Electrophilic nitro-fatty acids inhibit vascular inflammation by disrupting LPS-dependent TLR4 signalling in lipid rafts

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Received 13 April 2012; revised 20 December 2012; accepted 3 January 2013; online publish-ahead-of-print 17 January 2013

Aims

Electrophilic fatty acid nitroalkene derivatives, products of unsaturated fatty acid nitration, exert long-term cardiovascular protection in experimental models of metabolic and cardiovascular diseases. The goal of this study is to examine the effects of nitro-fatty acids in the regulation of upstream signalling events in nuclear factor-κB (NF-κB) activation and determine whether low-dose acute administration of nitro-fatty acids reduces vascular inflammation in vivo.

Methods and results

Using NF-κB-luciferase transgenic mice, it was determined that pre-emptive treatment with nitro-oleic acid (OA-NO2), but not oleic acid (OA) inhibits lipopolysaccharide (LPS)-induced NF-κB activation both in vivo and in isolated macrophages. Acute intravenous administration of OA-NO2 was equally effective to inhibit leukocyte recruitment to the vascular endothelium assessed by intravital microscopy and significantly reduces aortic expression of adhesion molecules. An acute treatment with OA-NO2 in vivo yielding nanomolar concentrations in plasma, is sufficient to inhibit LPS-induced Toll-like receptor 4 (TLR4)-induced cell surface expression in leukocytes and NF-κB activation. In vitro experiments reveal that OA-NO2 suppresses LPS-induced TLR4 signalling, inhibitor of NF-κB (IκB) phosphorylation and ubiquitination, phosphorylation of the IκB kinase (IKK), impairing the recruitment of the TLR4 and TNF receptor associated factor 6 (TRAF6) to the lipid rafts compartments.

Conclusion

These studies demonstrate that acute administration of nitro-fatty acids is effective to reduce vascular inflammation in vivo. These findings reveal a direct role of nitro-fatty acids in the disruption of the TLR4 signalling complex in lipid rafts, upstream events of the NF-κB pathway, leading to resolution of pro-inflammatory activation of NF-κB in the vasculature.

Keywords

Nitro-fatty acids • Inflammation • TLR4 • NF-κB • Lipid rafts

1. Introduction

During inflammatory responses, the nitrogen dioxide radical (NO2) is formed by multiple reactions of nitric oxide (NO), nitrite (NO2−), partially reduced oxygen species and heme peroxidases. Then, NO2 can react with cell components including DNA bases, protein tyrosine residues, and unsaturated fatty acids to form relatively stable nitro derivatives. It is viewed that protein tyrosine nitration during inflammation represents a shift from the signal transducing physiological actions of NO to more pro-oxidative and pathogenic reactions. In contrast, the nitration of unsaturated fatty acids represents a convergent redox signalling mechanism yielding electrophilic products that promote adaptive signalling responses. Nitro-fatty acids exert pluripotent cell signalling actions in the vasculature. Electrophilic nitro-fatty acids participate in kinetically rapid protein addition reactions by undergoing Michael additions, mainly with Cys and...
to a lesser extent His residues. In this manner, nitro-fatty acids form reversible adducts with critical electrophile-sensitive signalling mediators such as peroxisome proliferator-activated receptor-γ (PPARγ) and the Keap1 regulatory protein for Nrf2-dependent antioxidant response signalling. While nitroalkene derivatives of linoleic acid (LNO2) and oleic acid (OA-NO2) are potent partial agonists of PPARγ and activators of Nrf2-dependent gene expression, current data support additional anti-inflammatory properties independent of PPARγ and Nrf2. Notably, the electrophilic nature of nitro-fatty acids promote Michael addition to NF-κB signalling-related proteins, contributing to inhibition of inflammatory responses in vascular cells. However, the precise mechanism by which nitroalkenes mediates suppression of NF-κB activity remains to be fully defined.

