NLRX1 Ligand, Docosahexaenoic Acid, Ameliorates LPS-Induced Inflammatory Hyperalgesia by Decreasing TRAF6/IKK/IkB-a/NF-kB Signaling Pathway Activity

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Research Article

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Abstract

Objective 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 kB (IkB) kinase (IKK)/IkB-a/nuclear factor-κB (NF-κB) signaling pathway in the central nervous system.

Methods 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 activation of the TRAF6/IKK/IkB-a/NF-kB pathway in the brains and spinal cords of LPS-treated animals.

Results 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/IKKβ, IKK, and K63 U in the NLRX1-immunoprecipitated tissues, (2) IKK/IKKβ, K63 U, and K48 U in the IKK-immunoprecipitated tissues, and (3) IkB-α, NF-kB p65, and interleukin-1β associated with decreased IkB-α expression.

Conclusion Inhibition of IKK/IkB-a/NF-kB signaling by dissociation of NLRX1 from TRAF6 in response to LPS treatment may contribute to the protective effect of DHA against inflammatory hyperalgesia.

Introduction

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 [1-3]. 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 kB kinase (IKK)/nuclear factor-κB (NF-κB) pathway depending on experimental conditions [4-7].For example, NLRX1 activates NF-κB as a result of increased reactive oxygen species production in cells stimulated by tumor necrosis factor-a (TNF-a) [8].NLRX1 can also inhibit activation of the classical (canonical) NF-κB pathway via TLR4 by interacting with TRAF6 [4]. In unstimulated cells, NLRX1 interacts withTRAF6. Upon stimulation bylipopolysaccharide (LPS), however,NLRX1dissociates from TRAF6 andundergoes K63-linked polyubiquitination [7]. Subsequently, it interacts with theK48 U (facilitates NF-κB activation via inhibitor of kB [IκB]) and K63 U (controls the binding of TRAF6 to IKKgto activate NF-κB) bonds of lysine residues of ubiquitin in the regulatory IKKg subunit of the IKK complex leading to its polyubiquitination. This interaction leads to inhibition of phosphorylation of the IKKa/IKKβ catalytic subunits of the IKK complex and NF-κB activation, which isassociated withproinflammatory 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

studiessuggest that w-3 polyunsaturated fatty acids thathave the ability toenter the central nervous system (CNS) [9], such as docosahexaenoic acid (DHA), NLRX1 ligand, have anti-inflammatory effectsby inhibiting NF-κB activity in various neuroinflammation models with NLRX1-dependent mechanism[10-12].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 [13].However, there are no studies focusing on either the role of NLRX1 or the effect of NLRX1 ligands in perceived LPS-nduced inflammatory hyperalgesia at the spinal and/or supraspinal level.

The lipid A component of LPS, endotoxin, has been shown to enhance pain sensationin response to thermal stimuli at supraspinal and spinal levels,as demonstrated by the hot plate test, which is considered anintegration of supraspinal pathways and a supraspinal controlled acute pain test [14]. We have previously shown that LPS-induced inflammatory hyperalgesia is associated withdecreased expression and/or activity of inducible nitric oxide (NO) synthase, neuronal NO synthase, soluble epoxide hydrolase,NLRC3, and peroxisome proliferator-activated receptors a/b/gin the various tissues of mice including brain and spinal cord [15-19]. 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-κB/cyclooxygenase (COX)-2 pathway, and nicotinamide adenine dinucleotide phosphate oxidase, which areassociated with the production of pro-inflammatory cytokines,were also observed in the brain and spinal cord of LPS-treated mice [15-19]. Given the beneficial role of NLRX1 in inflammation, we test the hypothesisthat anti-inflammatory NLRX1 ligands such as DHA preventLPS-induced inflammatory hyperalgesia by inhibiting theTRAF6/IKK/IkB-a/NF-kB 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 undera 12-hours 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; Protocolnumber: 2019/16) and performedfollowingthe National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Inflammatory hyperalgesia model

The inflammatory hyperalgesia model was induced by intraperitoneal injection of LPS, and the latency of pain to nociceptive response was measured by the hotplate test as previously reported[15, 17-20]. To observethe pain behavior, mice were randomly divided into 7 groups, and the dose-response relationship was investigated with different DHA doses [21]: (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 injectedinto 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 ± 0.2°C (AHP 9601, Commat Ltd., Ankara, Turkey). The latency to paw withdrawal within 30 seconds was recordedusing 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 addessment of hyperalgesia according to our previous time-course studies [15-19]. Mice were killed after the test by cervical dislocation,and the brains and spinal cords of 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 IKKg with TRAF6, IKKa, IKKβ, phosphorylated IKKa/IKKβ (p-IKKa/IKKβ), IKKg, phosphorylated IKKg (p-IKKg), K63 U, or K48 U proteins according to previously described methods [4,7, 22, 23].In addition, the expression of IkB-a, phosphorylated IkB-a (p-IkB-a), NF-kB p65, phosphorylated NF-kB p65 (p-NF-kB p65), and interleukin-1β (IL-1β) 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 µg of protein for each sample was precleared with 20 µl of protein A/G-agarose beads for 1 hour at 4ºC, the beads were pelleted (1.000 x *g* for 5 minutes at 4°C), and the supernatants were incubated for 1 hour at 4ºC with 2 µg of antibodies specific forNLRX1 (sc-374514; Santa Cruz) or IKKg(sc-71331; Santa Cruz).20 µl of protein A/G-agarose beads were added and incubated for 12 hours at 4ºC 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 µl of HEPES and 40 µl of Laemmli sample buffer.The immunoprecipitated samples were kept at -80°C for measurement ofprotein expression of β-tubulin, TRAF6, IKKa, IKKβ, p-IKKa/IKKβ, IKKg, p-IKKg, K63 U, or K48 U. The totalamount of protein in the immunoprecipitated samples was determined by the Coomassie blue method [24]. Samples (10 mg 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 membraneswere 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) IKKa antibody (2682; Cell Signaling, Danvers, MA, USA); (4) IKKβ antibody (8943; Cell Signaling); (5) p-IKKa/IKKβ antibody (2078; Cell Signaling); (6) IKKg antibody (sc-71331; Santa Cruz); (7) p-IKKg 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) IkB-aantibody (sc-1643; Santa Cruz); (11) p-IkB-aantibody (sc-7977; Santa Cruz); (12) NF-kB p65 antibody (sc-8008; Santa Cruz); (13) p-NF-kB p65 antibody (sc-33020; Santa Cruz), and (14) IL-1β antibody (sc-52012; Santa Cruz).Secondary antibodies were sheep anti-mouse IgG-horseradish peroxidase (RPN4201; Amersham Life Sciences, Cleveland, OH,

USA) (for NLRX1, TRAF6, IKKg, p-IKKg, IkB-a, K63 U, NF-kB p65, p-NF-kB p65, and IL-1β, andgoat anti-rabbit IgG-horseradish peroxidase (RPN4301; Amersham Life Sciences) (for IKKa, IKKβ, p-IKKa/IKKβ, K48 U, and p-IkB-a)in 0.1% BSA (1:1.000). Membranes were reprobed and used foranti-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 the develop the blots. Immunoreactive band images were acquiredusing a gelimaging 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, IKKa, IKKβ, p-IKKa/IKKβ, IKKg, p-IKKg, K63 U, K48 U, IkB-a, p-IkB-a, NF-kB p65, p-NF-kB p65, and IL-1βproteins.

