognizes K63-linked polyubiquitin chains) (BML-PW0600; Enzo Life Sciences, Lausen, Switzerland); (9) K48 U antibody (recognizes polyubiquitin chains formed by lysine-48 [K48] residue linkage) (ab140601; Abcam, Waltham, MA, USA); (10) I κ B- α antibody (sc-1643; Santa Cruz); (11) p-I κ B- α antibody (sc-7977; Santa Cruz); (12) NF- κ B p65 antibody (sc-8008; Santa Cruz); (13) p-NF- κ B p65 antibody (sc-33020; Santa Cruz), and (14) IL-1 β antibody (sc-52012; Santa Cruz). Secondary antibodies were sheep anti-mouse IgG-horseradish peroxidase (RPN4201; Amersham Life Sciences, Cleveland, OH, USA) (for NLRX1, TRAF6, IKK γ , p-IKK γ , I κ B- α , K63 U, NF- κ B p65, p-NF- κ B p65, and IL-1 β , and goat anti-rabbit IgG-horseradish peroxidase (RPN4301; Amersham Life Sciences) (for IKK α , IKK β , p-IKK α /IKK β , K48 U, and p-I κ B- α) in 0.1% BSA (1:1.000). Membranes were reprobbed and used for anti- β -tubulin antibody (**D-10**) (sc-5274; Santa Cruz) (1:500 in 5% BSA) followed by incubation with sheep antimouse IgG-horseradish peroxidase (1:1000 in 0.1% BSA). Blots were developed using Enhanced Chemiluminescence (ECL Prime Western Blotting Detection Reagent) (RPN2232; Amersham) was used to develop the blots. Immunoreactive band images were acquired using a gel imaging system (EC3-CHEMI HR Imaging System; Ultra-Violet Products, UVP, Cambridge, UK). Image J densitometry analysis software (Image J 1.46r, Wayne Rasband, National Institute of Health, Bethesda, MD, USA) was used to quantify the relative densities of the immunoreactive bands. The ratio of each band/ β -tubulin was considered for the expression of NLRX1, TRAF6, IKK α , IKK β , p-IKK α /IKK β , IKK γ , p-IKK γ , K63 U, K48 U, I κ B- α , p-I κ B- α , NF- κ B p65, p-NF-B p65, and IL-1 β proteins.

Statistical analysis

Data are expressed as means \pm standard error of the mean (SEM). Parametric or nonparametric statistical analysis was performed with Student's *t*-test or Mann-Whitney *U*-test for normally or nonnormally distributed data, respectively. Statistical analysis was performed using GraphPad Prism version 7.04 for Windows (GraphPad Software, San Diego California USA; <http://www.graphpad.com>). A

P value < 0.05 was considered statistically significant.

Results

DHA treatment prevents hyperalgesia induced by LPS

To determine the contribution of NLRX1 to the LPS-induced hyperalgesia, the NLRX1 ligand, DHA, was administered to mice alone or in combination with saline or LPS. Consistent with our previous results (24-28), hot plate latency was decreased 6 hours after LPS injection compared with control group values (Figure 1) (*P*<0.05). DHA at doses of 3 and 10 mg/kg prevented the reduction in latency compared with LPS-injected mice (*P*<0.05). DHA at doses of 1 and 2 mg/kg was not effective in preventing the reduction in latency compared with LPS-injected

mice (*P*>0.05). At a dose of 3 mg/kg, treatment with DHA had no effect on hot plate latency in mice injected with saline (*P*>0.05). Also, no mortality was observed during the experiments. Therefore, tissues from mice injected with DHA at the minimum effective dose (3 mg/kg) in the LPS-induced hyperalgesia were used for further experiments.

DHA treatment does not prevent the LPS-induced decrease in the dissociation of NLRX1 from TRAF6

The results of previous studies show that NLRX1 dissociates from TRAF6 to inhibit NF- κ B signaling pathway activity after LPS stimulation (14,17,34). To test whether DHA has an effect on the dissociation of NLRX1 from TRAF6 in the CNS, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were immunoprecipitated with the NLRX1 antibody and then immunoblotted with the antibody for TRAF6. Decreased expression of TRAF6 was associated with increased NLRX1 expression in the brain (Figure 2a) and spinal cord (Figure 2b) of LPS-treated mice compared with levels in the control group (*P*<0.05). The expression of NLRX1 and TRAF6 in the tissues of DHA-treated mice was not different from the levels in the control (*P*>0.05).

DHA treatment prevents the LPS-induced increase in the IKK complex activity, but not expression, through inhibiting the association of NLRX1 with the active regulatory and catalytic complex subunits

Since NLRX1 inhibits NF- κ B signaling pathway activity through its dissociation from TRAF6 and interaction with the IKK complex in response to LPS stimulation *in vitro* (17), we aimed to test whether DHA affects the association of NLRX1 with the catalytic (IKK α and IKK β) and regulatory (IKK γ) subunits of the IKK complex in addition to IKK complex activity in the CNS. For this purpose, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were immunoprecipitated with the NLRX1 antibody and subsequently immunoblotted with the antibodies for IKK α , IKK β , p-IKK α /IKK β (on Ser¹⁷⁶ and Ser¹⁷⁷, respectively), IKK γ , and p-IKK γ (on Ser³⁷⁶).