Nitro-fatty acids are present as both free and nucleophile-adducted species in healthy human blood, with their endogenous levels at ~1 nM for OA-NO2 and isomers of LNO2. Recent studies indicate that fatty acid nitration products are generated in vivo in myocytes following ischaemic preconditioning and focal ischaemia–reperfusion that actively participate in protein reactions. From current pharmacological viewpoints, chronic administration of nitroalkenes, in particular OA-NO2, mediate beneficial effects in experimental models of cardiovascular, inflammatory, and metabolic diseases. Also, in mice treated with LPS, OA-NO2 administration attenuates systemic inflammation and limits multiorgan dysfunction. In many of these studies, therapeutic benefit was achieved by long-term administration of pre-emptive OA-NO2. Herein, we reveal that an acute, low-dose in vivo administration of OA-NO2 results in the rapid inhibition of NF-κB signalling. We show that OA-NO2 disrupts toll-like receptor-4 (TLR4)–mediated signalling by altering the recruitment of adaptor proteins and upstream regulatory proteins of NF-κB into membrane lipid rafts of macrophages and endothelial cells.

2. Methods

2.1 Animals

Animal procedures were conducted under the approval of the University Committee on Use and Care of Animals at the University of Michigan (Protocol Number #09398) and conform with the Guide for the Care and Use of Laboratory Animals, US National Institutes of Health (NIH Publication N˚ 85-23, revised 1996). C57BL/6 were from Jackson Laboratories. NF-κB-Luciferase transgenic mice and PPARE-green fluorescent protein transgenic mice have been previously described.

2.2. Tissue NF-κB-Luciferase activity measurement

NF-κB-Luciferase transgenic mice were anaesthetized with a single dose of intraperitoneal ketamine (125 mg/kg body weight) and xylazine (12.5 mg/kg body weight). All procedures were performed when mice no longer reacted to a toe pinch. Polyethylene glycol (PEG)-solvated OA or OA-NO2 was delivered to NF-κB-Luciferase transgenic mice by subcutaneously implanted osmotic pumps (Alzet, model 2002) at 5 mg/kg/day. After 3 days of OA or OA-NO2 delivery, mice were treated with 0.5 mg/kg LPS via tail-vein injection. After 3 h, mice were anaesthetized with intraperitoneal ketamine/xylazine and aortas harvested and homogenized in T-PEK lysis buffer at 4°C. Luciferase activity assay was performed following the manual instructions (Promega).

2.3 Haemodynamic measurements and intravital microscopy

OA-NO2 and OA were delivered to C57BL/6j mice by a single tail-vein injection of each fatty acid (0.5 μmole/kg) simultaneously with an induction of inflammation with LPS (0.5 mg/kg). Mice were anaesthetized with ketamine/xylazine and aortas harvested and homogenized in T-PEK lysis buffer at 4°C. Luciferase activity assay was performed following the manual instructions (Promega).

2.4 Mass spectrometric analysis of nitro-fatty acids in plasma

Plasma proteins (20 μL) were precipitated with 80 μL of cold (−20°C) acetonitrile in the presence of [13C13]OA-NO2. Quantification of nitrofatty acids was performed by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) using a triple quadrupole mass spectrometer in negative ion mode (API 5000, Applied Biosystems). Nitro-fatty acids were chromatographically resolved on a C18 reversed phase column using a water/acetonitrile solvent system containing 0.1% acetic acid. The following transitions were followed in multiple reaction monitoring (MRM) scan mode: 342.3/46 ([13C13]OA-NO2), 326.3/46 (OA-NO2), 328.3/46 (NO2-SA) 270.2/46 (NO2-14:1), 272.2/46 (NO2-14:0). OA-NO2 was synthesized via nitroselenation, giving equimolar 9- and 10-nitro-ocadecanoic acid regioisomers.

2.5 Cell surface immunofluorescence and FACS analysis

Mice were treated with LPS and OA or OA-NO2 as described earlier. After 3 h, monocytes from the peritoneal cavity were obtained by lavage with 1 mL PBS. Peritoneal cell populations were consistently composed of >90% monocytes as determined by Giemsa staining (Fluka). Monocytes were counted with a haemocytometer and incubated with 2 μg/mL TLR4-Alexa Fluor488 antibody (eBioscience) at 4°C for 1 h. IgG-Alexa Fluor488 was used as isotypic control and DAPI (1 μg/mL, 5 min) was added for cell death exclusion. Data acquisition was obtained using a Beckman Coulter MoFlo XDP (Beckton Dickinson) and analysed using a Beckman Coulter Summit program, version 4.3.