Statistical analysis

Data are expressed as means ± standard error of 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.A P value < 0.05 was considered statistically significant.

Results

DHA treatment prevents hyperalgesia induced by LPS

To determine the contribution of NLRX1 to LPS-induced hyperalgesia,the NLRX1ligand, DHA, was administered to mice alone or in combination with saline or LPS. Consistent with our previous results[15, 17-19], hot plate latency was decreased 6 hours after LPS injection compared with control group values(Fig. 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 theLPS-injected mice (P>0.05). At a dose of 3 mg/kg, treatment with DHAhad 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 DHAat the minimum effective dose (3 mg/kg) in LPS-induced hyperalgesia were used for further experiments.

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

The results of previous studies show that NLRX1dissociates from TRAF6 to inhibitNF-kB signaling pathwayactivity after LPS stimulation [4, 7, 25]. To test whether DHA has an effect on the dissociation of NLRX1 from TRAF6, the brains and spinal cordsof mice treated with saline-, LPS- and/or DHA were immunoprecipitated with the NLRX1 antibody and then immunoblotted with the TRAF6 antibody. Decreased expression of TRAF6 was associated with increased NLRX1 expression in the brain (Fig. 2A) and spinal cord (Fig. 2B) ofLPS-treated mice compared with the levels in the control group

values($P<0.05$). The expression of NLRX1 and TRAF6 in the tissues of DHA-treated mice was not different from the levels in the control group ($P > 0.05$).

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

Since NLRX1 inhibits NF-kB signaling pathway activity through its dissociation from TRAF6 and interaction with the IKK complex in response to LPS stimulation *in vitro* [7], weaimed to test whether DHA affects the association of NLRX1 with the catalytic (IKKa and IKKβ) and regulatory (IKKg) subunits of the IKK complex in addition to IKK complex activity. For this purpose, the brains and spinal cordsofmice treated with saline-, LPS- and/or DHA were immunoprecipitated with the NLRX1 antibody and subsequently immunoblotted with the antibodies for IKKa, IKKβ, p-IKKa/IKKβ (on Ser^{176} and Ser^{177}, respectively), IKKg, and p-IKKg (onSer^{376}). Increased expression of IKKa, IKKβ, and IKKg, as well as IKKa/IKKβ and IKKgphosphorylation,was detectedin the brains (Fig. 3A) and spinal cords (Fig. 3B) of LPS-treated mice compared with the levels in the control group ($P<0.05$). Treatment with DHA inhibited the LPS-induced increase in IKKa/IKKβ and IKKg phosphorylation, but not the expression of IKKa, IKKβ, and IKKg,in the tissues compared with the LPS-injected mice ($P<0.05$). The expression of IKKa, IKKβ, and IKKg, andIKKa/IKKβ and p-IKKgphosphorylation,in the tissues of DHA-treated mice was not different from the levels in the control group ($P > 0.05$).

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

It wasfound that NLRX1 undergoes rapid polyubiquitinationvia the K63 U, but not K48 U, linkage 10-15 minutes after treatment of mouse embryonic fibroblasts with LPS, but is then reduced [7]. To test whether DHA affects the polyubiquitination of NLRX1viathe K63 U and K48 U linkages, 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 K63 U or K48 U antibody. The expression K63 Udecreased in the brains (Fig. 4A) and spinal cords (Fig. 4B) of LPS-treated mice compared with the levels in thecontrol 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 Uin the tissues of DHA-treated mice was not different from the levels in the control group ($P > 0.05$).

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

It has also been shown that polyubiquitination of NLRX1 via K63 U linkage in response to LPS treatment of RAW264.7 cellsleads to inhibition of IKKa/IKKβ phosphorylationand recruitment of IKKg and its IKK complex to form a stable complex[7]. Therefore, we aimed to test whether DHA has an effect on the association of IKKg with the catalytic subunits of IKK complex, IKKa and IKKβ,in addition to IKKa/IKKβ activity. For this purpose, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were

immunoprecipitated with the IKKg antibody and then immunoblotted with the antibodies for IKKa, IKKβ, or p-IKKa/IKKβ (at Ser^{176} and Ser^{177}, respectively). The expression of IKKa and IKKβ, as well as IKKa/IKKβ phosphorylation,increased in the brains (Fig. 5A) and spinal cords (Fig. 5B) of LPS-treated mice compared with the levels in the control group (*P<0.05*). Treatment with DHA inhibited the LPS-induced increase in IKKa/IKKβ phosphorylation, but not the expression of IKKa and IKKβ, in tissues compared with LPS-injected mice (*P<0.05*). The expression of IKKa and IKKβ, as well asIKKa/IKKβphosphorylation,in the tissues of DHA-treated mice was not different from the levels in the control group ($P > 0.05$).

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

Polyubiquination of IKKgvia K63 U, but not K48 U, linkage in response to LPS treatment in mouse peritoneal macrophageshas also been reported [26].To test whether DHA affects the polyubiquitination of IKKgvia K63 U and K48 U linkages, the brains and spinal cords of mice treated with saline-, LPS- and/or DHA were immunoprecipitated with IKKg antibody and then immunoblotted with K63 U or K48 U antibody.Increased expression of K63 U and K48 Uwas observed in the brains (Fig. 6A) and spinal cords (Fig. 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 Uin the tissues of DHA-treated mice was not different from the levels in the control group ($P > 0.05$).

DHA treatment prevents LPS-induced decrease in the IkB-α expression and increase in the IkB-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 IkB-α, cause NLRX1-associated IKK complexes to lose phosphorylation[7]. To test the effect of DHA on LPS-induced changes in expression of IkB-α and p-IkB-α (at Ser^{32}) proteins in the brains and spinal cords of mice treated with saline- LPS- and/or DHA. Decreased expression of IkB-αwas associated with increased IkB-αphosphorylation in the brains (Fig. 7A) and spinal cords (Fig. 7B) of mice treated with LPS compared with the levels in the control group (*P<0.05*). Treatment with DHA inhibited the LPS-induced changes in the expression of IkB-αand IkB-αphosphorylation in the tissues compared with LPS-injected mice (*P<0.05*). The expression of IkB-α and IkB-αphosphorylationin the tissues of DHA-treated mice was not different from the levels in the control group ($P > 0.05$).

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

It has been shown that NLRX1 negatively regulates TLR4-induced NF-kB signaling in various cell types [7].To test the effect of DHA on the changes induced by LPSin the expression of NF-kB p65, NF-kB p65, and p-NF-kB p65 (at Ser^{536}) proteins in the brains and spinal cords of mice treated with saline-, LPS- and/or DHA. Consistent with our previous results[18, 19],increasedexpression of NF-kB p65 and NF-kB p65phosphorylation was observed in the brains (Fig. 8A) and spinal cords (Fig. 8B) of LPS-treated mice

compared with the the levels in thecontrol group (*P<0.05*). Treatment with DHA inhibited the LPS-induced increase in the expression of NF-kB p65 and NF-kB p65phosphorylation in the tissues compared with the LPS-injected mice (*P<0.05*). The expression of NF-kB p65 and NF-kB p65phosphorylation in the tissues of DHA-treated mice was not different from the levels in the control group ($P > 0.05$).