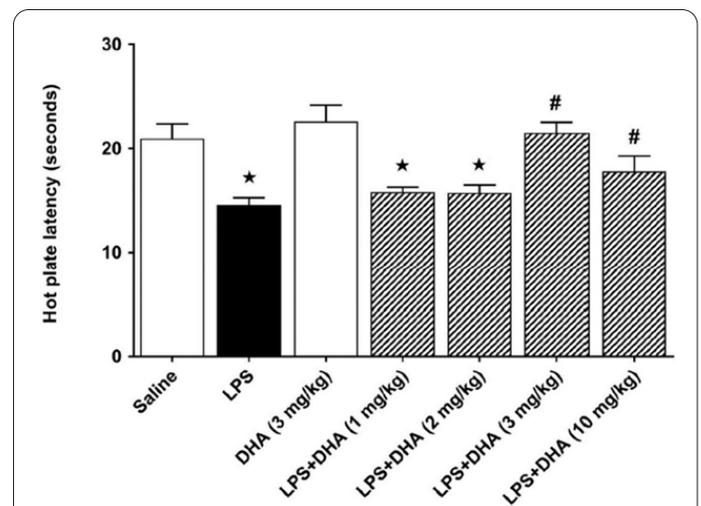


Figure 1. Effect of DHA on LPS-induced hyperalgesia. Response time to thermal stimuli within 30 seconds was determined 6 hours after injection of saline (10 ml/kg; i.p.), LPS (10 mg/kg; i.p.), or DHA (1, 2, 3, or 10 mg/kg; i.p.) in mice using the hot plate test. Data are expressed as means \pm SEM from 6-10 animals. * *P*<0.05 vs. saline-injected group; # *P*<0.05 vs. LPS-injected group.

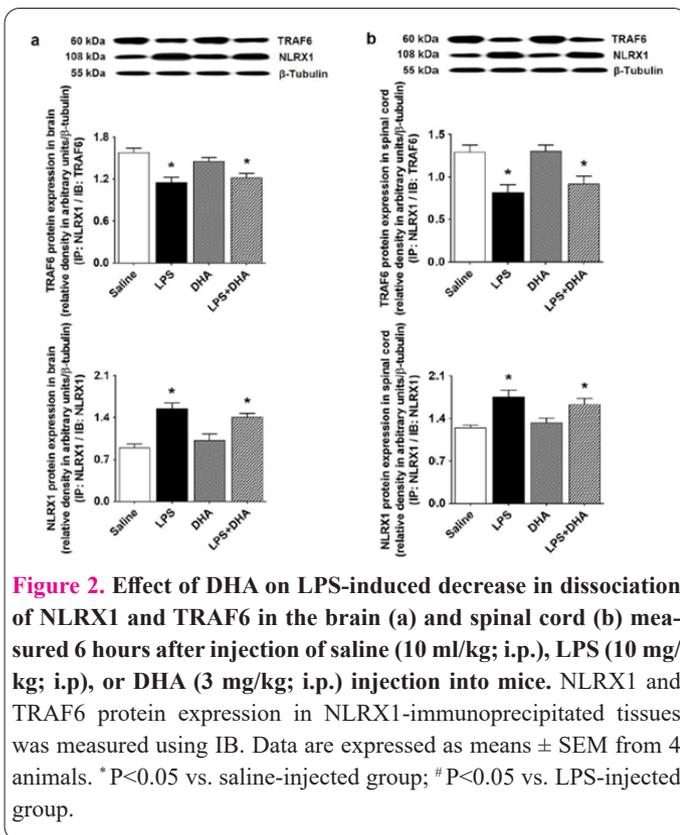


Figure 2. Effect of DHA on LPS-induced decrease in dissociation of NLRX1 and TRAF6 in the brain (a) and spinal cord (b) measured 6 hours after injection of saline (10 ml/kg; i.p.), LPS (10 mg/kg; i.p.), or DHA (3 mg/kg; i.p.) injection into mice. NLRX1 and TRAF6 protein expression in NLRX1-immunoprecipitated tissues was measured using IB. Data are expressed as means \pm SEM from 4 animals. * P <0.05 vs. saline-injected group; # P <0.05 vs. LPS-injected group.

Increased expression of IKK α , IKK β , and IKK γ , as well as IKK α /IKK β and IKK γ phosphorylation, was detected in the brains (Figure 3a) and spinal cords (Figure 3b) of LPS-treated mice compared with levels in the control group (P <0.05). Treatment with DHA inhibited the LPS-induced increase in IKK α /IKK β and IKK γ phosphorylation, but not the expression of IKK, IKK β , and IKK γ , in the tissues compared with LPS-injected mice (P <0.05). The expression of IKK α , IKK β , and IKK γ , and IKK α /IKK β and p-IKK γ phosphorylation, in the tissues of DHA-treated mice was not different from the levels in the control group (P >0.05).