2.6 Total RNA preparation and RT-qPCR analysis

Total RNA was isolated from homogenized whole aortas of mice treated as above using TRIzol reagents (Invitrogen). RNA extraction and primer sequences are described in Supplementary material online, Methods and Supplementary material online, Table S1.

2.7 Lipid rafts preparation

Lipid rafts fractions were separated from RAW 264.7 macrophages grown to confluency in 100 mm dishes. The homogenates in TNE buffer (Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) containing 1% Triton X-100 were adjusted to 40% Optiprep (Sigma) in TNE lacking detergent and placed at the bottom of an ultracentrifuge tube. A 5–30% discontinuous Optiprep gradient was formed above (4 mL of 5% Optiprep/4 mL of 30% Optiprep, both in TNE lacking detergent) and centrifuged at 134 000g for 4 h in a TH-641 rotor (Sorvall). Starting from the top, 12 subfractions were collected, precipitated in 10% TCA, and the distribution of individual
proteins in the gradient was detected by western blotting. Lipid rafts were identified with an anti-Flotillin antibody (BD Biosciences).

2.8 Western blot
Cell lysates were subjected to immunoblot as previously described. Antibodies against phospho-IκBα (Ser32/36) mouse monoclonal, phospho-IκKα/β (Ser176/180) rabbit were from Cell Signaling Technology. Antibodies against TLR4, IκB, ubiquitin, TRAF6, and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All primary antibodies were used at 1:1000 dilutions.

2.9 Confocal microscopy
HUVEC (Lonza) were cultured in four-chamber coverglass (Lab-Tek) and treated with OA or OA-NO2 (2 μM) for 30 min. LPS (1 μg/mL) was added for 10 min before washing with PBS. Gangliosides enriched in lipid rafts were stained with Alexa594-labelled cholera toxin subunit-B (1 μg/mL, 1 h) (Molecular Probes). For dual-staining and colocalization of lipid rafts with the TLR4, HUVEC were further incubated with mouse anti-TLR4-Alexa Fluor 488 conjugated antibody (1 μg/mL, 1 h) (Santa Cruz). Nuclei were stained with DAPI (1 μg/mL, 10 min, Pierce). Immunofluorescence was visualized with an Olympus Fluoview 500 Laser Scanning Confocal Microscope. Images were obtained by an investigator blinded for the experimental treatments. Three independent fields were considered.

2.10 Statistics
Values are expressed as mean ± SEM. The data were analysed using ANOVA with the Newman–Keuls’ test. Values of P < 0.05 were considered statistically significant.

3. Results
3.1 Nitro-fatty acids inhibit LPS-induced aortic NF-κB activation
The physiological relevance of OA-NO2-mediated actions was tested in vivo by evaluating alterations in NF-κB reporter gene expression.

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/98/1/116/312163)
Transgenic mice carrying a firefly luciferase reporter driven by a tandem repeat of three NF-κB binding sites were used. Thioglycollate-derived peritoneal macrophages were isolated from NF-κB-luciferase transgenic mice to test the ability of OA-NO2 to inhibit LPS-stimulated NF-κB activity in vitro (Figure 1A). After 6 h treatment with OA-NO2 (2 μM), the firefly luminescence was significantly reduced in NF-κB-luciferase macrophages stimulated with LPS.

To test whether OA-NO2 is similarly effective in reducing vascular NF-κB activity in vivo, OA-NO2 or OA were delivered to NF-κB-luciferase transgenic mice via subcutaneously implanted osmotic minipumps. On the third day, mice were treated via tail-vein injection with LPS (0.5 mg/kg) for 3 h and NF-κB activity was directly measured from aortic tissue extracts. Low intravenous treatment with LPS caused a mild and transient decrease in cardiac +dP/dt and reduction of LV pressure, which is rapidly recovered within the first 10 min (Supplementary material online, Figure S1), thus averting many of the physiological effects of chronic endotoxaemia, which requires higher doses of LPS. However, it is sufficient to induce vascular NF-κB activation (Figure 1B). Systemic delivery of OA-NO2 significantly inhibited LPS-induced aortic NF-κB activation, whereas vehicle (PEG) and native OA had no effect (Figure 1B). These data demonstrate that pre-emptive delivery of OA-NO2 potently inhibits vascular NF-κB activation upon exposure to pro-inflammatory stimuli.

