Peroxidized unsaturated fatty acids stimulate Toll-like receptor 4 signaling in endothelial cells

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Abstract

Aim: Although unsaturated fatty acids are assumed to be protective against inflammatory disorders that include a pathway involving Toll-like receptor 4 (TLR4) activation, they might actually be toxic because of their high susceptibility to lipid peroxidation. Here we studied the effects of peroxidized unsaturated fatty acids on the TLR4–nuclear factor (NF)-κB pathway in endothelial cells.

Main methods: Confluent cultured endothelial cells from bovine aorta were incubated for 1 h with fatty acids integrated into phosphatidylcholine vesicles. Lipopolysaccharide (LPS) or phosphatidylcholine vesicles without fatty acids were also applied as a positive control or a control for fatty acid groups, respectively. Activation of TLR4 and downstream signaling was assessed by membrane fractionation and Western blotting or immunofluorescent staining.

Key findings: In the same way as LPS, application of sufficiently peroxidized unsaturated fatty acids like oleic acid or docosahexaenoic acid, acutely caused TLR4 translocation to caveolae/raft membranes, leading to activation of NF-κB signaling in endothelial cells. In contrast, saturated fatty acids did not show such effects. Applying well-peroxidized unsaturated fatty acids, but not saturated fatty acids, acutely activates the TLR4/NF-κB pathway.

Significance: Peroxidation of unsaturated fatty acid is essential for the acute activation of TLR4 by the fatty acids that follow the same pathway as the activation by LPS. Unsaturated fatty acids have been assumed to be protective against inflammatory disorders, and drugs containing unsaturated fatty acids are now developed and provided. Our result suggests that, for inflammatory disorders involving TLR4 signaling, using unsaturated fatty acids as anti-inflammatory drugs may cause contrary effects.

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Introduction

Since 1970, when studies were conducted in an Eskimo population (Bang et al., 1971; Kromann and Green, 1980), possible benefits of intake of polyunsaturated fatty acids (PUFAs) for the prevention of atherosclerotic cardiovascular diseases have been a focus of research. In fact, a series of randomized clinical trials did show that PUFA intake reduces cardiovascular event risk, albeit modestly (Burr et al., 1989; Marchioli et al., 2002). Thus, the aim of the present study was to determine whether peroxidized unsaturated fatty acids stimulate TLR4 signaling, resulting in secretion of pro-inflammatory cytokines and chemokines (Holland et al., 2011; Kim et al., 2007; Shi et al., 2006; Suganami et al., 2007). In contrast, PUFAs reportedly suppress such TLR4-mediated inflammatory reactions (Lee et al., 2001, 2003). However, it is noteworthy that PUFAs may also have toxic effects, as they are highly susceptible to lipid peroxidation, which is implicated in the pathogenesis of various diseases (Guillen and Goicoechea, 2008; Serini et al., 2011). And indeed, there is reported experimental and clinical evidence that suggests an association of lipid peroxides from PUFAs with atherosclerosis (Esterbauer et al., 1992; Clavind et al., 1952; Jira et al., 1998; Stringer et al., 1989). Thus, the aim of the present study was to determine whether peroxidized unsaturated fatty acids stimulate TLR4 signaling.

Materials and methods

Cell culture

Fetal bovine aortic endothelial cells (ECs) were purchased from Japanese Collection of Research Bioresources (JCRB, Osaka, Japan) or were isolated from bovine fetuses, as previously described (Mutoh et al., 2008). ECs were cultured in Medium 199 (GIBCO, 0024-3205/$ – see front matter © 2013 Elsevier Inc. All rights reserved. http://dx.doi.org/10.1016/j.lfs.2013.03.019
Life technologies Japan Ltd., Tokyo, Japan) supplemented with 20% complement-depleted fetal bovine serum (FBS) (Nichirei Biosciences Inc. Tokyo, Japan) in a humidified CO2 incubator at 37 °C, were passaged before full confluence, and were used before passage 15 for all experiments. Normal cell functions (eNOS phosphorylation and nitric oxide production) of cultured ECs isolated from bovine fetuses were confirmed at passage 20, and therefore it was decided that it was reasonable to use ECs up to passage 15 in the current study.