DHA treatment prevents the LPS-induced decrease in the polyubiquitination of NLRX1 through the K63 U, but not K48 U, linkage

It was found that NLRX1 undergoes rapid polyubiquitination via the K63 U, but not K48 U, linkage 10-15 minutes after treatment of mouse embryonic fibroblasts with LPS, but is then reduced (17). To test whether DHA affects the polyubiquitination of NLRX1 via the K63 U and K48 U linkages in the CNS, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were immunoprecipitated with the NLRX1 antibody and then immunoblotted with the antibody for K63 U or K48 U. The expression K63 U was decreased in the brains (Figure 4a) and spinal cords (Figure 4b) of LPS-treated mice compared with levels in the control group (P <0.05). Treatment with DHA inhibited the LPS-induced decrease in K63 U expression in tissues compared with LPS-injected mice (P <0.05). On the other hand, K48 U expression was not different in the tissues of mice injected with saline, LPS, and/or DHA. The expression of K63 U and K48 U in the tissues of DHA-treated mice was not different from the levels in the control group (P >0.05).

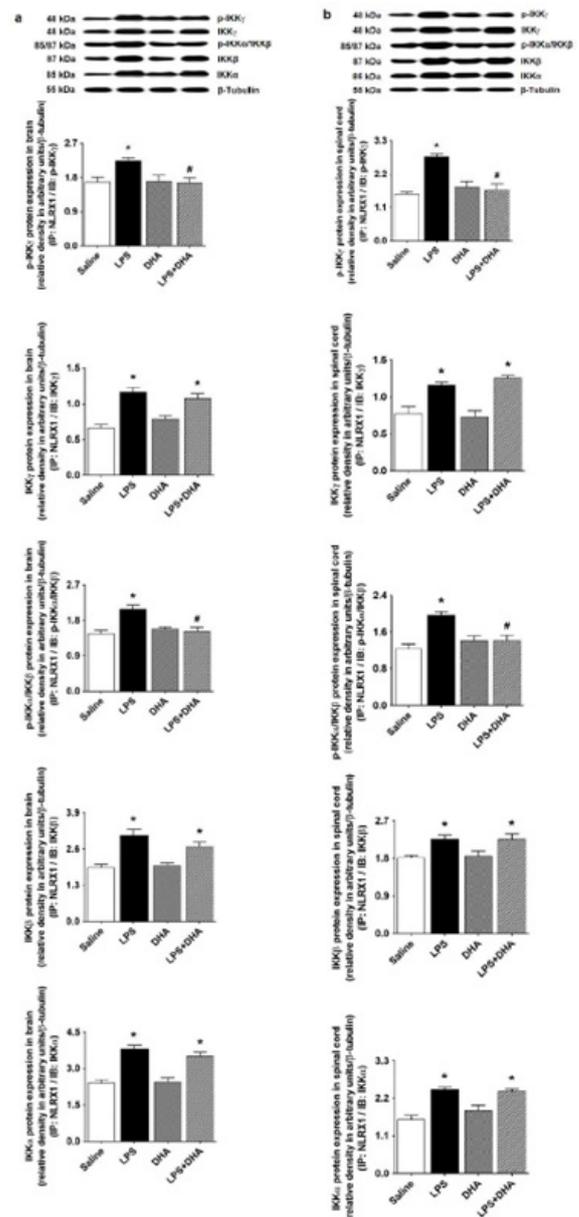
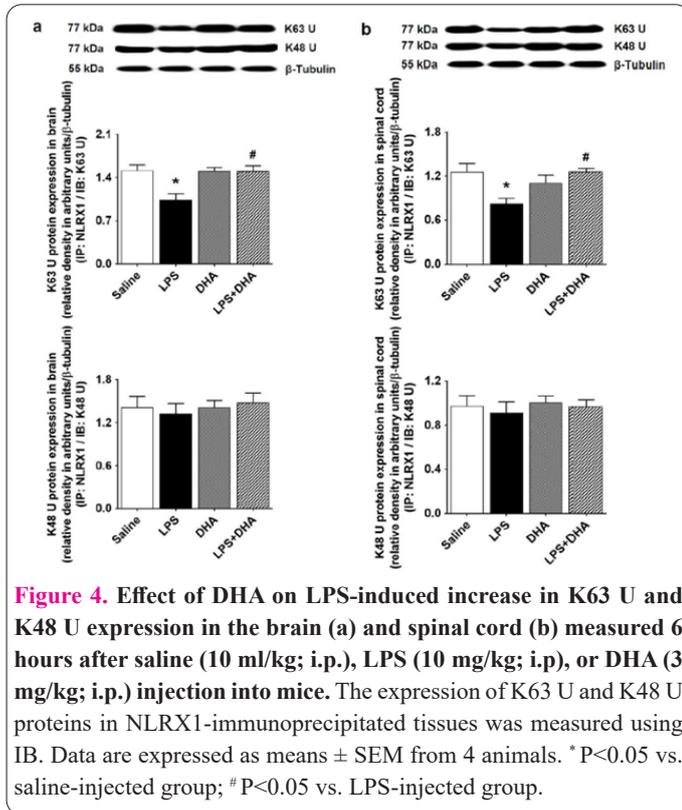


