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**Neuroprotective effects of DHA-derived peroxidation product 4(RS)-4-F4t-Neuroprostane on microglia**

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## Abstract

The abundance of docosahexaenoic acid (DHA) in brain membrane phospholipids has stimulated studies to explore its role in neurological functions. Upon released from phospholipids, DHA undergoes enzymatic reactions resulting in synthesis of bioactive docosanoids and prostanoids. However, these phospholipids are also prone to non-enzymatic reactions leading to more complex pattern of metabolites. A non-enzymatic oxidized product of DHA, 4(*RS*)-4-F<sub>4t</sub>-Neuroprostane (44FNP), has been identified in cardiac and brain tissues. In this study, we examined effects of the 44FNP on oxidative and inflammatory responses in microglial cells treated with lipopolysaccharide (LPS). The 44FNP attenuated LPS-induced production of reactive oxygen species (ROS) in both primary and immortalized microglia (BV2). It also attenuated LPS-induced inflammation through suppressing NFκB-p65 and levels of iNOS and TNFα. In addition, 44FNP also suppressed LPS-induced mitochondrial dysfunction and upregulated the Nrf2/HO-1 antioxidative pathway. In sum, these findings with microglial cells demonstrated neuroprotective effects of this 44FNP and shed light into the potential of nutraceutical therapy for neurodegenerative diseases.

Keywords: 4(*RS*)-4-F<sub>4t</sub>-Neuroprostane, microglia, antioxidant, and anti-inflammatory

## 1. Introduction

Docosahexaenoic acid (DHA) is a polyunsaturated fatty acid (PUFA) abundant in the phospholipids in brain, and studies have shown a critical role for it to maintain brain health [1]. Intake of DHA has been correlated to lower cognitive impairment in Alzheimer's disease [2, 3]. There is growing evidence that some of the neuroprotective effects of DHA is due to its conversion to oxidative metabolites such as neuroprotectin D1, resolvins and maresins [4].

Besides enzymatic reactions, DHA is also vulnerable to non-enzymatic reactions to produce peroxidation products, such as neuroprostanooids [5-10]. Among these, 4(RS)-4-F4t-Neuroprostane (44FNP) is one of the most abundant [11-13], and has been proposed to be an oxidative damage biomarker of neurological diseases [14]. To date, studies have unveiled different health effects of 44FNP, e.g., on arrhythmia [15], decreasing infarct and ventricular tachycardia in ischemia reperfusion [16], inducing production of heme oxygenase-1 (HO-1) in SH-SY5Y cells [17], and proliferation of human breast cancer cells [18]. However, whether 44FNP can exert neuroprotective effects in microglia has not been investigated in detail.

In order to provide information to further our understanding of mechanism(s) underlying the neuroprotective effects of 44FNP, this study examined oxidative stress, inflammation, and mitochondrial membrane function in microglial cells stimulated with the bacteria toxin lipopolysaccharide (LPS). In addition, this study also evaluated whether 44FNP may confer antioxidation through the Nrf2/HO-1 pathway.

## 2. Materials and Methods

### 2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin (P/S, 10,000 units/ml) were purchased from Life Technologies (Grand Island, NY), fetal bovine serum (FBS) and lipopolysaccharide (LPS) from Cayman Chemical (Ann Arbor, MI), dimethyl sulfoxide (DMSO), cOmplete™ protease inhibitor cocktail, PhosSTOP™ phosphatase inhibitor cocktail from Sigma-Aldrich (St. Louis, MO), radioimmunoprecipitation assay (RIPA) buffer, bicinchoninic acid (BCA) protein assay kit, SuperSignal™ West Pico plus chemiluminescent substrate, Restore™ PLUS Western blot stripping buffer, TNF- $\alpha$  mouse uncoated ELISA kit, and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCF) from Thermo Fisher Scientific (Waltham, MA). Primary antibodies against HO-1, phospho-NF- $\kappa$ B p65 (p-p65) was purchased from Cell Signaling (Beverly, MA), monoclonal anti- $\beta$ -actin peroxidase antibody from Sigma-Aldrich (St. Louis, MO), anti-inducible nitric oxide synthase (iNOS) antibody and TMRM Assay Kit (Mitochondrial Membrane Potential) from Abcam (Cambridge, MA), and anti-Nrf2 antibody from GeneTex (Irvine, CA). Neural Tissue Dissociation kit (P), MACS buffer, and CD11b microbeads were purchased from Miltenyi Biotec (Auburn, California). 44FNP (1 mg/ml in methanol) was synthesized in Dr. T. Durand's laboratory at IBMM, France [19].