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

The results of previous studies in the LPS-induced septic shock model in NLRX1 KO and WT mice also show that NLRX1 inhibits theNF-kB signaling pathway-dependentformation of pro-inflammatory cytokines(e.g., IL-6) and prevents mortality [7]. To test the effect of DHA on the expression of one of the major pro-inflammatory cytokines, IL-1β, in the brains and spinal cords of mice treated with saline-, LPS- and/or DHA. Consistent with our previous findings [16-18], increasedexpression of IL-1β was observed in the brains (Fig. 9A) and spinal cords (Fig. 9B) of LPS-treated mice compared with the levels in the control group (*P<0.05*). Treatment with DHA inhibited the LPS-induced increase in IL-1β expression in tissues compared with LPS-injected mice (*P<0.05*). The expression of IL-1β 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 IKKa/β/g, IkB-a, and NF-kB in addition to IL-1β expression as a result of binding to the IKK complex after increased polyubiquitination via K63 U linkage after dissociation of NLRX1 from TRAF6 in the CNS contributes to the preventive effect of the NLRX1 ligand, DHA, against inflammatory hyperalgesia in response to LPS (Fig. 10).

TRAF6has been shown to be closely associated with NLRX1 in unstimulated cells, while possesing ubiquitin ligase activity and rapidly leading to its own and NLRX1 ubiquitination after LPS stimulation [7, 25].According to the results of the studies conducted to further identify the involvement of NLRX1 in TLR4-mediated NF-kB signaling pathway activity in response to LPS *in vitro* and *in vivo*[4, 7],NLRX1 negatively regulates NF-κB-induced TLR4 activity by dynamically interacting with TRAF6 and the IKK complex and promoting the formation pro-inflammatory cytokines.In addition,suppression of NLRX1 expressionhas been reported to increase susceptibility toseptic shock induced by LPS in association with elevated plasma levels of IL-6in mice [7].NLRX1has also been shown to attenuateLPS-induced apoptosis and inflammation in chondrocytes via negative regulation of NF-κB signaling in an *in vitro* model of osteoarthritis [25]. In a study conducted in NLRX1-knockout mice [23], data on exacerbation of LPS-induced cardiac injury were obtained as a result of increased reactive oxygen species formation with the IKKα/IKKβ/IkB-α/NF-kB 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-1β associated with mortality was prevented in the LPS-induced sepsis model in mice [27]. In addition Zhao et al. [26] demonstrated that LPS caused phosphorylation of IKKα/IKKβ, IkB-α, and NF-kB

p65 proteins and K63-linked polyubiquitination of IKKg, without affecting the total amount of IKKg protein, which was associated with increased formation of inflammatory cytokines (e.g., IL-1β, 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 andK63 U, but not K48 U, and phosphorylation of IKKa, IKKβ, and IKKgin NLRX1-immunoprecipitated mouse brain and spinal cords.Increasedphosphorylation of IKKa and IKKβ was observed in association with K63 U and K48 U protein expression in the IKKg-immunoprecipitated tissues of LPS-injected mice.Decreased protein expression of IkB-α was also associated with increased expression ofNF-kB p65 and phosphorylation of IkB-α and NF-kB p65in the tissues. Therefore, in agreement with the studies mentioned above [4, 7, 23, 25-27] and our previous findings [15-19],increasedformation of pro-inflammatory mediators as a result of increased activity of the TRAF6/IKK/IkB-a/NF-kB signaling pathwayin conjunction with up-regulation of NLRX1in the CNS of mice appears to be involved in LPS-induced inflammatory hyperalgesia.

It has also been shown that intraperitoneal injection of the NLRX1 ligand DHAreduces brain volume loss and improves neurological functionsin a perinatal hypoxia-ischemia rat model potentiated by systemic inflammationinduced by LPS [21]. As shown by Lu et al. [28], intrathecal injection of DHAin miceexerts an anti-nociceptive effect on inflammatory pain induced by intraplantar injection of carrageenan byinhibiting 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 [10, 29]. 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 CFAinto mice by inhibiting the up-regulationof TRAF6 in the spinal cord and allograft inflammatory factor 1 (a microglial marker) [13].In the present study, treatment with DHA showed a signinicant improvement in latency compared with endotoxemic mice. DHAalso prevented theLPS-induced increase in K63 Uprotein expression, as well as phosphorylation ofIKKa, IKKβ, and IKKgproteins,in NLRX1-immunoprecipitated tissues. On the other hand, treatment with DHA failed to prevent the LPS-induced changes in NLRX1 and TRAF6 protein expression.In the IKKg-immunoprecipitated tissues of LPS-injected mice treated with DHA, IKKa and IKKβ phosphorylation,as well as K63 U and K48 U protein expression,weredecreased.In addition, the LPS-induced decrease in protein expression of IkB-α associated with the increased expression of p-IkB-α,NF-kB p65, p-NF-kB p65,and IL-1βproteins in the tissues was also prevented by treating the mice with DHA.Based on the results of studies in the literature [4, 7,10, 12, 13, 21,23, 26-28] and our previous findings on the model of inflammatory hyperalgesia induced by LPS injection [15-19],it appears that reduced formation of pro-inflammatory mediators as a result of inhibition of the TRAF6/IKK/IkB-a/NF-kB signaling pathway at the transcriptional and/or post-transcriptional level in the CNS of mice is involved in the analgesicand 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 IKKg leading to suppression of IKK/IkB-a/NF-kB signaling

pathway activity contribute to the protective effects of DHA against inflammatory hyperalgesia in response to LPS.

Alimitation of this study is that we didnot exploredthe 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/IkB-a/NF-kB signaling pathway, 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 ofhyperalgesia-related inflammatory diseases.

Conclusion

In this study, 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/IkB-a/NF-kB 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 painconditions in which inflammation plays a role in the pathophysiology, as well as acute inflammatory diseases associated with pain that may result from bacterial infections.

Abbreviations

ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CNS, central nervous system; COX, cyclooxygenase; DHA, docosahexaenoic acid; IkB, inhibitor of kB; IB, immunoblotting; IKK, inhibitor of kB kinase;inhibitor of kB; IL-1β; interleukin-1β; IP, immunoprecipitation; LPS, lipopolysaccharide; LRR, leucine rich repeat; MyD88, myeloid differentiation factor 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; NLR, nucleotide-binding oligomerization domain-like receptor; NLRX1, nucleotide-binding oligomerization domain-like receptor X1; NO, nitric oxide; NLRP3, nucleotide binding domain and leucine-rich repeat protein; p-IkB-a, phosphorylated inhibitor of kB-a; p-IKKg, phosphorylated inhibitor of kB kinase g; p-IKKa/IKKβ; phosphorylated inhibitor of kB kinase a/inhibitor of kB kinase β; p-NF-kB p65, phosphorylated nuclear factor-κB p65; SEM, standard error of mean; TLR, toll-like receptor; TNF-a, tumor necrosis factor-a; TRAF6, tumor necrosis factor receptor-associated factor 6.

Declarations

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Conflict of interest The authors declare that they have no competing of interests.

Author contributions statement B.T. conceptualized and conceived the research design, analyzed the data, and drafted the manuscript. B.T., D.E.Y., S.P.S., and M.T.R. carried out the experiments. S.S.F. contributed to the finalizing of the manuscript. All authors read and approved the final manuscript.