Figure 3. Effect of DHA on LPS-induced increase in IKK complex expression and activity in the brain (a) and spinal cord (b) measured 6 hours after saline (10 ml/kg; i.p.), LPS (10 mg/kg; i.p.), or DHA (3 mg/kg; i.p.) injection into mice. The expression of unphosphorylated and phosphorylated IKK α , IKK β , and IKK γ proteins in the NLRX1-immunoprecipitated tissues was measured using IB. Data are expressed as means \pm SEM from 4 animals. * P <0.05 vs. saline-injected group; # P <0.05 vs. LPS-injected group.

DHA treatment prevents the LPS-induced increase in the association of IKK γ with the active catalytic subunits of the IKK complex

It has also been shown that polyubiquitination of NLRX1 via K63 U linkage in response to LPS treatment of RAW264.7 cells leads to inhibition of IKK α /IKK β phosphorylation and recruitment of IKK γ and its IKK complex to form a stable complex (17). Therefore, we aimed to test whether DHA affects the association of IKK γ with the catalytic subunits of IKK complex, IKK α and IKK β , in addition to IKK/IKK β activity in the CNS. For this purpose, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were immunoprecipitated with the IKK γ antibody and then immunoblotted with the antibodies for IKK α , IKK β , or p-IKK α /IKK β (at Ser¹⁷⁶ and



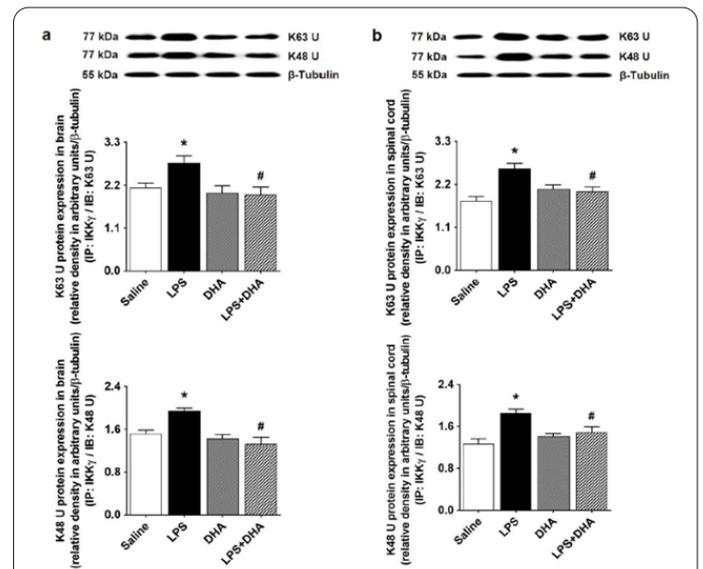
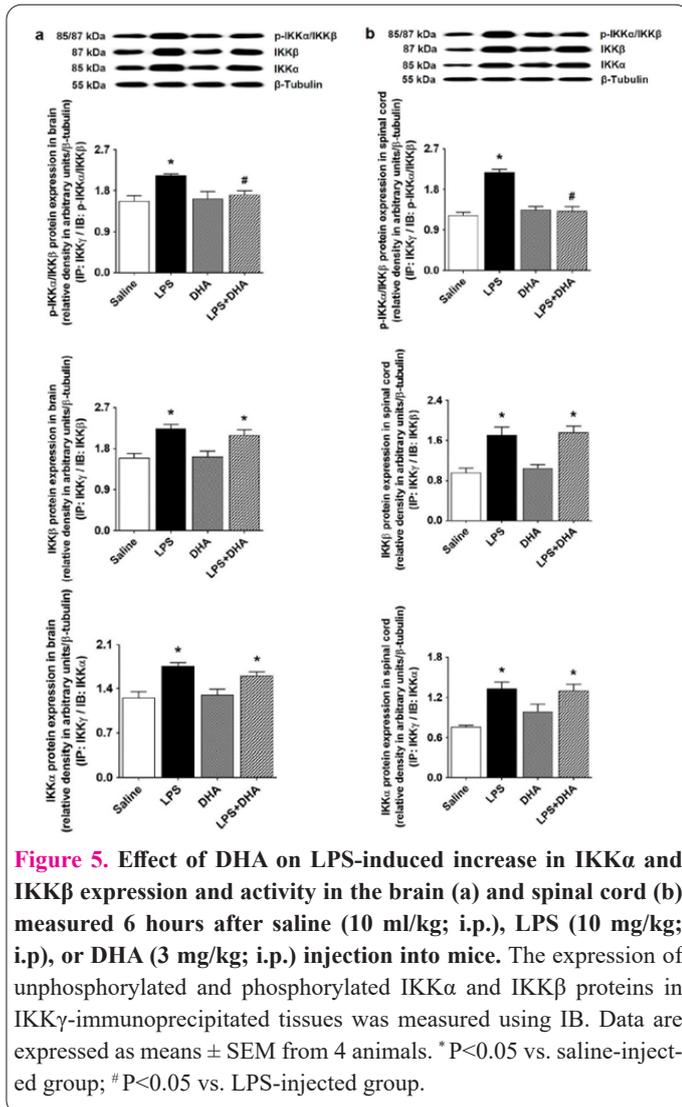
in the brains (Figure 5a) and spinal cords (Figure 5b) of LPS-treated mice compared with levels in the control group (P<0.05). Treatment with DHA inhibited the LPS-induced increase in IKK α /IKK β phosphorylation, but not the expression of IKK α and IKK β , in tissues compared with LPS-injected mice (P<0.05). The expression of IKK α and IKK β , as well as IKK α /IKK β phosphorylation, in the tissues of DHA-treated mice was not different from the levels in the control group (P>0.05).