### 3.2 Nitro-fatty acids inhibit LPS-induced leukocyte adhesion in vivo

Next, it was evaluated whether an acute treatment with OA-NO2 exert similar in vivo anti-inflammatory effects. An inflammatory response was induced in mice by treatment with LPS, with a simultaneous single dose of either OA-NO2 or OA (0.5 μmoles/kg) co-administered in some groups. At 3 h, mice were anaesthetized and the cremaster vasculature exposed for intravital videomicroscopy to evaluate the in vivo adhesion of leukocytes to the vessel wall (Figure 1C, see accompanying Supplementary material online, Videos). As shown in Figure 1D, a single dose of OA-NO2 significantly reduced the number of leukocytes adhered to the venular endothelium. An equivalent amount of non-nitrated OA had no effect.

We have previously shown that OA-NO2 delivery via osmotic minipump implantation induced, ~20 nM steady state free nitro-fatty acid concentrations in plasma. To address whether acute intravenous administration of OA-NO2 yielded circulating levels sufficient to inhibit monocyte adhesion to the endothelium in vivo, blood samples were collected 3 h after mice were treated with LPS (0.5 mg/kg), LPS+OA or LPS+OA-NO2 (0.5 μmoles/kg each). The concentration of OA-NO2 and its major metabolites were quantified revealing plasma levels of 25 nM OA-NO2 (Figure 2). In vivo metabolism of OA-NO2 includes β-oxidation and inactivation of its electrophilic reactivity via the reduction of the nitroalkene OA-NO2 to the nitroalkane product nitro-stearic acid (NO2-SA, the 2e reduction product of OA-NO2). Plasma NO2-SA reached 55 nM at 3 h (Figure 2A). Detectable levels of the β-oxidation metabolites NO2-14:0 and NO2-14:1 were also observed (Figure 2B), consistent with previous studies on the metabolism of nitro-fatty acids. Free OA-NO2 and NO2-SA were below quantifiable levels in the LPS and LPS+OA groups (Figure 2C).
3.3 Nitro-fatty acids inhibit LPS-induced TLR4 cell surface expression and vascular adhesion in vivo

Under these acute conditions, TLR4 cell surface expression was determined by FACS analysis in monocytes isolated from the peritoneal cavity (Figure 3A). LPS-induced TLR4 cell surface recruitment was impaired after a simultaneous intravenous injection of OA-NO2, whereas control-administration of OA had no effect (Figure 3B). Moreover, aortic mRNA expression of vascular (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, and inducible NO synthase (iNOS) was significantly reduced upon OA-NO2 treatment (Figure 3C). Notably, TLR4 mRNA levels were unchanged. Collectively, these data demonstrate that an acute treatment with OA-NO2, yielding nanomolar concentrations, exert a robust anti-inflammatory response and is sufficient to inhibit vascular NF-κB activation and recruitment of the upstream TLR4 to the cell membrane. These particular responses are not attributed to transcriptional regulation events, e.g. PPARγ activation, because of the rapidity of the response kinetics and the fact that PPARγ activity was not significantly induced upon acute treatment with OA-NO2 (Supplementary material online, Figure S2).

3.4 Nitro-fatty acids suppress IKK/IκB signalling in endothelial cells and macrophages

The observed inhibition of NF-κB signalling led us to propose a direct interference of OA-NO2 with upstream signalling reactions leading to NF-κB activation. OA-NO2 dose-dependently inhibited LPS-induced phosphorylation of IκBα in RAW264.7 macrophages, whereas control treatments with OA did not alter IκB phosphorylation (Figure 4A). This was further supported by HUVEC responses, where OA-NO2 inhibited LPS-induced phosphorylation and subsequent degradation of IκBα in a time-dependent manner (Figure 4B). Moreover, OA-NO2 interfered with LPS-induced IκBα ubiquitination (Supplementary material online, Figure S3). Notably, phosphorylation of IKK was also suppressed by OA-NO2 (Figure 4C). These data reveal that OA-NO2 suppresses pro-inflammatory activation of IκB/IKK, upstream events in the NF-κB signalling pathway. Confirmatory experiments also showed that nitro-fatty acids similarly represses upstream signalling events of the NF-κB pathway in endothelial cells (Supplementary material online, Figure S4).