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Figures

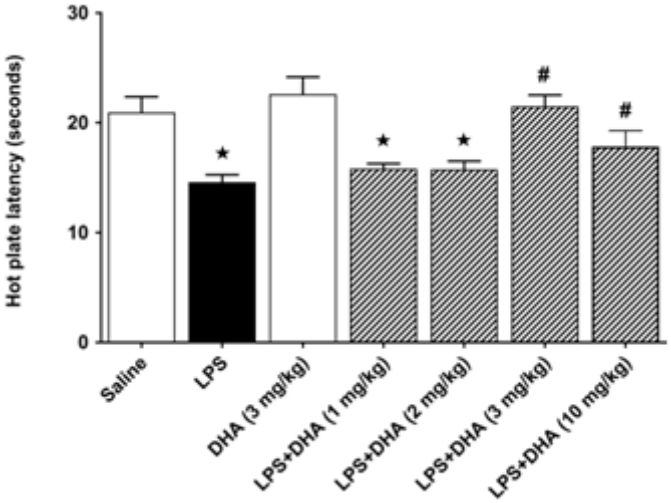

Figure 1

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±SEM from 6-10 animals. $^{*}P<0.05$ vs. saline-injected group; $^{\#}P<0.05$ vs. LPS-injected group.

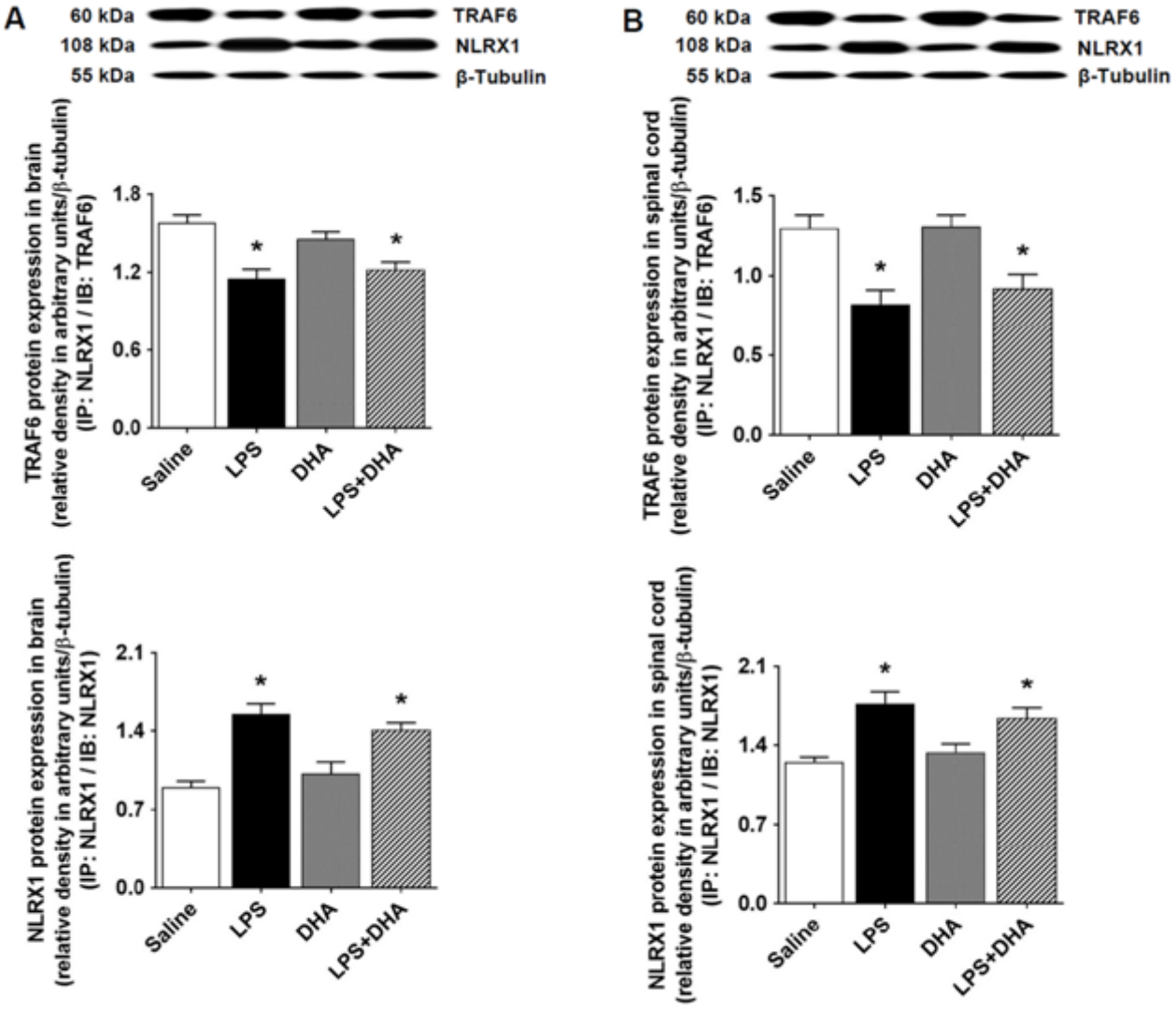

Figure 2

Effect of DHA on LPS-induced decreasein dissociation of NLRX1 and TRAF6 in **(A)** brains and**(B)**spinal cords 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-immuno precipitated tissues was measured usingIB. Data are expressed as means ± SEM from 6-10 animals. $^{*}P < 0.05$ vs. saline-injected group; $^{\#}P < 0.05$ vs. LPS-injected group.