DHA treatment prevents the LPS-induced increase in the polyubiquitination of IKK γ through K63 U and K48 U linkages

Polyubiquitination of IKK γ via K63 U, but not K48 U, linkage in response to LPS treatment in mouse peritoneal macrophages has also been reported (35). To test whether DHA affects the polyubiquitination of IKK γ via K63 U and K48 U linkages in the CNS, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were immunoprecipitated with the IKK γ antibody and then immunoblotted with the specific antibodies for K63 U or K48 U. Increased expression of K63 U and K48 U was observed in the brains (Figure 6a) and spinal cords (Figure 6b) of LPS-treated mice compared with levels in the control group (P<0.05). Treatment with DHA inhibited the LPS-induced increase in K63 U and K48 U expression in tissues compared with LPS-injected mice (P<0.05). Expression of K63 U and K48 U in the tissues of DHA-treated mice was not different from the levels in the control group (P>0.05).

DHA treatment prevents the LPS-induced decrease in the I κ B- α expression and increase in the I κ B- α activity

Under physiological conditions, NLRX1, particularly its leucine rich repeat (LRR) domain, is thought to interact with the imputed kinase domain of p-IKK. Certain phosphatases, in addition to their kinase activity for phosphorylation of I κ B- α , cause NLRX1-associated IKK complexes to lose phosphorylation (17). To test the effect of DHA



Ser¹⁷⁷, respectively). The expression of IKK α and IKK β , as well as IKK α /IKK β phosphorylation, was increased

on the LPS-induced changes in expression and activity of I κ B- α in the CNS, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were immunoblotted with the antibodies for I κ B- α and p-I κ B- α (at Ser³²). Decreased expression of I κ B- α was associated with increased I κ B- α phosphorylation in the brains (Figure 7a) and spinal cords (Figure 7b) of mice treated with LPS compared with levels in the control group ($P < 0.05$). Treatment with DHA inhibited the LPS-induced changes in the expression of I κ B- α and I κ B- α phosphorylation in the tissues compared with LPS-injected mice ($P < 0.05$). The expression of I κ B- α and I κ B- α phosphorylation in the tissues of DHA-treated mice was not different from levels in the control group ($P > 0.05$).

DHA treatment prevents the LPS-induced increase in the NF- κ B p65 expression and activity

It has been shown that NLRX1 negatively regulates TLR4-induced NF- κ B signaling in various cell types (17). To test the effect of DHA on the changes induced by LPS in the expression and activity of NF- κ B p65 in the CNS, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were immunoblotted with the antibodies for NF- κ B p65 and p-NF- κ B p65 (at Ser⁵³⁶). Consistent with our previous results (26,27), increased expression of NF- κ B p65 and NF- κ B p65 phosphorylation was observed in the brains (Figure 8a) and spinal cords (Figure 8b) of LPS-treated mice compared with levels in the control group ($P < 0.05$). Treatment with DHA inhibited the LPS-induced increase in the expression of NF- κ B p65 and NF- κ B p65 phosphorylation in the tissues compared with LPS-injected mice ($P < 0.05$). The expression of NF- κ B p65 and NF- κ B p65 phosphorylation in the tissues of DHA-treated mice was not different from the levels in the control group ($P > 0.05$).