Figure 3 OA-NO2 inhibits LPS-induced TLR4 expression and vascular inflammation in vivo. (A) Representative histogram of the flow cytometry expression of TLR4 on peritoneal monocytes from mice treated with LPS (0.5 mg/kg), LPS+OA, or LPS+OA-NO2 (0.5 μmoles/kg) for 3 h. (B) Quantitative data are shown as mean fluorescence intensity ± SEM. P-values are indicated in the figure. n = 6. (C) Aortic mRNA expression of TLR4, VCAM-1, ICAM-1, E-selectin, iNOS. Relative levels were normalized using 18S mRNA expression as control. Data are shown as relative expression ± SEM. n = 6. *P < 0.05 vs. control, **P < 0.05 vs. OA+LPS or LPS.
3.5 Nitro-fatty acids disrupt the assembly of the TLR4 signalling complex in lipid rafts

Lipid raft microdomains serve as discrete membrane regions that coordinate initial signalling events essential for TLR4-mediated inflammatory responses.27,28 Thus, we isolated buoyant lipid-raft fractions from RAW264.7 macrophages by flotation on a discontinuous sucrose-density gradient and explored whether OA-NO2 affect LPS-induced trafficking of TLR4-associated signalling mediators to lipid rafts. In unstimulated macrophages, TLR4 and TRAF6 preferentially localized in high-density non-lipid raft fractions, whereas LPS induced a rapid recruitment of TLR4 and TRAF6 to flotillin-1-rich fractions, a protein constitutively expressed in lipid rafts28 (Figure 5A). In contrast, OA-NO2, but not OA, significantly inhibited LPS-induced migration of TLR4 and TRAF6 to the light lipid raft fractions (Figure 5A). To further evaluate colocalization of TLR4 with lipid raft microdomains, TLR4 recruitment into lipid rafts was visualized in HUVECs by confocal microscopy, using cholera toxin B as a surrogate marker of lipid rafts. LPS treatment induced TLR4 colocalization into lipid rafts, which was significantly inhibited by treatment with OA-NO2 but not OA (Figure 5B). For comparison, methyl-β-cyclodextrin (MβCD), an inhibitor of lipid raft formation by depleting membrane cholesterol was used29 (Supplementary material online, Figure S5). To corroborate these findings, we compared the LPS-induced IκBα phosphorylation in macrophages treated with OA-NO2 and MβCD. MβCD inhibited LPS-mediated IκBα phosphorylation in a dose-dependent manner, whereas phosphorylation of IκBα was significantly inhibited by OA-NO2 (Supplementary material online, Figure S5). In aggregate, these results indicate that OA-NO2 inhibits the lipid raft assembly of TLR4 signalling and adaptor molecules, upstream events of the NF-κB pathway.

Taken together, these studies provide the first evidence that OA-NO2 impairs lipid raft-dependent clustering of pro-inflammatory initial signalling in vascular cells leading to resolution of pro-inflammatory vascular activation of NF-κB.

4. Discussion

Exogenously administered nitro-fatty acids display protective anti-inflammatory actions in a variety of models of cardiovascular disease. In ApoE knockout mice treated with nitro-fatty acids, the
onset and progression of atherosclerosis and the expression of cell adhesion molecules are significantly reduced. This potent anti-inflammatory role of nitro-fatty acids on the adhesion of monocytes was also examined in vitro when applying physiological flow-mediated forces to endothelial cells. Subsequent experiments using phorbol 12-myristate 13-acetate and N-formyl-Met-Leu-Phe-activated human neutrophils and bone marrow-derived macrophages from ApoE deficient mice indicated that OA-NO2 reduced LPS-induced superoxide generation and inflammation in vitro. In most of these studies, the reduction of the inflammatory response was observed when nitro-fatty acids are pre-emptively delivered, with the relevant exception regarding the ability of OA-NO2 to form adducts with the angiotensin II receptor, significantly reducing pre-existing hypertension. Herein, we show that (i) a single dose of OA-NO2 reduces the adhesion of inflammatory cells to the vasculature and (ii) acute nanomolar concentrations of OA-NO2 are sufficient to suppress NF-κB activity in vivo in response to pro-inflammatory LPS stimulation.