## 2.2. Preparation of Primary Mouse Microglia

Timed pregnant C57BL/6 mice were purchased from Charles River (Wilmington, MA). All animal care and experimental protocols were carried out with permission from the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Chicago. Preparation of primary mouse microglia from mouse pups (P1–P4) were accomplished using the Miltenyi Biotec MACS cells separation system. Briefly, cerebral cortices were dissected, and meninges removed. Brain tissue was

homogenized using the Neural Tissue Dissociation Kit. Cells were collected and resuspended in ice-cold MACS buffer containing CD11b microbeads and then passed through the magnetized LS columns to harvest the primary microglia according to the manufacturer's protocol. Microglial cells were collected and plated as previously described [20].

### 2.3. BV2 Cell Culture

BV2 cells were originally obtained from Dr. Rosario Donato (University of Perugia, Italy) and subsequently propagated and stored in Dr. Grace Sun's laboratory (University of Missouri, MO, USA). BV2 mouse microglial cells were cultured in DMEM supplied with 5% FBS and 1% P/S. For experiments, cells were subcultured to 80% confluence in multiple well plates and serum starved for 3h prior to treatment with 44FNP for 1h and followed by stimulation with 50 ng/mL LPS. 44FNP was dissolved in DMSO diluted in DMEM to the indicated concentrations.

### 2.4. Measurement of ROS Production

Cells were suspended in 96-well plates, pretreated with 44FNP for 1 h, and followed by LPS treatment for 12h. After treatment, 10  $\mu$ M DCF was added to the cells for 45 min. The fluorescent intensity of DCF in cells was measured using a Synergy H1 Plate Reader (BioTek, St. Louis, MO) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

### 2.5. Western Blot Analysis

After treatment, the culture medium was removed, and cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. Cell lysates were collected and centrifuged at  $14,000\times g$  for 15 min at 4 °C, and supernatants were collected. Protein concentration in cell lysate was determined using BCA assay. Samples were loaded onto SDS-PAGE for electrophoresis. Afterwards, proteins were transferred to 0.2- $\mu$ m PVDF membranes at 100 V for 1 h at 4 °C. Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h, and incubated with antibodies overnight at 4 °C. After washing with TBS-T, blots were incubated with secondary antibody for 1 h. Signals were developed using SuperSignal™ West Pico plus chemiluminescent substrate and captured with a myECL imager (Thermo Scientific). The optical density of bands was measured with the Image Studio Lite 5.2 (LI-COR Biotechnology, Lincoln, NE).

## 2.6. TNF- $\alpha$ ELISA Assay

TNF- $\alpha$  in culture medium was determined by sandwich ELISA. Briefly, cells were treated, and medium was collected and centrifuged at  $4000\times g$  for 5 min. The levels of TNF- $\alpha$  were assessed using an ELISA kit following manufacturer's instruction.

## 2.7. Mitochondrial membrane potential measurement

After treatment, cells were incubated with 200 nM TMRM in Hank's balanced salt solution (HBSS) for 30 min at 37 °C. Fluorescence was analyzed use Synergy H1 Plate Reader with an excitation wavelength of 548 nm and an emission wavelength of 575 nm. For positive control, p-trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP), a mitochondrial uncoupler known to cause mitochondria depolarization, was used to validate our technique.

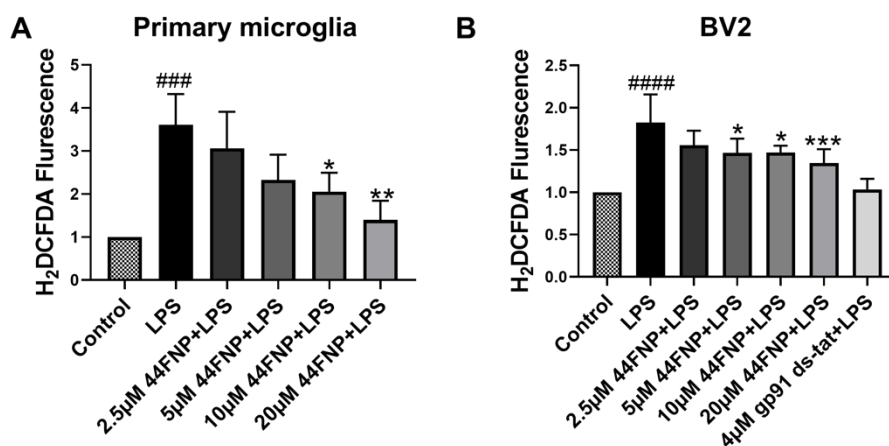
## 2.8. Statistical Analysis

Data are expressed as mean  $\pm$  standard deviation (SD) from at least three independent experiments. Statistical analysis between multiple groups was carried out using one-way ANOVA followed by Tukey's post hoc HSD test in GraphPad Prism (version 8.10). A *p* value  $< 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Effects of 44FNP on LPS-induced ROS production.