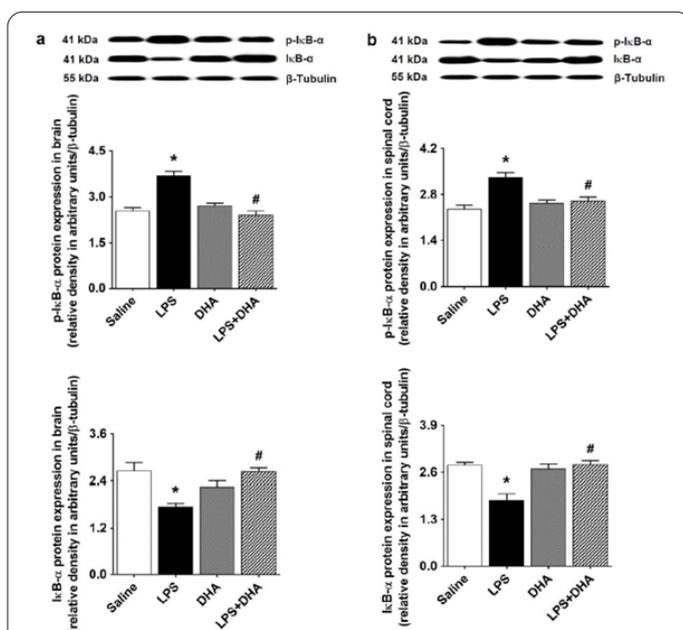


Figure 7. Effect of DHA on LPS-induced increase in the I κ B- α expression and activity in the brain (a) and spinal cord (b) measured 6 hours after saline (10 ml/kg; i.p.), LPS (10 mg/kg; i.p.), or DHA (3 mg/kg; i.p.) injection into mice. The expression of unphosphorylated and phosphorylated I κ B- α proteins in tissues was measured using IB. Data are expressed as means \pm SEM from 4 animals. * $P < 0.05$ vs. saline-injected group; # $P < 0.05$ vs. LPS-injected group.

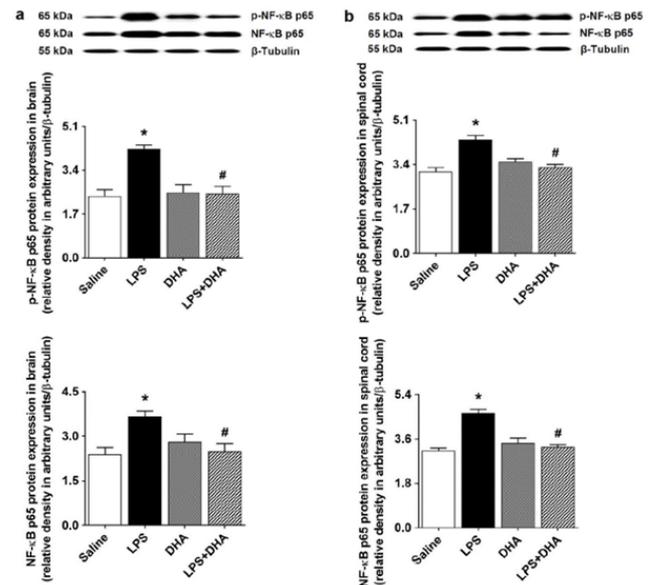


Figure 8. Effect of DHA on LPS-induced increase in NF- κ B p65 expression and activity in the brain (a) and spinal cord (b) measured 6 hours after saline (10 ml/kg; i.p.), LPS (10 mg/kg; i.p.), or DHA (3 mg/kg; i.p.) injection into mice. The expression of unphosphorylated and phosphorylated NF- κ B p65 proteins in tissues was measured using IB. Data are expressed as means \pm SEM from 4 animals. * $P < 0.05$ vs. saline-injected group; # $P < 0.05$ vs. LPS-injected group.

DHA treatment prevents the LPS-induced increase in IL-1 β expression

The results of previous studies in the LPS-induced septic shock model in NLRX1 KO and WT mice also demonstrated that NLRX1 inhibits the NF- κ B signaling pathway-dependent formation of pro-inflammatory cytokines (e.g., IL-6) and prevents mortality (17). To test the effect of DHA on the changes induced by LPS in the expression of one of the major pro-inflammatory cytokines, IL-1 β , in the CNS, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were immunoblotted with the IL-1 β antibody. Consistent with our previous findings (24-27), increased expression of IL-1 β was observed in the brains (Figure 9a) and spinal cords (Figure 9b) of LPS-treated mice compared with levels in the control group ($P < 0.05$). Treatment with DHA inhibited the LPS-induced increase in IL-1 β expression in tissues compared with LPS-injected mice ($P < 0.05$). The expression of IL-1 β in the tissues of DHA-treated mice was not different from the levels in the control group ($P > 0.05$).

Discussion

The results of the present study provide the first evidence that a decrease in the activity of IKK α / β / γ , I κ B- α , and NF- κ B in addition to IL-1 β expression as a result of the dissociation of NLRX1 from TRAF6, increased polyubiquitination of NLRX1 via K63 U linkage, and binding to the IKK complex in the CNS contributes to the preventive effect of the NLRX1 ligand, DHA, against inflammatory hyperalgesia in response to LPS (Figure 10).