NF-κB is sequestered in the cytoplasm by its binding to inhibitory proteins, e.g. IκB. In response to inflammatory cytokines, mitogens, and LPS, activation of NF-κB is mediated by a signalling cascade that results in phosphorylation of IκB by an IκB kinase (IKK) complex composed of IKKα, IKKβ, and IKKγ, the activity of which results in the release and proteasomal degradation of IκB. These phosphorylation events are highly dependent on the initial formation of specialized lipid raft microdomains in the membrane. Lipid rafts are rich in cholesterol and sphingolipids, and serve as platforms for conducting a variety of cellular functions. Lipid rafts and their subsets, caveolae, play a central role in the regulation of the cardiovascular function, inflammation, and immune responses. Lipid raft clustering contributes to endothelial dysfunction and enhances redox signalling mediated by NAD(P)H oxidases, all of which are early events in the onset of atherosclerosis. For instance, TLR4-mediated inflammatory responses significantly contribute to early atherosclerosis development. T-cell receptor-induced NF-κB activation involves lipid raft
In mice deficient in sphingomyelin synthase 2, in which lipid raft formation is impaired, NF-κB activation and its target gene expression are attenuated in macrophages. In the present study, we demonstrate that the electrophilic nitroalkene derivatives of oleic acid, OA-NO₂, preferentially impair the recruitment of the TLR4 into lipid raft compared with their non-nitrated counterparts and disrupt the assembly of adaptor proteins TRAF6/IKKβ/IκBα complex. As a consequence, vascular activation of the NF-κB upstream kinases IKKβ and IκB is impaired. Fatty acid composition plays a critical role in lipid raft formation and initiation of inflammatory signalling. Unsaturated fatty acids prevent anti-inflammatory signalling recruitment into lipid rafts. It has been proposed that saturated but not unsaturated fatty acids bind to the TLR4 receptor, promoting receptor dimerization and subsequent downstream signalling. On the other hand, unsaturated fatty acid supplementation alters the acyl composition of lipid rafts. In our experimental conditions, OA had no significant effect in the recruitment of TLR4 and adaptor proteins into lipid rafts and did not inhibit NF-κB signalling. Thus, redox-dependent modification of these fatty acids confers them with electrophilic properties advantageous to prevent the initial generation of a focal inflammatory ‘signalosome’. Alternatively, the electrophilic nature of nitro-fatty acids may directly neutralize ligands for TLR-signalling. Thus, nitro-fatty acids are able to disrupt TLR4 initial signalling and the assembly of TRAF6/IKK recruitment into lipid rafts, which may facilitate further nitroalkylation of NF-κB proteins, ultimately leading to inactivation of vascular pro-inflammatory and adhesion molecules.

In aggregate, we provide experimental evidence that an acute treatment with electrophilic OA-NO₂, yielding nanomolar plasma levels reduces vascular inflammatory responses, adhesion of immune cells to the vascular endothelium, and NF-κB activation. These in vivo observations reinforce the concept that nitro-fatty acids are potent signalling mediators that act to resolve inflammation and that the nitration or oxygenation of unsaturated fatty acids to yield electrophilic products during inflammation can transduce the actions of unsaturated fatty acids. These actions occur via both the acute responses described herein and as a consequence of the transcriptional activation of other cellular responses. Thus, synthetic homologues of this lipid class holds promise as a therapeutic strategy for treating cardiovascular disease.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

### Funding

This work was partially supported by the National Institutes of Health [R01-HL68878, R01-HL89544, R01-HL105114 to Y.E.C., R01-HL103455, R01-HL058115, R01-HL064937 to B.A.F., R01-AT006822-01 to F.J.S.]; the American Heart Association National Scientist Development Grant [10SDG4150085 to L.V., 09SDG2230270 to L.C.], and the American Heart Association National Career Development Grant [0835237N to J.Z.]. Y.E.C. is an Established Investigator of American Heart Association [084002SN].
Conflict of interest: F.J.S. and B.A.F. acknowledge financial interest in Complexa, Inc.

References


   989–1003.

   45–52.


   20450–20463.


   865–867.

   12321–12333.


   35686–35698.


   1225–1235.


   28:1519–1526.


32. Kim W, Fan YY, Barhoumi R, Smith SM, McMurray DN, Chaplin RK. n-3 polysaturated fatty acids suppress the localization and activation of signaling proteins at the immu

   17:331–367.


   243924–243950.