We examined the effects of 44FNP (2.5- 20  $\mu$ M) on ROS production in primary mouse microglia and BV2 cells stimulated with LPS (50 ng/ml). In both types of microglia, 44FNP inhibited LPS-stimulated ROS in a dose-dependent manner with a significant decrease at 5  $\mu$ M (Fig. 1). To verify ROS production through NADPH oxidase, we used the gp91ds-tat peptide, known to block ROS production through disruption of NADPH subunits [21]. Addition of gp91ds-tat abolished LPS-induced ROS in these cells, suggesting that ROS production was mainly through NADPH oxidase. Our treatments in all experimental groups did not impose any ill effects on cell viability (data not shown).



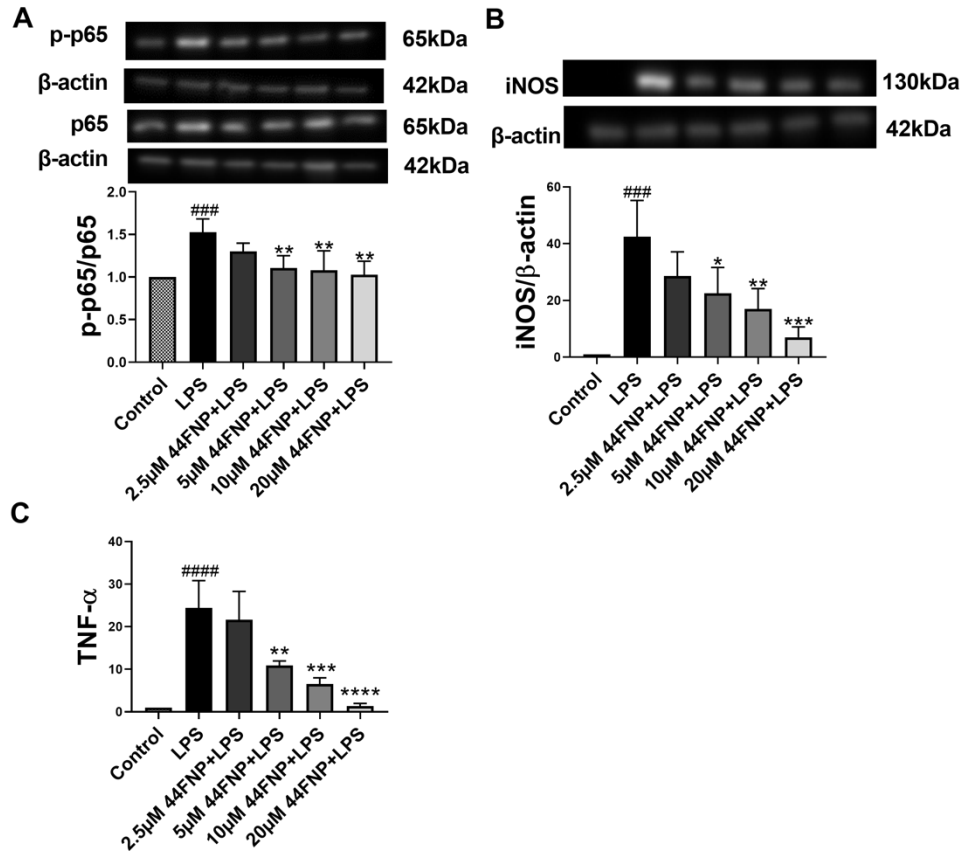


**Fig. 1. 44FNP attenuated LPS-induced reactive oxygen species (ROS) production in primary mouse microglia and BV2 cells.** Primary microglia (A), BV2 cells (B) were pretreated with 44FNP for 1 h, followed by stimulation with 50ng/ml LPS for 12 h. Data are represented in mean  $\pm$  standard deviation (SD) (n = 3). Statistical analysis was carried out with one way ANOVA followed by Bonferroni post-tests. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared with the LPS group; ###p<0.001, ####p<0.0001 compared with the control group.

### 3.2. Effects of 44FNP on LPS-induced inflammatory responses in BV2 cells.

LPS has been reported to activate microglial inflammation through the NF $\kappa$ B-p65 pathway [20]. In this study, we examined effects of 44FNP on p65 and p-p65 expression in control and LPS-stimulated microglial cells. Results show that LPS-induced phosphorylation of p65 expression was attenuated by 44FNP in a dose-dependent manner (Fig. 2A).