Fig. 1. Effect of fatty acid peroxidation on TLR4/NF-κB signaling in endothelial cells. Experiments presented in a, b and c were performed simultaneously, twice (using the same vesicle preparation sets divided into halves), immediately following preparation and after aging, 5 days later. ECs were incubated with no stimulation (None), 10 ng/ml LPS, PC, PCFA (2.5 × 10⁻⁶ M as fatty acids), or UV-irradiated PC or PCFA (UV-PC, UV-PCFA), respectively for 1 h. (a) Distribution of TLR4 in plasma membranes determined by sucrose ultracentrifugation. The fractions are along the lines of sucrose gradient that start from lowest density (fraction No. 1; 5% sucrose) to highest density (No. 9; 42.5% sucrose). Caveolae/raft membranes, detected by Flot-1 and Cav-1, found in fractions 3 and 4. TLR4 blots showed ECs incubated with freshly prepared (A) or 5-day-old (B) vesicle samples. (b) MDA concentration in vesicles used in a and c analyzed by the TBARS method. (c) Expression of IκB-α in EC whole cell lysates by Western blotting in duplicate. The same vesicles were used in both panels, with the difference being the freshness (indicating the level of peroxidation of fatty acids), as showed in a and b.
Fatty acid preparation

In cell cultures, fatty acids integrated into phosphatidylcholine (PC) vesicles were used instead of bovine serum albumin (BSA)-conjugated fatty acids because of a concern about contamination with lipopolysaccharide (LPS) in BSA and resultant non-specific BSA effects on EC function, including increased activity of endothelial nitric oxide synthase (eNOS) (Maniatis et al., 2006). Hydrogenated phosphatidylcholine (PC), chloroform, palmitic acid (PA), and oleic acid (OA) were purchased from Wako Pure Chemicals Industries, Ltd., Osaka, Japan. Docosahexaenoic acid (DHA) was purchased from Cayman Chemical Company (MI, USA). Fatty acids were integrated into PC vesicles using a protocol that was developed by reference to previously published reports (Anel et al., 1993; Huang and Thompson, 1974; Nojima, 1988), as follows: 50 mg PC was dissolved in 1 ml chloroform in a 50-ml round-bottom recovery flask. To prepare vesicles containing fatty acids, stock solutions in chloroform were added to the flask as follows: 200 μl of 1 × 10^{-1} M PA (to yield PC–PA vesicles; because of its relative insolubility, twice as much PA as other fatty acid was loaded) or 100 μl of 1 × 10^{-1} M OA or DHA (to yield PC–OA and PC–DHA vesicles). In addition, to prepare vesicles containing a 1:1 molar ratio mixture of PA and OA (to yield PC–FA vesicles), 100 μl of each stock solution was placed in the flask. The flask was plugged using a silicone stopper having connections to an N2 gas supply and a vacuum pump. Chloroform was evaporated by intermittent addition of N2 gas. When visible liquid chloroform had almost disappeared, the flask was maintained under vacuum for a few hours until completely dry. The resultant thin layer on the inner wall of this flask was reconstituted in Hank’s balanced salt solution (HBSS, GIBCO) at 70 °C and used for vesicle formation by vigorous stirring with a teaspoon of glass beads in an N2 atmosphere. The flask was placed in a sonication bath for 1 min and the vesicle slurry was centrifuged at 10,500 × g for 60 min at 20 °C. Supernatants containing the vesicles were passed through a 0.45 mm cellulose acetate filter (Sartorius Stedim Biotech, Goettingen, Germany) and were then stored in sealed glass tubes filled with N2 gas, and were protected from light.

Concentrations of fatty acids in the vesicles were determined using LabAssay™ NEFA (Wako Pure Chemical Industries, Ltd.). Average fatty acid concentrations (n = 4–6) in the resultant vesicles were 0.004 mM (undetected) in PC, 0.043 mM in PC–PA, 0.145 mM in PC–OA, 0.047 mM in PC–DHA and 0.082 mM in PC–FA. Malondialdehyde (MDA) in the vesicles was measured by the TBARS assay kit (Cayman Chemical Company).