TRAF6 has been shown to be closely associated with NLRX1 in unstimulated cells while possessing ubiquitin ligase activity and rapidly leading to its own and NLRX1 ubiquitination after LPS stimulation (17,34). According to

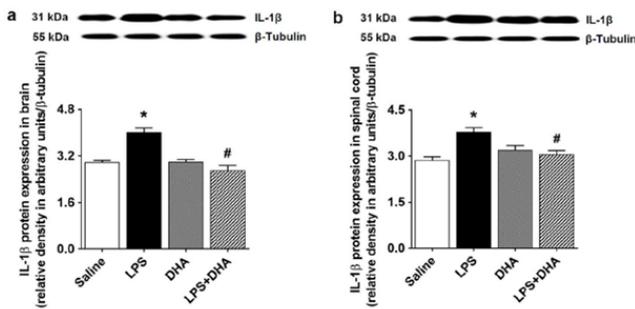


Figure 9. Effect of DHA on LPS-induced increase in IL-1 β expression in the brain (a) and spinal cord (b) measured 6 hours after saline (10 ml/kg; i.p.), LPS (10 mg/kg; i.p.), or DHA (3 mg/kg; i.p.) injection into mice. IL-1 β protein expression in tissues was measured using IB. Data are expressed as means \pm SEM from 4 animals. *P<0.05 vs. saline-injected group; #P<0.05 vs. LPS-injected group.

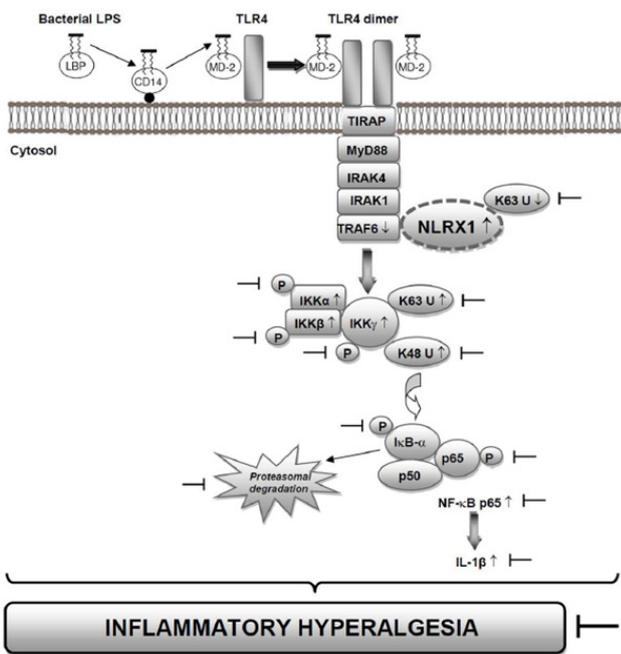


Figure 10. Diagram showing the effect of NLRX1 ligand, DHA, on LPS-induced changes in TRAF6/IKK/NF- κ B signaling pathway and pro-inflammatory mediator formation during inflammatory hyperalgesia in mice based on the results of the present study in addition to our previous findings (23-28). Presumably, a decrease in the activity of IKK α /IKK β , IKK γ , I κ B- α , and NF- κ B in addition to IL-1 β protein expression as a result of binding to the IKK complex after increased polyubiquitination via K63 U linkage following dissociation of NLRX1 from TRAF6 in response to LPS in the CNS contributes to the preventive effect of DHA against inflammatory hyperalgesia. (\uparrow) Increased by LPS; (\downarrow) decreased by LPS; (—) prevented by DHA.

NF- κ B signaling in an *in vitro* model of osteoarthritis (34). In a study conducted in NLRX1-knockout mice (32), data on exacerbation of LPS-induced cardiac injury were obtained as a result of increased reactive oxygen species formation with the IKK α /IKK β /I κ B- α /NF- κ B signaling pathway and NLRP3 inflammasome activity. Moreover, the results of a recent study suggest that after administration of the conjugate of the LRR domain of NLRX1 with the peptide "C10", which efficiently transports cargo molecules to macrophages, the increase in blood levels of IL-6 and IL-1 β associated with mortality was prevented in the LPS-induced sepsis model in mice (36). In addition, Zhao et al. (35) demonstrated that LPS caused phosphorylation of IKK α /IKK β , I κ B- α , and NF- κ B p65 proteins and K63-linked polyubiquitination of IKK γ , without affecting the total amount of IKK protein, which was associated with increased formation of inflammatory cytokines (e.g., IL-1 β , IL-6, and TNF- α). In the current study, we demonstrated that intraperitoneal injection of LPS into mice resulted in hyperalgesia associated with decreased TRAF6 protein expression, increased protein expression of NLRX1 and K63 U, but not K48 U, and phosphorylation of IKK α , IKK β , and IKK γ in NLRX1-immunoprecipitated mouse brain and spinal cords. Increased phosphorylation of IKK α and IKK β was observed in association with K63 U and K48 U protein expression in the IKK γ -immunoprecipitated tissues of LPS-injected mice. Decreased protein expression of I κ B- α was also associated with increased expression of NF- κ B p65 and phosphorylation of I κ B- α and NF- κ B p65 in the tissues. Therefore, in agreement with the studies mentioned above (14,17,32,34-36) and our previous findings (23-28), increased formation of pro-inflammatory mediators as a result of increased activity of the TRAF6/IKK/I κ B- α /NF- κ B signaling pathway in conjunction with up-regulation of NLRX1 in the CNS of mice appears to be involved in the LPS-induced inflammatory hyperalgesia.

