Anti-inflammatory effects of EPA and DHA are dependent upon time and dose-response elements associated with LPS stimulation in THP-1-derived macrophages

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Abstract

The long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) of fish oil, eicosapentanoic (EPA) and docosahexanoic (DHA) acids are considered cardioprotective. Inflammation elicited by macrophages is increasingly recognised in the aetiology of metabolic syndrome. This study investigated the differential anti-inflammatory potential of EPA and DHA through cytokine production and nuclear factor (NF)-κB signalling in a human macrophage model. We investigated the dependency of LC n-3 PUFA immune-modulation on concentration and duration of lipopolysaccharide (LPS) activation. Interleukin (IL)-1β, IL-6 and tumor necrosis factor-α secretion from EPA, DHA and control cells were differentially limited by LPS concentration. In all cases, there was no benefit in activating cells with >0.1 μg/ml LPS. LC n-3 PUFA decreased proinflammatory cytokines production, an effect modulated by LPS concentration. Expression of the transcription factor NF-κB p65 was significantly reduced in the nucleus and retained in the cytoplasm of EPA- and/or DHA-treated macrophages during 5-h activation with 0.1 μg/ml LPS. Nuclear binding of p65 was significantly reduced in EPA- and DHA-treated cells at 2-h LPS activation. Over the time course, expression of nuclear IκBα was significantly reduced, cytoplasmic NF-κB p50 significantly increased and cytoplasmic cleaving enzyme IκB inhibitor complex significantly reduced in LC n-3 PUFA-treated cells. EPA and DHA down-regulated the production of proinflammatory cytokines associated with the aetiology of metabolic syndrome, NF-κB transcriptional activity and upstream cytoplasmic signalling events. Immune responses are dynamic, and the present study suggests a nutrient sensitive window of LPS activation at which EPA and DHA are strongly anti-inflammatory.
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1. Introduction

Fish oils, rich in long chain *n*-3 polyunsaturated fatty acids (LC n-3 PUFA) eicosapentanoic (EPA) and docosahexanoic (DHA) acids show promise in the treatment of conditions associated with the metabolic syndrome. Fish oils have a variety of potential cardioprotective effects, including reduction of plasma triacylglycerol concentrations, regulation of blood pressure, amelioration of cardiac arrythmia and reduction in incidence of sudden cardiac death [1–7]. LC n-3 PUFA supplementation improved insulin sensitivity in normal and insulin-resistant rodents [8–11]. High concentrations of LC n-3 PUFA in plasma and muscle membranes are inversely associated with insulin resistance and protect against the metabolic syndrome in obese human subjects [12,13]. However, the effects of LC n-3 PUFA supplementation on insulin sensitivity in man are controversial and require further clarification [14]. The specific molecular pathways through which EPA and DHA may exert beneficial health effects, in isolation from fish oil blends and outside the context of PUFA ratios, needs to be elucidated.

Chronic low-grade inflammation is a characteristic feature and potential aetiological factor of the metabolic syndrome. Obesity is associated with progressive infiltration of macrophages into adipose tissue [15]. In obesity, macrophages can constitute up to 40% of the cell population of adipose tissue [16] and may account for the large repertoire of inflammatory genes expressed by adipose tissue [17–19]. The activated macrophage and its proinflammatory products, including the cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, are thought to be critical to the induction of insulin resistance and the development of Type 2 diabetes mellitus (T2DM) [16]. In obesity, elevated adipose tissue TNF-α is strongly correlated with insulin resistance, the key marker of T2DM [20]. TNF-α alters the phosphorylation status of the insulin receptor and insulin receptor substrate-1, directly impeding insulin action and glucose uptake [14]. It is estimated that one third of circulating IL-6 comes from adipose tissue and that IL-6 inhibits insulin transduction [21]. Recent studies have shown that IL-1β also promotes insulin resistance [22,23].

Transcription of IL-1β, IL-6 and TNFα is partially regulated by nuclear factor (NF)-κB and associated proteins. The most intensively studied NF-κB dimer is p50/p65. p65 is capable of directly inducing transcription of target genes [24,25], and p65-deficient mice lose the ability to induce NF-κB regulated genes, including TNFα [26]. Over-expression of p50 blocks the transcription of TNF-α in LPS-stimulated macrophages [27]. A key point in the activation of the NF-κB signalling pathway involves the natural inhibitor of κB (IκB) which binds to NF-κB dimers, masks their nuclear localisation sequences and retains the entire complex in the cytoplasm [28,29]. Cytoplasmic dissociation of NF-κB from IκB is regulated by the activation of IκB inhibitor complex

(IKK). Activated IKK phosphorylates IκBα on serines 32 and 36 [29–31], which frees the NF-κB dimers to translocate to the nucleus. NF-κB then interacts with κB elements in the promoter region of several inflammatory genes to activate their transcription [32].