To further explore the anti-neuroinflammatory effects of 44FNP, results also showed that 44FNP was able to attenuate LPS-induced increase in iNOS (Fig. 2B) and TNF $\alpha$  secretion (Fig. 2C).

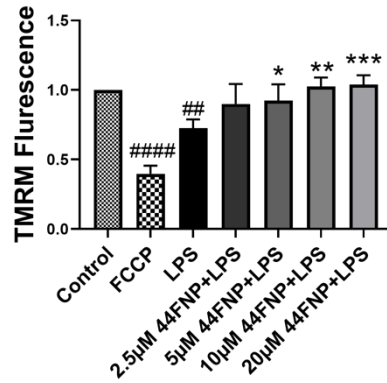


**Fig. 2. 44FNP attenuated LPS-triggered the p65 pathway (A), iNOS (B), TNF- $\alpha$  (C) in BV2 cells.** BV2 cells were pretreated with 44FNP for 1 h followed by stimulation with 50 ng/ml LPS for 1 h (A) or 18 h (B, C). Data are represented in mean  $\pm$  SD (n = 3). Statistical analysis was carried out with one way ANOVA followed by Bonferroni post-tests. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  compared with the LPS group; ### $p < 0.001$ , #### $p < 0.0001$  compared with the control group.

### 3.3. Effects of 44FNP on LPS-induced changes in mitochondrial membrane potential.

A leaky mitochondrial membrane is a key characteristic of mitochondrial dysfunction. Loss of mitochondrial membrane potential ( $\Delta\psi_m$ ) is related to mitochondrial depolarization. To demonstrate the effects of 44FNP on LPS-induced mitochondrial dysfunction, we applied TMRM to monitor  $\Delta\psi_m$  in BV2 cells. As shown in Fig. 3, exposing cells to LPS significantly decreased TMRM intensity, indicating the loss of  $\Delta\psi_m$ . The loss of  $\Delta\psi_m$  due to LPS was also attenuated by pretreatment of cells with

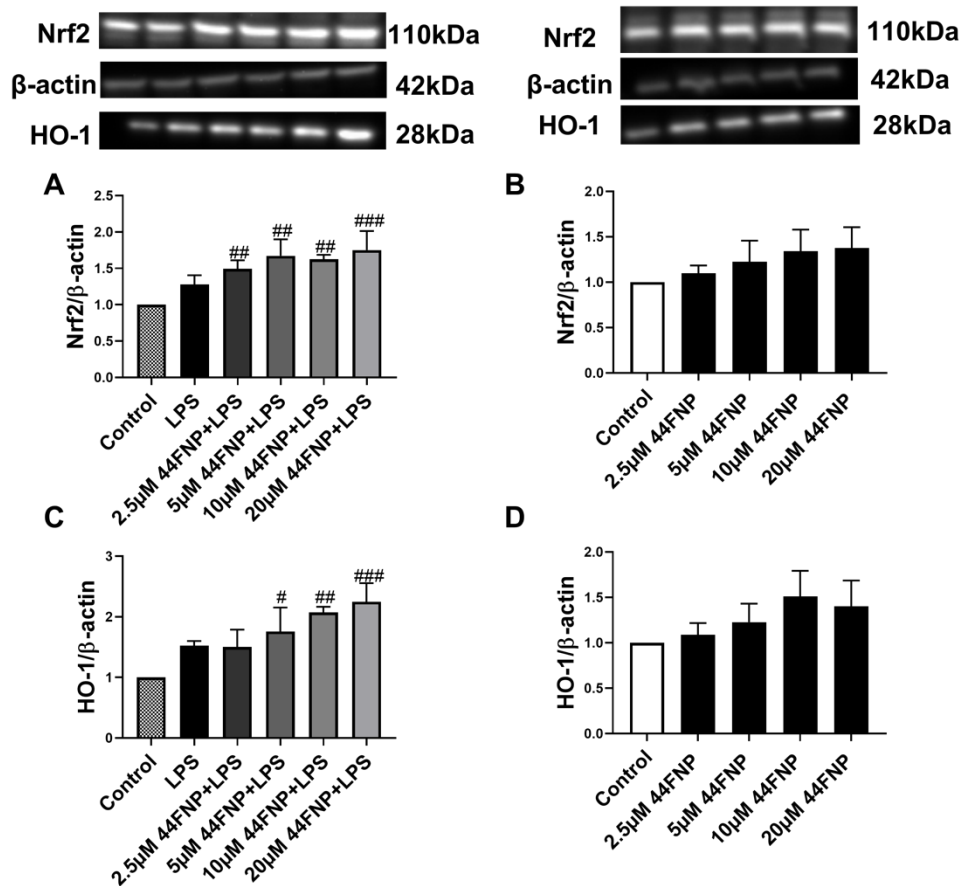
44FNP. Consequently, these results demonstrated the ability for 44FNP to suppress LPS-induced mitochondrial potential loss in microglial cells.