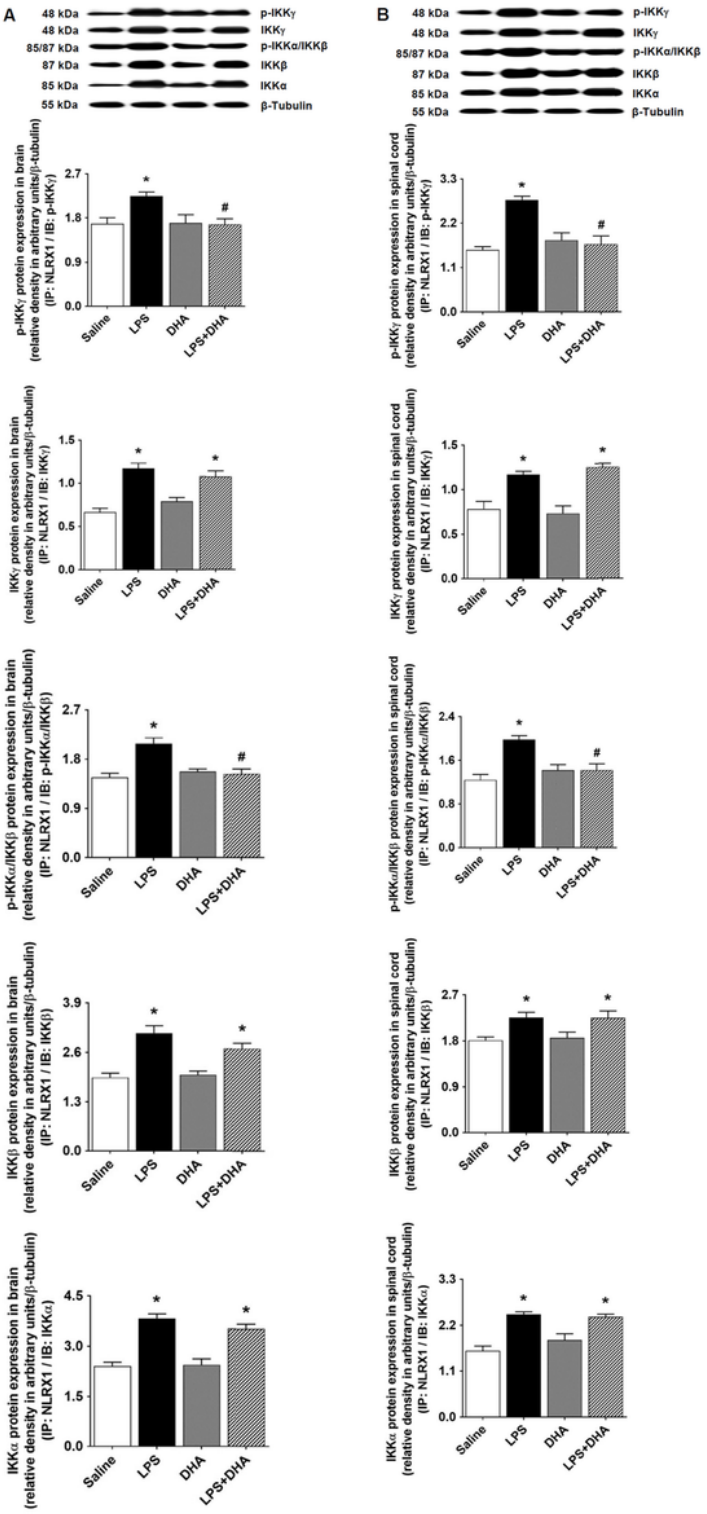

Figure 3

Effect of DHA on LPS-induced increase in IKK complex expression and activityin **(A)** brains and**(B)**spinal cords 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 IKKa, IKKb, and IKKgproteins in the NLRX1-immuno precipitated tissues was measured usingIB. Data are expressed as means ± SEM from 6-10 animals. $^{*}P < 0.05$ vs. saline-injected group; $^{\#}P < 0.05$ vs. LPS-injected group.

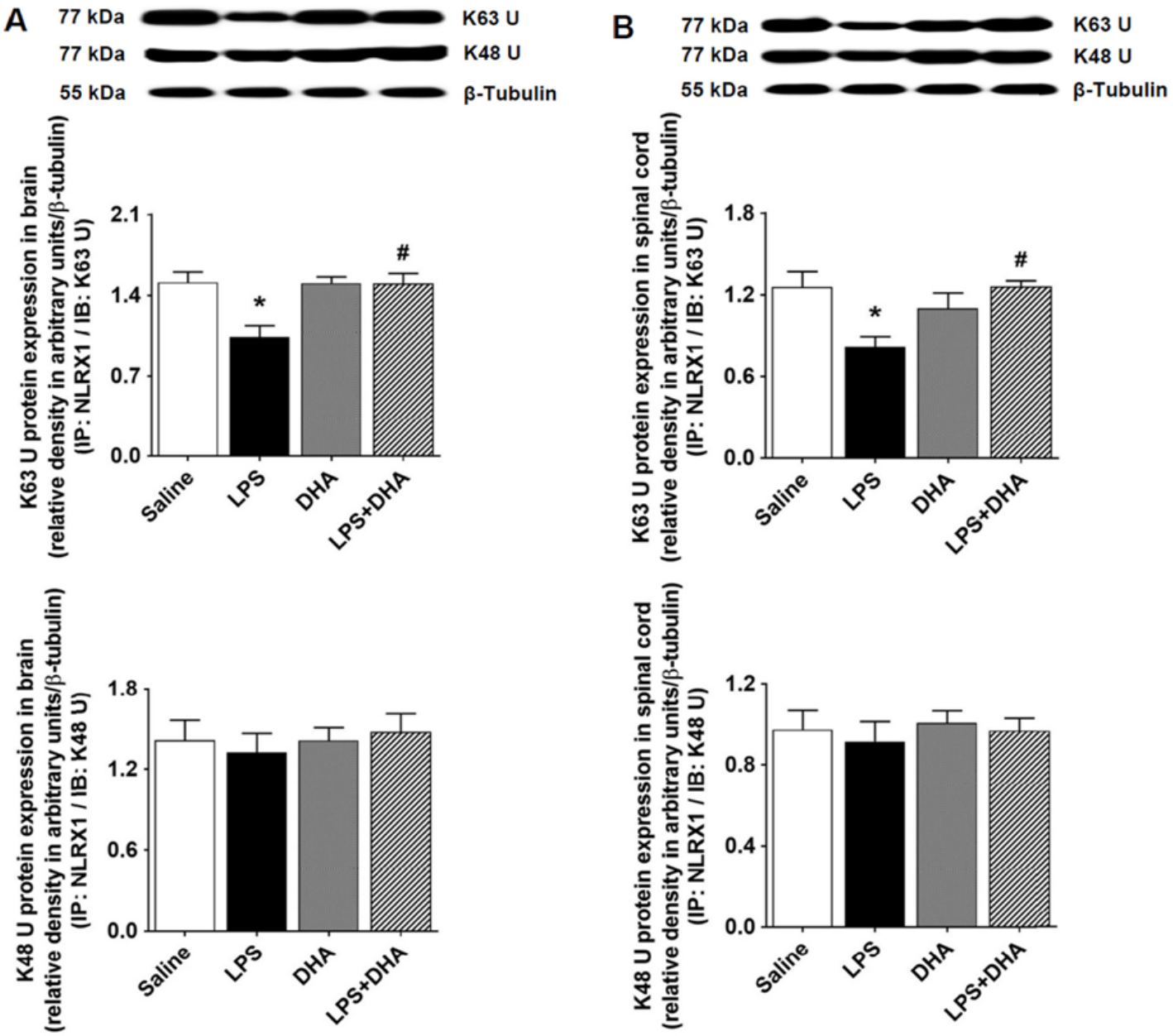

Figure 4

Effect of DHA on LPS-induced increase in K63 U and K48 U expression in **(A)** brains and**(B)**spinal cords 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 K63 U and K48 U proteins in NLRX1-immuno precipitated tissues was measured usingIB. Data are expressed as means ± SEM from 6-10 animals. $^{*}P < 0.05$ vs. saline-injected group; $^{\#}P < 0.05$ vs. LPS-injected group.