Western blotting

Confluent ECs in 3.5 cm dishes were washed and culture medium was replaced with HBSS, cells were incubated for 1 h, and then LPS, PC vesicles only, or PC vesicles containing fatty acid were added to the medium and incubated for 1 h. SDS-PAGE and Western blotting were performed as previously described (Mutoh et al., 2008). Immunoblotting of the membranes was done by incubation with IκB-α antibody or NF-κB p65 antibody (1:2000) (Cell Signaling Technology Japan, K.K., Tokyo, Japan) overnight at 4 °C. Immunoreactive proteins were detected by HRP-conjugated secondary antibodies (1:20,000) (GE Healthcare Japan, Tokyo, Japan) and chemiluminescent reagent (ImmunoStar LD, Wako Pure Chemical Industries, Ltd.) by following the manufacturer’s instructions. Detection of beta-actin was performed by incubating the membranes with HRP-conjugated anti-beta-actin (1:90,000) (Abcam, Cambridge, UK) for 20 min at room temperature followed by carrying out the chemiluminescent reaction as described above.

TLR4 activation assay by membrane fractionation

Isolation of detergent-resistant membrane fractions was performed as previously described (Sonnino and Prinetti, 2008). Briefly, ECs were cultured in 10 cm dishes. After treatment with LPS or vesicles as described above, ECs were washed and scrapped off the plates with ice-cold PBS containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich Japan Co., LLC, Tokyo, Japan) and then centrifuged.

The assay (≤0.005 EU/ml), whereas 2% BSA (RIA Grade, Sigma Chemical Co., MO, US) HBSS (GIBCO) solution contained 0.57 EU/ml.
ECs pellets were suspended in lysis buffer containing 0.5% Triton X-100 (ICN Biomedicals, Inc., Ohio, USA), 10 mM Tris–HCl (Invitrogen), 150 mM NaCl (Wako), 5 mM EDTA (GIBCO) and protease and phosphatase inhibitors, and then left on ice for 1 h. ECs were disrupted by passing through a 27 G needle 6 times and then centrifuged at 1300 × g for 5 min at 4 °C to obtain the post nuclear supernatant (PNS). Each 1 ml PNS was mixed with 1 ml 85% sucrose in a 13PA tube (Hitachi Koki, Co., Ltd., Tokyo, Japan) and then a 7 ml 30%–5% continuous sucrose gradient was layered on the PNS samples (now in a 42.5% sucrose solution) using a gradient maker (Sanplatec Corporation, Osaka, Japan). The tubes were ultracentrifuged in a CP56G II (Hitachi Koki) equipped with a swing rotor (P40 S) at 200,000 × g for 18 h. The gradient was separated into 1 ml fractions from the top to obtain fraction Nos.1 (the lowest density fraction in the sucrose gradient) to 9 (the highest density fraction). Proteins in the fractions were purified by trichloroacetic acid (TCA) precipitation and then subjected to SDS-PAGE and Western blotting, as described above. Membranes were immunoblotted with polyclonal (1:1000) (Abnova Corporation, Taipei, Taiwan) or monoclonal (1:1000) (BD Biosciences, CA, USA) anti-TLR4 antibody, or anti-caveolin-1 or anti-flotillin-1 antibody (1:5000) (BD Biosciences).

**Immunofluorescent staining for phospho-p65**

ECs were cultured on 15 mm in diameter micro coverslips. After stimulation with LPS (10 ng/ml, as the positive control) or vesicles, as described in the Western blotting section, ECs were washed with saline and fixed with 1% TCA for 15 min. TCA was removed by washing with TBST (an aqueous solution containing 137 mM NaCl, 2.7 mM KCl, 24.7 mM Tris and 0.1% Tween 20, pH 7.4), and then ice cold 0.1% Triton X-100 in TBST was applied to the coverslips on ice for 5 min, followed by further TBST washing. After blocking with 5% BSA in TBST for 30 min, phospho-Ser536 p65 rabbit monoclonal antibody (1:200) (Cell Signaling Technology) was applied overnight at 4 °C. The coverslips were washed 3 times with 0.5% BSA in TBST, followed by incubation for 1 h at room temperature with the secondary antibody,
Alexa Fluor 488-conjugated anti-rabbit IgG (1:100) (Invitrogen) and 1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI, Wako) for nuclear staining. The coverslips were washed 3 times with TBST, rinsed with pure water, and then mounted on glass slides using PermaFluor aqueous mounting medium (Thermo Fisher Scientific Inc., MA, USA). Fluorescent images of Alexa Fluor 488 and DAPI labeling were obtained with a Leica confocal laser scanning microscope system (Leica Microsystems, Wetzlar, Germany). X–y fluorescent images were stacked along the z-axis from top to bottom in 5 planes and then merged into 1 image.