The beneficial effects of fish oils in conditions associated with the metabolic syndrome may be due, in part, to anti-inflammatory properties of the constituent LC n-3 PUFA. LC n-3 PUFA suppressed TNF-α expression in in vitro murine RAW 264.7 macrophages [33,34]. EPA and DHA reduced TNF-α and IL-1β in LPS-activated human monocytes and murine macrophages [35–37]. LC n-3 PUFA affect several components of the NF-κB transcriptional complex [34–36,38,39]. Nevertheless, the use of fish oil and LC n-3 PUFA blends has limited our understanding of the individual effects of EPA and DHA. Preliminary work from our research group suggested that DHA may be more potent than EPA in reducing TNF-α, IL-1β and IL-6 expression in LPS-activated THP-1-derived macrophages [39]. As nutrients, the anti-inflammatory effects of EPA and DHA are likely to be subtle and may depend upon the strength of the inflammatory stimulus. Therefore, the aim of this study was to investigate how EPA and DHA modulated the dose- and time-dependent effects of LPS stimulation on macrophage cytokine response. Also, the individual effects of EPA and DHA on key regulatory elements of the NF-κB system, including p50 and IKK, required investigation.

2. Methods

2.1. Reagents and cell lines

The monocytic THP-1 cell line was purchased from the European Collection of Animal Cell Cultures (ECACC No. 88081201, Salisbury, UK). Reagents, unless stated
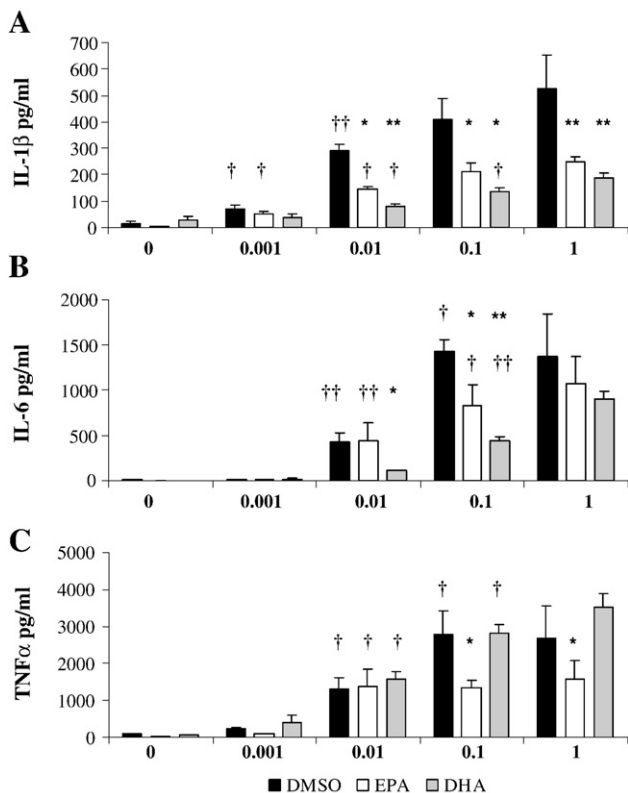

Fig. 1. Secretion of IL-1β (A), IL-6 (B) and TNFα (C) from macrophages activated by a range of LPS concentrations (0.001–1 μg/ml). Results represent the mean+S.E.M. of 5–6 independent experiments. *P≤.05; **P≤.001 relative to DMSO within each LPS concentration; †P≤.05; ††P≤.001 between sequential LPS concentrations within a treatment group.
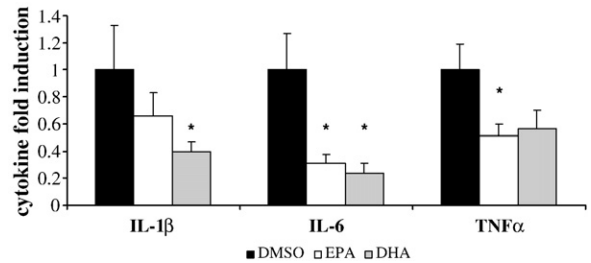

Fig. 2. Transcription of IL-1β, IL-6 and TNFα in macrophages activated by 0.1 μg/ml LPS. Results represent mean+S.E.M. of 4–6 independent experiments and normalised to DMSO. *P≤.05.

otherwise, were purchased from Sigma (Dorset, UK). Working solutions of bacterial LPS and phorbol-12-myristate-13-acetate (PMA) were prepared in RPMI 1640 medium (Gibco, Grand Island, New York). Working solutions of EPA and DHA were prepared in dimethyl sulfoxide (DMSO).