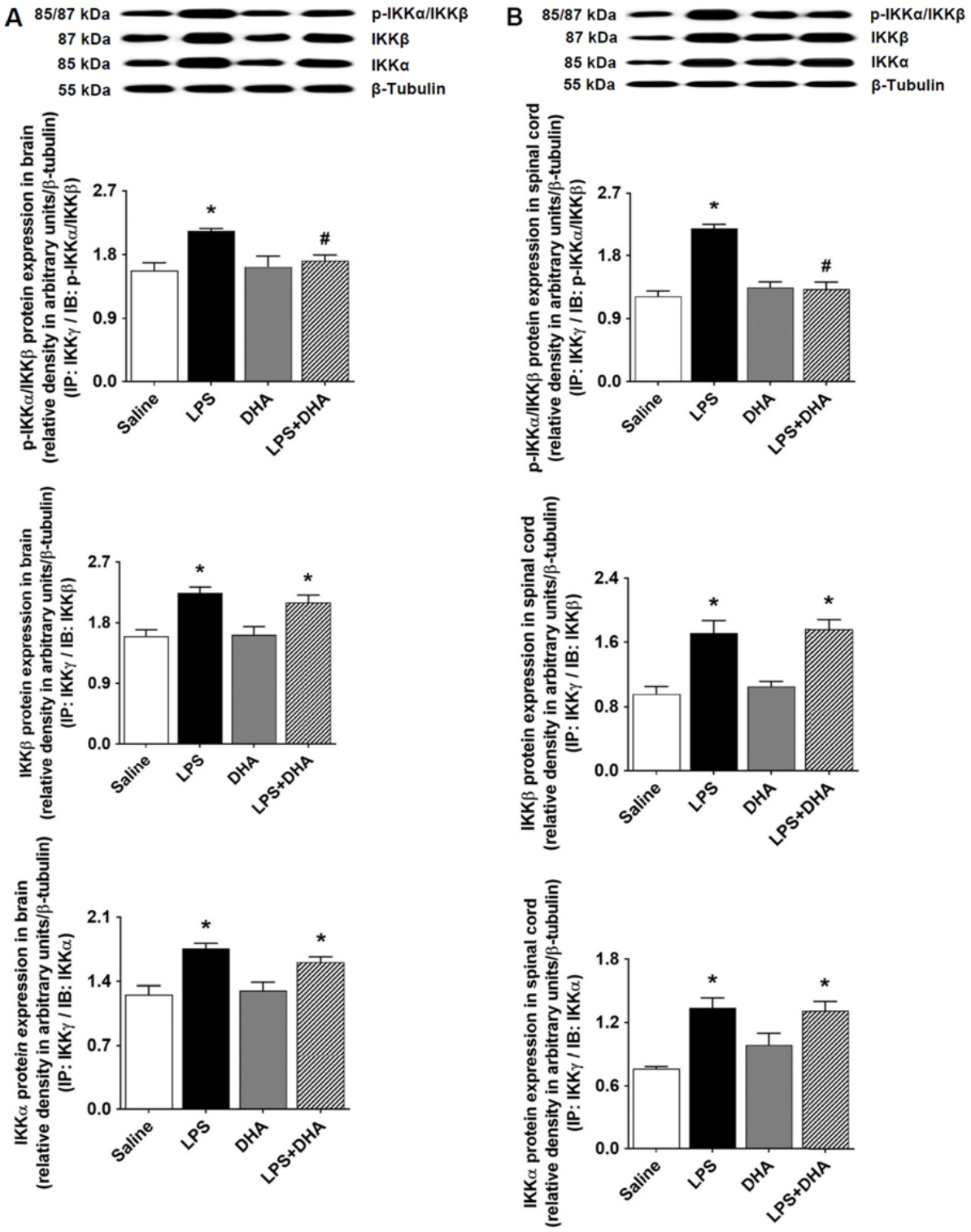

Figure 5

Effect of DHA on LPS-induced increase in IKKa and IKKbexpression and activity in **(A)** brains and**(B)**spinal cords 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 IKKa and IKKb proteins in IKKg-immuno precipitated tissues was measured usingIB. Data are expressed as means ± SEM from 6-10 animals. $^{*}P < 0.05$ vs. saline-injected group; $^{\#}P < 0.05$ vs. LPS-injected group.

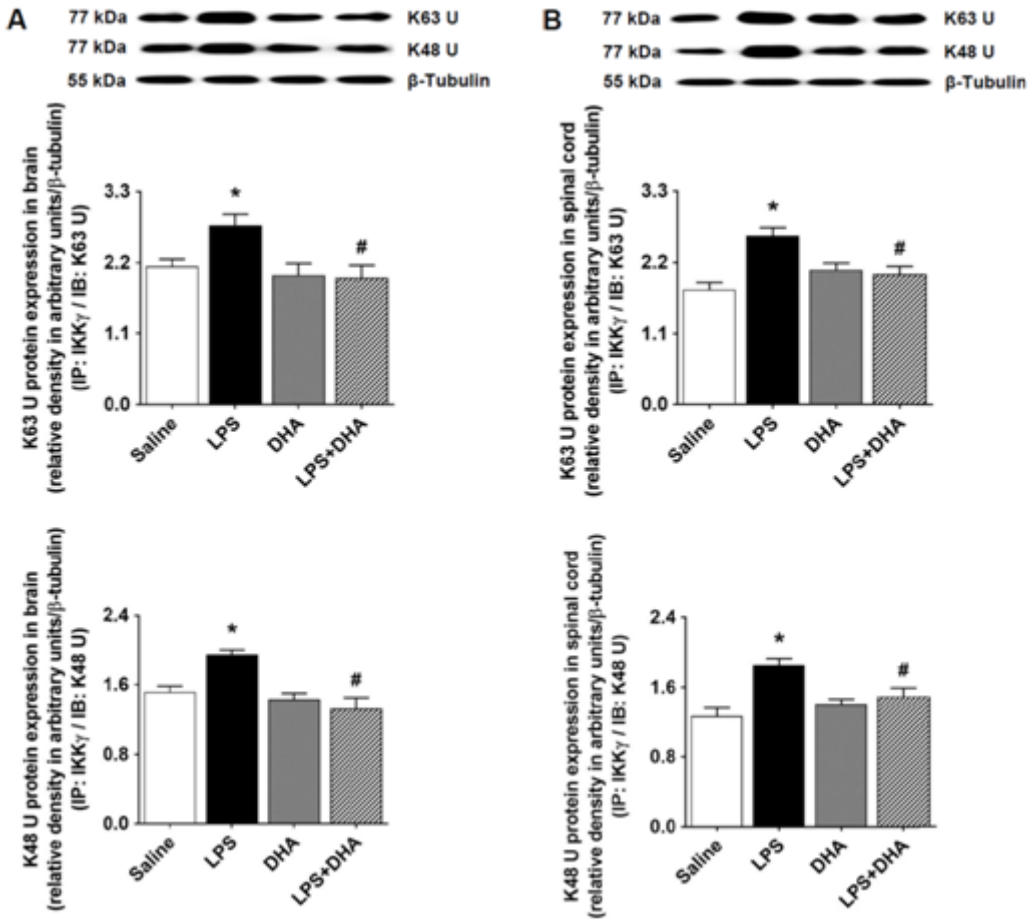

Figure 6

Effect of DHA on LPS-induced increase in the K63 U and K48 U expression in (A) brains and (B) spinal cords 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 K63 U and K48 U proteins in IKKg-immuno precipitated tissues was measured usingIB. Data are expressed as means ± SEM from 6-10 animals. $^{*}P < 0.05$ vs. saline-injected group; $^{\#}P < 0.05$ vs. LPS-injected group.