**Fig. 3. 44FNP attenuated LPS-induced mitochondrial membrane potential loss in BV2 cells.** Cells were pretreated with 44FNP for 1 h followed by stimulation with 50 ng/ml LPS for 24 h. Data are represented in mean  $\pm$  SD (n = 3). Statistical analysis was carried out with one way ANOVA followed by Bonferroni post-tests. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared with the LPS group; ##p<0.01, ####p<0.0001 compared with the control group.

#### 3.4. Effects of the 44FNP on Nrf2/HO-1

Our previous study demonstrated ability for exogenous DHA and its oxidative derivatives to upregulate the antioxidant pathway involving Nrf2/HO-1 in microglia. In this study, 44FNP dose-dependently upregulated Nrf2 and HO-1 in cells stimulated with LPS, but not in controls without stimulation with LPS (Fig 4).



**Fig. 4. 44FNP enhanced LPS-induced Nrf2/HO-1 pathway in BV2 cells.** Cells were pretreated with 44FNP for 1 h followed by stimulation with 50 ng/ml LPS for 6 h. Data are represented in mean  $\pm$  SD (n = 3). Statistical analysis was carried out with one way ANOVA followed by Bonferroni post-tests. #p<0.05, ##p<0.01, ###p<0.001 compared with the control group.

#### 4. Discussion

Free radical-induced peroxidation of arachidonic acid, another PUFA found in membrane phospholipids, results in the formation of isoprostanes [22]. These isoprostanes are elevated in brain and other body organs due to oxidant injury and are related to inflammatory mechanisms [23]. On the contrary, oxidative derivatives of DHA result in production of neuroprostanes which tend to show protective effects. In agreement with recent studies showing evidence for protective effects of DHA-

derived 44FNP on the peripheral organs [16], results in this study demonstrated similar effects of this neuroprostane on microglial cells in the central nervous system.

In this study, we demonstrate the ability for 44FNP to suppress ROS production and inflammatory responses in both primary mouse microglia and immortalized microglial BV2 cells (Fig. 1A). By using gp91ds tat, results also demonstrated ROS production in microglia is mediated mainly through NADPH oxidase.

LPS is known to stimulate the Toll-like Receptor (TLR4) which is responsible for activation of the NF- $\kappa$ B transcriptional pathway [24]. Binding of the NF- $\kappa$ B dimer to the DNA consensus sequence of target genes leads to production of proinflammatory cytokines, such as TNF $\alpha$  [25]. Consistent with the anti-inflammatory effects of 44FNP demonstrated in human macrophage [26], our results with BV2 microglia also demonstrated the ability for 44FNP to suppress LPS-induced iNOS and TNF $\alpha$  through the NF- $\kappa$ B pathway (Fig. 2).

Mitochondrial membrane potential ( $\Delta \psi_m$ ) is generated from proton pump across the mitochondrial inner membrane and involves complexes I, III, and IV [27]. The stability of the  $\Delta \psi_m$  is essential for many microglia functions [28]. In this study, we provided important new evidence showing that 44FNP was capable of suppressing LPS-induced loss of  $\Delta \psi_m$  in microglia.

Nrf2 is an important transcription factor involving the maintenance of cellular redox state and alleviates expression of antioxidant genes such as HO-1. Some compounds that suppress NF- $\kappa$ B inflammation could also upregulate Nrf2/HO-1 [24, 29]. In a recent study, 44FNP was found to elevate HO-1 mRNA expression in primary neurons [17]. However, study with microglia here showed effects of 44FNP

on Nrf2/HO-1 only when cells were stimulated with LPS. These results suggest a role for 44FNP as an antioxidative agent in activated microglia.

In sum, this study demonstrated that 44FNP, a non-enzymatic peroxidation product of DHA, to exert neuroprotective effects on LPS-stimulated microglial cells through its abilities to attenuate oxidative stress, inflammatory responses, and mitochondrial dysfunction. Although future studies should include *in vivo* testing in animal models, this study has provided a better understanding on the mechanism(s) underlying the neuroprotective effects of 44FNP and better insights into its potential for the development of nutraceutical therapy for neurodegenerative diseases.

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