Statistical analysis

The Fisher’s PLSD test was applied to determine significant differences between pairs after performing analysis of variance (ANOVA). Differences having $p < 0.05$ were considered significant.

Results

Fig. 1 summarizes results of an exploratory study to find out important factors in TLR4 activation by fatty acids. Fig. 1a, b, and c shows results of TLR4 translocation assay; MDA concentration assay, and IκB-α degradation, respectively. These experiments were performed simultaneously using half of one set of vesicles (PC, PCFA, UV-PC, UV-PCFA) just after being prepared as a “fresh” set. When a period of 5 days had elapsed after the vesicles’ preparation, the same experiments were repeated using the other half as an “aged” set.

The results of Fig. 1a clarify conditions determining whether fatty acids act in a manner similar to LPS to activate TLR4 in ECs. The activation of TLR4 was assessed by translocation of TLR4 from the non-caveolae/raft to the caveolae/raft domain, as reported by Triantaflou et al. (2002) and Walton et al. (2003). Fig. 1a shows TLR4 localization in detergent-resistant membrane fractions (low numbers indicate low-density membrane fractions). Fractions 3 and 4, which contained abundant flotillin-1 (Flot-1) and caveolin-1 (Cav-1), are caveolae/raft membrane fractions. In unstimulated ECs, TLR4 was mainly distributed in non-caveolae/raft membranes (Fig. 1a, top left). Incubation of ECs with 10 ng/ml LPS for 1 h led to accumulation of TLR4 in caveolae/raft membranes (Fig. 1a, top right), consistent with previous reports. In a similar fashion, fatty acids were applied to EC for 1 h. A freshly prepared blend of PA and OA (PCFA) ($2.5 \times 10^{-6}$ M as fatty acid) did not activate TLR4 (Fig. 1a, middle right). However, the PCFA peroxidized either by aging for 5 days (aged
PCFA (Fig. 1a, middle right) or by UV irradiation for 10 min (UV-PCFA) (Fig. 1a, lower right) translocated TLR4 to the caveolae/raft domain. Identically treated control PC vesicles had no effects (Fig. 1a, middle and lower left). In Fig. 1 results of Flot-1 and Cav-1 blots are shown only for the sample to which “fresh” vesicles were applied. Application of “aged” vesicles resulted in the same distribution. To confirm lipid peroxidation after aging or UV radiation, concentrations of MDA in PCFA were measured by the TBARS method, and were found to be elevated significantly, compared to control, after aging for 5 days (Fig. 1b).

The next experiments were done to assess whether translocation of TLR4 by peroxidized fatty acids could lead to alterations downstream of TLR signaling. Fig. 1c shows effects of PCFA on expression of IkB-α in ECs as assessed by Western blotting. PCFA enhanced degradation of IkB-α after UV-radiation and aging (Fig. 1c), consistent with the TLR4 translocation response shown in Fig. 1a, suggesting that fatty acids have effects similar to those of LPS on TLR4 activation, but only when peroxidized.

Titration of peroxidized PCFA on TLR4 translocation is shown in Fig. 2a. TLR4 significantly translocated to caveolae/raft membranes in PCFA concentrations greater than $0.5 \times 10^{-6}$ M. Dose dependency was also observed in the degradation of IkB-α, as shown in Fig. 2b. PCFA at the dose of $1 \times 10^{-6}$ M which caused apparent translocation of TLR4 and enhanced degradation of IkB-α, phosphorylated serine 536 of p65 of NF-κB as shown by immunofluorescent staining (Fig. 2c). The MDA concentration in the $1 \times 10^{-6}$ M peroxidized PCFA used in these experiments was estimated to be $0.030 (±0.009) \times 10^{-6}$ M.

The next experiment was designed to ascertain differential effects of SFA and unsaturated fatty acids, including PUFA, on the TLR4/NF-κB

### Table 1

<table>
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<th>PC</th>
<th>PC-PA</th>
<th>PC-OA</th>
<th>PC-DHA</th>
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<tr>
<td>MDA concentration (×10^{-6} M) in 1×10^{-6} M of each fatty acids</td>
<td>Fresh</td>
<td>N.D.</td>
<td>0.020 (0.018)</td>
<td>0.067 (0.040)</td>
</tr>
<tr>
<td></td>
<td>Aged</td>
<td>N.D.</td>
<td>0.036 (0.015)</td>
<td>0.191 (0.031)</td>
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MDA concentrations in 1×10^{-6} M of each freshly prepared or aged (5 days old to 3 weeks) fatty acid as determined by the TBARS method. Mean (SD). (n = 3). N.D.: not detected.