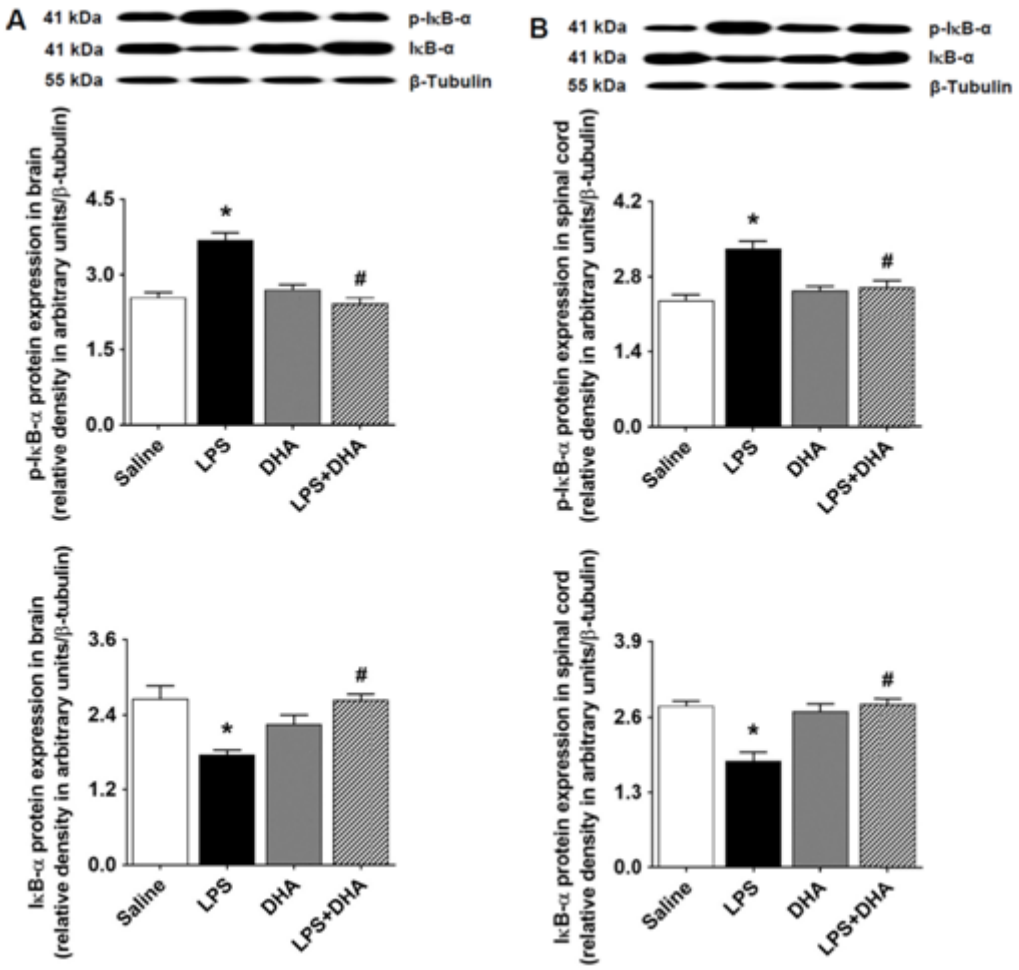

Figure 7

Effect of DHA on LPS-induced increase in the IkB-α expression and activity in (A) brains and (B) spinal cords 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 IkB-α proteins in tissues was measured usingIB. Data are expressed as means ± SEM from 6-10 animals. $^{*}P < 0.05$ vs. saline-injected group; $^{\#}P < 0.05$ vs. LPS-injected group.

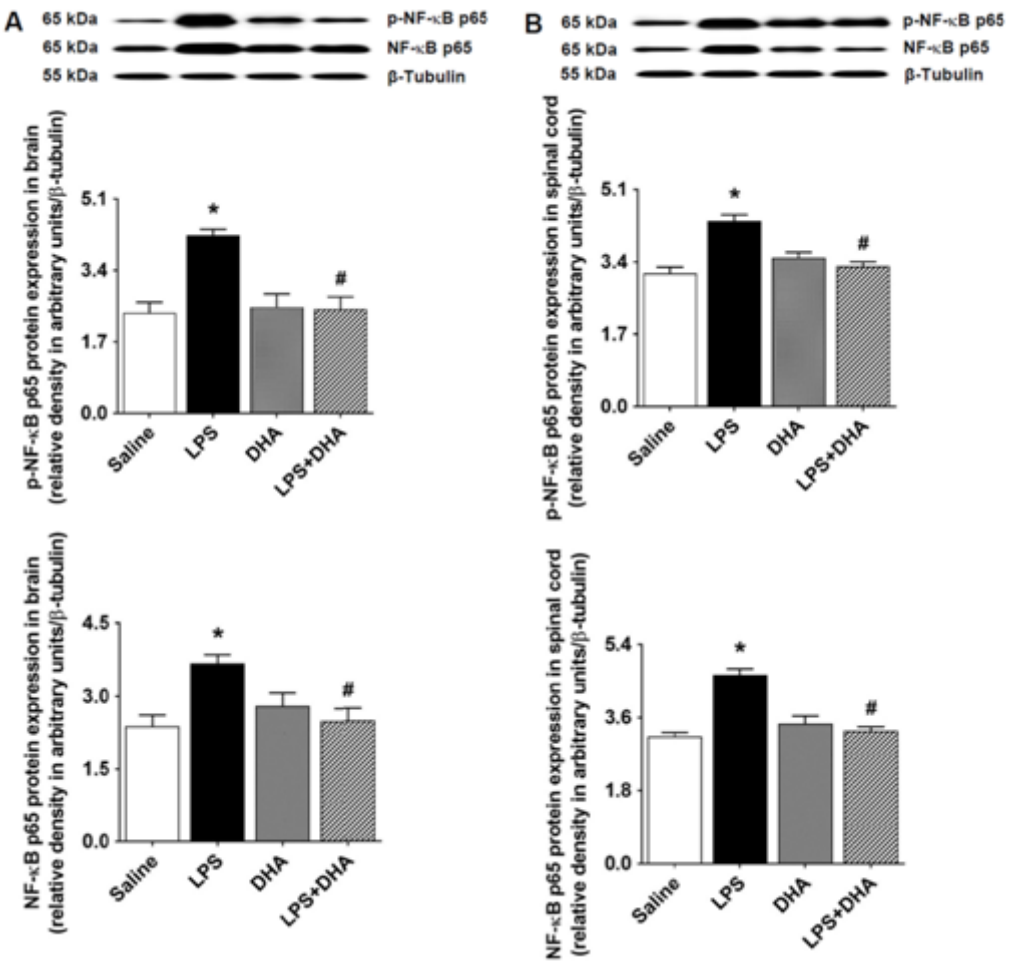

Figure 8

Effect of DHA on LPS-induced increase in NF-kB p65 expression and activity in (A) brains and (B) spinal cords 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-kB p65 proteins in tissues was measured usingIB. Data are expressed as means ± SEM from 6-10 animals. $^{*}P < 0.05$ vs. saline-injected group; $^{\#}P < 0.05$ vs. LPS-injected group.