2.2. Experimental conditions

THP-1 cells cultured in RPMI supplemented with L-glutamine (2 mmol/L), streptomycin (100 mg/ml), penicillin (100 mg/ml) and 10% heat-inactivated foetal calf serum (Invitrogen, Dublin, Ireland) were differentiated into macrophages by exposure to 0.1 μg/ml PMA for up to 72 h. Macrophages were washed twice with pre-warmed HBSS and incubated in free-serum culture medium prior to treatment with 25 mM EPA, DHA or equivalent volume DMSO for 48 h. After this incubation with EPA, DHA or DMSO, macrophages were activated with LPS.

2.3. Dose-response effects of LPS activation on THP-1-derived macrophages

THP-1-derived ($1×10^6$) macrophages pretreated with 25 mM EPA, DHA or DMSO were incubated with LPS in a concentration range of 0–1 mg/ml for 24 h. Supernatants were collected and stored at −80C until quantification of IL-1β, IL-6 and TNF-α secretion, as described below.

2.4. Cytokine secretion

The concentrations of IL-1β, IL-6 and TNF-α in culture supernatants were measured by commercially available enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Oxon, UK) according to the manufacturer's instructions.

2.5. Cytokine transcription

Total RNA was isolated and reverse transcribed from $1×10^6$ THP-1-derived macrophages pretreated with 25 mM EPA, DHA or DMSO and activated with 0.1 mg/ml LPS for 6 h. Semiquantitative real-time RT-PCR for IL-1β, IL-6 and TNF-α using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control was performed, as previously described [39], using Assays-on-Demand gene expression products and the ABI prism 7700 sequence detection system (Applied Biosystems, Warrington, UK).

2.6. Western-blotting for nuclear and cytoplasmic NF-κB family proteins

Nuclear and cytoplasmic proteins were extracted from $4×10^6$ THP-1-derived macrophages pretreated with 25 mM EPA, DHA or DMSO and activated with 0.1 mg/ml LPS for 0, 0.5, 1, 2 and 5 h using a method based on that of Osborn et al. [40]. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA); 5 μg of nuclear and 25 μg of cytoplasmic protein were separated by SDS-PAGE through an 8% acrylamide gel and immunoblotted to polyvinylidene fluoride transfer membrane (Pall Corporation, Pensacola, FL, USA) as previously described [39]. After blocking, immunoblots were incubated with primary antibodies for NF-κB p65, IκBα, NF-κB p50 and IKK (Santa-Cruz Biotechnology) and peroxidase-conjugated detection antibody (polyclonal goat anti-mouse IgG). Antigen detection by chemiluminescence (Supersignal West Dura, Pierce) on high-performance film (Amersham Biosciences, Buckinghamshire, UK) was developed using AGFA CURIX 60 (AGFA-Gevaert, AG Munich, Germany). Imaging and densitometric analysis was performed using GeneSnap and GeneTools Analysis Software (Syngene, Cambridge, UK).

2.7. Immunostaining of NF-κB p65 and confocal microscopy

THP-1-derived macrophages were cultured on sterile coverslips prior to treatment with 25 mM EPA, DHA or DMSO and activation with 0.1 mg/ml LPS for 0 and 2 h. Cells were washed with phosphate-buffered saline and fixed in 4% paraformaldehyde for 30

min. Cells were blocked with 5% bovine serum albumin for 20 min to avoid nonspecific binding prior to permeabilisation in 0.1% Triton X-100 for 10 min. Cells were incubated with primary antibody for NF-κB p65 for 1 h and with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 h (Abcam, Cambridge, UK). Coverslips were washed and mounted in Ultracruz mounting medium (Santa-Cruz Biotechnology) containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Confocal images were acquired with a laser-confocal scanning microscope (Olympus) using Fluoview 1.5 software. Quantitative image analysis was performed using the Volocity 4.01 software (Improvision).