Fig. 3. Fatty acids with unsaturated bond(s) activate TLR4/NF-κB signaling. ECs incubated for 1 h with no stimulation (None), 10 ng/ml LPS, PC, or PC containing a single fatty acid, as indicated. Aged vesicles used as described in Table 1 legend (i.e., PC-OA and PC-DHA are peroxidized). (a) TLR4 activation by aged fatty acid samples. Percentage of TLR4 in caveolae/raft membrane (fractions 3 and 4) relative to TLR4 level in all fractions. *p < 0.05 vs. PC. n = 4–7. (b) Western blotting to analyze phosphorylation of p65 and degradation of IkB-α. Representative blots. Mean (SD). n = 4. *p < 0.05, †p < 0.001 vs. control or PC.
pathway. As shown in Table 1, the MDA concentration was significantly elevated in aged OA or DHA, but not in aged PC or PA. Using these aged samples, the following experiments were performed. Incubation of ECs with PC–OA or PC–DHA for 1 h, caused translocation of TLR4, whereas PC–PA did not (Fig. 3a). PC–DHA significantly enhanced phosphorylation of p65 and degradation of IκB-α (Fig. 3b). However, PC–OA had no apparent effect on phosphorylation of p65 and only a modest effect on degradation of IκB-α. PC–PA did not have any effects on downstream components of TLR4.

Discussion

The present study clearly showed that peroxidized fatty acids having unsaturated bond(s), but not SFAs, dose-dependently translocate TLR4 to the caveolae/raft domain with downstream signal activation, as evidenced by enhancement of IκB-α degradation and phosphorylation of serine 536 of NF-κB p65. Triantafillou and colleagues showed that receptor molecules implicated in LPS- or bacteria-induced cellular events, including TLR4, accumulate in lipid rafts after stimulation with LPS or bacteria, and that disruption of the rafts inhibits TLR4 signaling (Triantafillou et al., 2002). TLR4 accumulation in the lipid microdomain appears to be a key event to initiate LPS- or bacteria-induced cell activation; such accumulation may be more direct evidence of activated TLR4 than alterations of downstream signals. To the best of our knowledge, this is the first report of fatty acids causing translocation of TLR4 as direct evidence for activated TLR4 than alterations of downstream signals. To the best of our knowledge, this is the first report of fatty acids causing translocation of TLR4 in the lipid microdomain appears to be a key event to initiate LPS- or bacteria-induced cell activation; such accumulation may be more direct evidence of activated TLR4 than alterations of downstream signals. To the best of our knowledge, this is the first report of fatty acids causing translocation of TLR4 as direct evidence for activated TLR4 than alterations of downstream signals. To the best of our knowledge, this is the first report of fatty acids causing translocation of TLR4 than alterations of downstream signals.

Activation through the non-TLR pathway cannot be experimentally excluded, because there is no TLR-specific antagonist. However, the dose–effect relationship of peroxidized fatty acids with %TLR4 in the caveolae/raft membranes, IκB-α degradation, and phosphorylation of p65 is consistent with our interpretation of the present results.

It should be noted that translocation of TLR4 occurred after a relatively short incubation (1 h) of cells with a relatively low concentration (0.5–2.5 × 10^{-6} M) of fatty acids. Given that LPS rapidly stimulates TLR4 at 10 ng/ml (within a few minutes) (Hambleton et al., 1996), these findings support our hypothesis. On the other hand, previous studies demonstrated that much longer incubations with SFAs (for 3 to 16 h) at much higher concentrations (ranging from 1 × 10^{-4} to 1 × 10^{-3} M) were required to activate the TLR4/NF-κB pathway in various tissues and cells (Holland et al., 2011; Kim et al., 2007; Shi et al., 2006; Suganami et al., 2007). Lack of effects of SFA (PA) on TLR4 signaling in the current experiments, therefore, may be explained by the possibility that there was less exposure of cells to the SFA than there was in previous studies. This might also suggest that SFAs, unlike LPS, enhances TLR4-related signaling through chronic displacement of fatty acids in membrane phospholipids rather than by acting as an agonist to acutely stimulate TLR4.