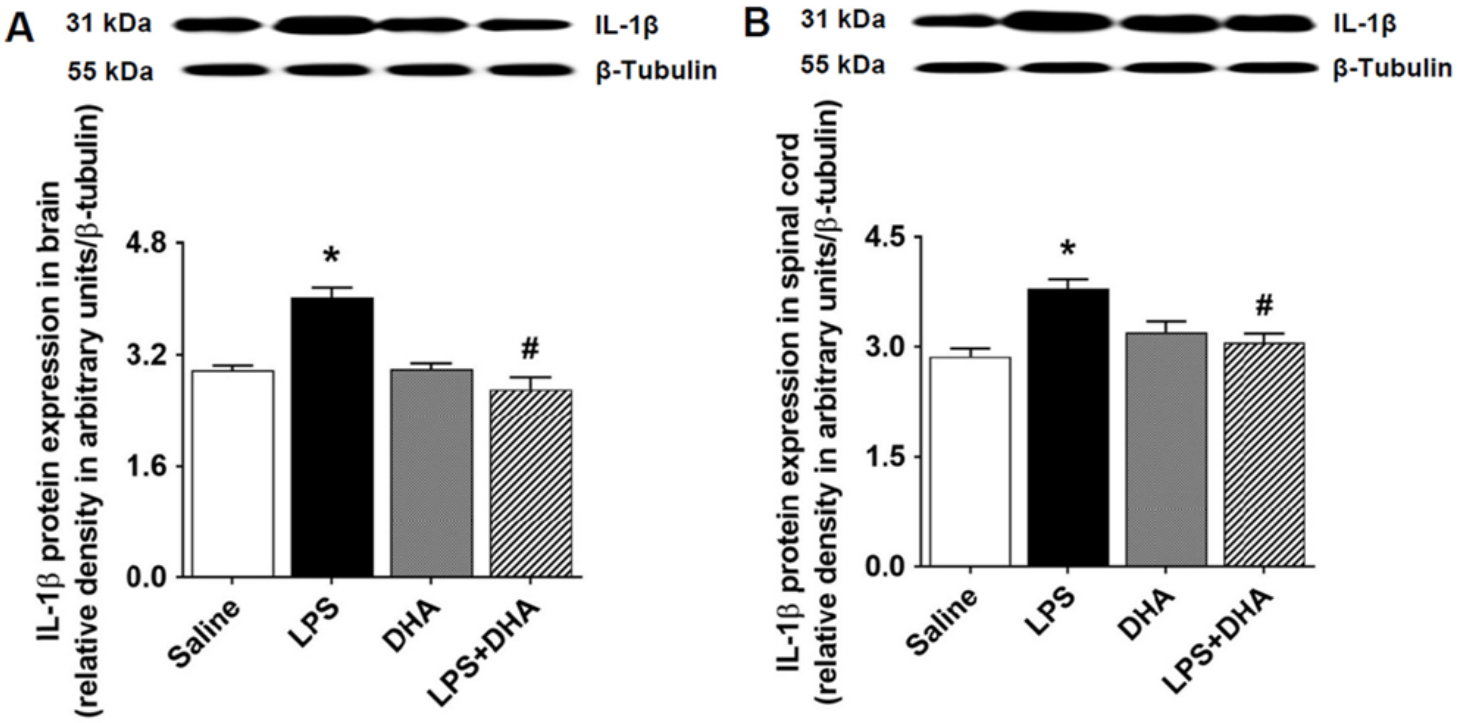

Figure 9

Effect of DHA on LPS-induced increase in IL-1β expression in (A) brains and (B) spinal cords 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-1β protein expression in tissues was measured usingIB. Data are expressed as means ± SEM from 6-10 animals. $^{*}P < 0.05$ vs. saline-injected group; $^{\#}P < 0.05$ vs. LPS-injected group.

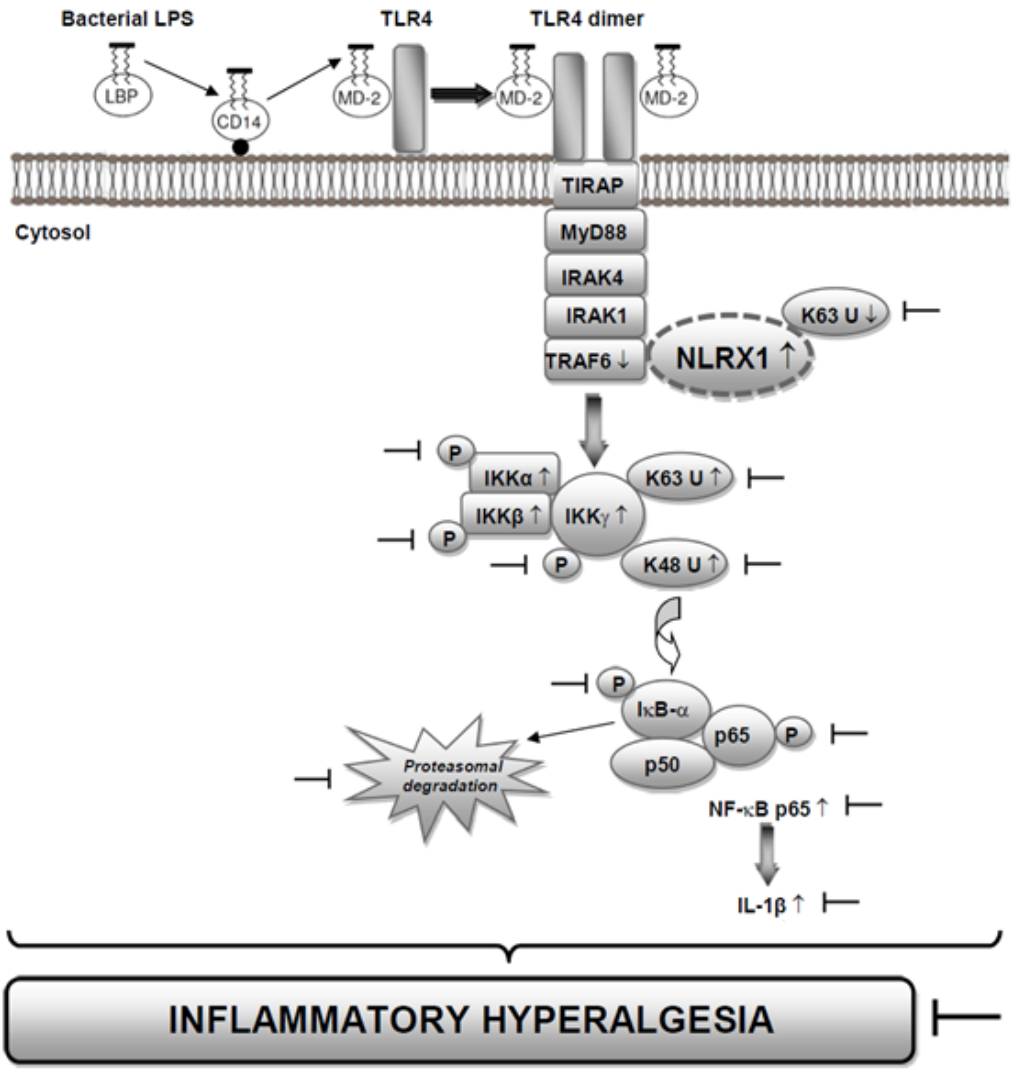

Fig. 10 Diagram showing the effect of NLRX1 ligand, DHA, on LPS-induced changes in TRAF6/IKK/NF-κB signaling pathway and pro-inflammatory mediator formation during inflammatory hyperalgesia in mice based on the results of the present study in addition to our previous findings [14, 16-18]. Presumably, a decrease in the activity of IKKα/IKKβ, IKKγ, IκB-α, and NF-κB in addition to IL-1β 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. *(↑) Increased by LPS; (↓) decreased by LPS; (⊣) prevented by DHA.*

Figure 10

See image above for figure legend