It has also been shown that intraperitoneal injection of the NLRX1 ligand DHA reduces brain volume loss and improves neurological functions in a perinatal hypoxia-ischemia rat model potentiated by systemic inflammation induced by LPS (30). As shown by Lu et al. (37), intrathecal injection of DHA in mice exerts an anti-nociceptive effect on inflammatory pain induced by intraplantar injection of carrageenan by inhibiting the activity of p38 mitogen-activated protein kinase in the spinal cord. Moreover, both the NLRX1 ligand DHA itself and specialized pro-resolving mediators synthesized from DHA, such as maresins, resolvins, and protectins and have been shown to exert analgesic and anti-inflammatory effects in various acute and chronic inflammatory pain models (19,38). On the other hand, there is only one study showing that intrathecal injection of DHA reduces chronic inflammatory pain as determined by the mechanical allodynia test induced by intrathecal injection of LPS or intraplantar injection of CFA into mice by inhibiting the up-regulation of TRAF6 in the spinal cord and allograft inflammatory factor 1 (a microglial marker) (22). In the present study, treatment with DHA showed a significant improvement in latency compared with endotoxemic mice. DHA also prevented the LPS-induced increase in K63 U protein expression, as well as phosphorylation of IKK α , IKK β , and IKK γ proteins, in NLRX1-immunoprecipitated tissues. On the other hand, treatment with DHA failed to prevent the LPS-induced changes in NLRX1 and TRAF6 prote-

the results of the studies conducted to further identify the involvement of NLRX1 in TLR4-mediated NF- κ B signaling pathway activity in response to LPS *in vitro* and *in vivo* (14,17), NLRX1 negatively regulates NF- κ B-induced TLR4 activity by dynamically interacting with TRAF6 and the IKK complex and promoting the formation pro-inflammatory cytokines. In addition, suppression of NLRX1 expression has been reported to increase susceptibility to septic shock induced by LPS in association with elevated plasma levels of IL-6 in mice (17). NLRX1 has also been shown to attenuate LPS-induced apoptosis and inflammation in chondrocytes via negative regulation of

in expression. In the IKK γ -immunoprecipitated tissues of LPS-injected mice treated with DHA, IKK α and IKK β phosphorylation, as well as K63 U and K48 U protein expression, were decreased. In addition, the LPS-induced decrease in protein expression of I κ B- α associated with the increased expression of p-I κ B- α , NF- κ B p65, p-NF- κ B p65 and IL-1 β proteins in the tissues was also prevented by treating the mice with DHA. Based on the results of studies in the literature (14,17,19,21,22,30,32,35,36,37) and our previous findings on the model of inflammatory hyperalgesia induced by LPS injection (23-28), it appears that reduced formation of pro-inflammatory mediators as a result of inhibition of the TRAF6/IKK/I κ B- α /NF- κ B signaling pathway at the transcriptional and/or post-transcriptional level in the CNS of mice is involved in the analgesic and anti-inflammatory effects of DHA. It is also possible that increased polyubiquitination via the K63 U, but not the K48 U, linkage after dissociation of NLRX1 from TRAF6 and/or decreased K63 U- and K48 U-linked polyubiquitination of IKK γ leading to suppression of IKK/I κ B- α /NF- κ B signaling pathway activity contribute to the protective effects of DHA against inflammatory hyperalgesia in response to LPS.

A limitation of this study is that we did not explore the molecular mechanisms of the analgesic and anti-inflammatory effects of DHA in LPS-induced hyperalgesia. For example, we did not investigate whether DHA exerts its beneficial effects (1) either directly, by acting as an endogenous NLRX1, or indirectly, by increasing endogenous NLRX1 expression and/or decreasing the expression/activity of factors/enzymes involved in the TRAF6/IKK/I κ B- α /NF- κ B signaling pathway in the specific cell types of the CNS expressing NLRX1 such as neuronal and non-neuronal cells, including astrocytes and microglia, and/or (2) due to its synergistic effects on signal transduction pathways involved in the pathogenesis of LPS-induced inflammatory hyperalgesia. Therefore, further investigation will contribute to the preclinical and clinical studies currently underway to develop NLRX1 ligands, such as DHA, as drugs for the treatment of hyperalgesia-related inflammatory diseases.

In conclusion, we demonstrated for the first time that the NLRX1 ligand DHA can prevent inflammatory hyperalgesia and increase the activity of the LPS-induced TRAF6/IKK/I κ B- α /NF- κ B signaling pathway in the CNS of mice. Our results indicate that NLRX1 ligands such as DHA, which can also enter the CNS when administered systemically, may be useful as analgesic/anti-inflammatory drugs in the prevention and treatment of chronic pain conditions in which inflammation plays a role in the pathophysiology, as well as acute inflammatory diseases associated with pain that may result from bacterial infections.

Interest conflict

The authors declare no conflict of interest/competing interests.

Author's contribution

BT conceptualized and conceived the research design, analyzed the data, and drafted the manuscript. BT, DEY, SPS, and MTR carried out the experiments. SSF contributed to the finalizing of the manuscript. All authors read and approved the final manuscript.

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