Although TLRs serve in the innate immune system as sensors of non-self pathogenic molecules, more than 20 molecules, including SFAs, have been proposed as endogenous ligands. However, whether these molecules can act as ligands for TLR4 has been questioned recently because of a concern that stimulation of TLRs by such candidate ligands might actually be due to minor LPS contamination in the albumin to which they were complexed (Erridge and Samani, 2009). In fact, Erridge and Samani showed that SFA–BSA complexes, but not SFA alone, activated TLR-dependent signaling (Erridge, 2010). In the present experiments, the PC vesicle method was adopted to exclude the likelihood of contamination by LPS in BSA. This could explain the lack of effect of PA on TLR4 signaling in this study.
Elevated free fatty acid levels are implicated in the development of metabolic and atherosclerotic diseases. While SFAs seem to serve as “the bad guys,” facilitating inflammatory reactions through TLR4 activation, causing insulin resistance and atherosclerosis, PUFAs serve as “the good guys,” protecting against adverse effects of SFAs. Such central dogma has been fairly well established by substantial experimental and clinical evidence (Bang et al., 1971; Burr et al., 1989; Holland et al., 2011; Kim et al., 2007; Krommann and Green, 1980; Marchioli et al., 2002; Shi et al., 2006; Suganami et al., 2007; Yokoyama et al., 2007). However, it is of note that PUFAs are highly susceptible to peroxidation, which has also been implicated in various diseases. Lipid peroxides were first detected in atherosclerotic human aorta tissue more than 50 years ago (Glavind et al., 1952). Several groups have since reported elevated plasma lipid peroxides in patients with atherosclerosis, dyslipidemia and diabetes (Dormandy et al., 1973; Sato et al., 1979; Stringer et al., 1989; Uysal et al., 1986). More recently, Walter et al. demonstrated that circulating lipid hydroperoxides have a predictive value for adverse cardiovascular outcome in patients with stable angina (Walter et al., 2008). TLR4 activation by peroxidized fatty acids shown in the present study, as well as possible effects mediated through the non-TLR pathway, may be related to such links between lipid peroxidation and cardiovascular diseases. In fact, TLR4 was expressed in macrophages and endothelial cells of atherosclerotic lesion (Edelfeldt et al., 2002). It can be assumed that secretion of cytokines and chemokines following the activation of TLR4 receptors in such cells contribute to the development of atherosclerosis and plaque activation. Considered together with previous reports, the results presented here suggest that PUFAs are not necessarily protective against atherosclerosis. Several clinical trials showed modest effects of n–3 fatty acids on cardiovascular events. A recent meta-analysis, however, of carefully designed clinical trials of n–3 fatty acids has not demonstrated any benefit for cardiovascular outcome (Kwak et al., 2012). A very recent double-blinded, placebo-controlled study showed that daily supplementation with 1 g of n–3 fatty acids did not reduce the rate of cardiovascular events in patients with dysglycemia (The ORIGIN Trial Investigators, 2012). The present results partly explain such lack of efficacy of n–3 fatty acids for cardiovascular event prevention. Interestingly, it was recently reported that omega-3 fatty acids, such as DHA, were rather harmful in patients with acute lung injury (Rice et al., 2011), in which TLR4 signaling is known to be involved (Xiang and Fan, 2010).

In this work there are some limitations to be noted. First of all, our experiments were performed on cultured cells. Strictly speaking, our experimental results do not tell anything definite about the effects of PUFAs on the vascular function of human whole body. Another limitation is that we have studied only acute effect of peroxidized unsaturated fatty acids on TLR4 signaling. Chronic effects also need to be studied, if one wants to elucidate the development of atherosclerosis.

In conclusion, peroxidation of fatty acids having unsaturated bond(s) is essential for inducing their acute action on TLR4, which follows the same pathway as LPS. This would suggest that lipotoxicity on vascular function requires fatty acids having unsaturated bond(s) and an oxidative microenvironment. Additionally, use of PUFAs as an anti-inflammatory agent may cause opposite effects in patients with inflammatory disorders involving TLR4 signaling.

Conflict of interest statement

The authors report no conflicts of interest.

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