2.8. NF-κB activity assay

NF-κB p65 nuclear binding at 0.2 and 5 h of LPS activation was investigated with the TransAM NF-κB Chemi assay (Active Motif), as per manufacturer instructions. 0.5 µg of nuclear protein was incubated in a 96-well plate containing the NF-κB consensus site (5′-GGGACTTTCC-3′) for 1 h. Following incubation with primary and peroxidase-conjugated secondary antibodies, signal substrate was applied and chemiluminescence was quantified (SPECTRAFluor Plus and XFLUOR Version 3.21, TECAN, Reading, UK).

2.9. Statistical analysis

Statistical analysis was performed with DataDesk 6.0 (Data Description, Ithaca, NY) and SPSS 12.0 (SPSS, Chicago, IL, USA). The distribution of data for each variable was assessed and variables transformed to normalise the distribution of data if necessary. Multiple comparisons were performed by one-way analysis of variance (ANOVA). Individual differences were subsequently tested by Fisher's least significant difference test after demonstration of significant intergroup differences by ANOVA. A probability of $P \leq .05$ was considered statistically significant.

3. Results

3.1. Dose-response effects of LPS on THP-1-derived macrophage cytokine secretion

The LPS induced dose-response effects of EPA and DHA on THP-1 macrophage cytokine secretion are presented in Fig. 1. IL-1β secretion was increased in a LPS dose-dependent fashion in cells treated with DMSO and was significantly attenuated by EPA and DHA at 0.01–1 µg/ml LPS (Fig. 1A). IL-1β secretion was significantly attenuated by EPA wherein the effect was most evident with 0.01 µg/ml LPS, and IL-1β secretion was not further enhanced with higher LPS concentrations. DHA also attenuated IL-1β secretion up to 0.1 µg/ml LPS.

IL-6 secretion was significantly increased according to LPS dose up to a maximum with 0.1 µg/ml LPS, this response was attenuated in cells treated with DHA more so than EPA (Fig. 1B). Differential effects between EPA and DHA on IL-6 secretion were apparent at 0.01 µg/ml LPS activation, wherein IL-6 secretion was significantly lower in DHA-treated macrophages compared to the control and EPA-treated cells. TNF-α secretion was significantly increased by 0.01 µg/ml LPS in control, EPA- and DHA-treated cells (Fig. 1C). EPA-treated cells secreted significantly less TNF-α following at least 0.1 µg/ml LPS, compared to control and DHA-treated cells. DHA had no effect on TNF-α secretion. The results above demonstrated that 0.1 µg/ml LPS activated macrophages at a level that was sensitive to the anti-inflammatory effects of EPA and DHA. Therefore, this concentration of LPS was used to activate macrophages for subsequent experiments.

3.2. Differential effects of EPA and DHA on cytokine transcription

The effect of EPA and DHA on IL-1β, IL-6 and TNFα transcription in macrophages after 6 h of LPS activation is presented in Fig. 2. IL-1β transcription was significantly decreased by DHA and nonsignificantly by EPA, relative to DMSO. IL-6 transcription was significantly reduced by both EPA and DHA relative to DMSO. TNF-α transcription was significantly decreased by EPA and nonsignificantly by DHA, relative

Table 1
Cytoplasmic expression of p65, IκBα, p50 and IKK from 0 to 5 h of LPS activation. IKK was undetectable on 1 and 5 h immunoblots (ND)

	0 h			0.5 h			1 h			2 h			5 h		
	DMSO	EPA	DHA	DMSO	EPA	DHA	DMSO	EPA	DHA	DMSO	EPA	DHA	DMSO	EPA	DHA
p65	1.0 (0.03)	0.89 (0.08)	0.77 (0.15)	1.0 (0.16)	0.60 (0.27)	0.86 (0.06)	1.0 (0.01)	1.52† (0.05)	1.48† (0.01)	1.0 (0.03)	1.02 (0.03)	1.14† (0.01)	1.0 (0.17)	1.63 (0.33)	34.03† (7.01)
IκBα	1.0 (0.13)	0.63 (0.18)	0.67 (0.10)	1.0 (0.20)	0.53* (0.23)	0.51* (0.16)	1.0 (0.13)	6.22 (2.59)	8.77* (1.38)	1.0 (0.02)	1.11 (0.04)	1.40† (0.10)	1.0 (0.10)	1.30 (0.10)	1.32 (0.03)
p50	1.0 (0.02)	0.99 (0.01)	0.87 (0.08)	1.0 (0.02)	1.06 (0.01)	0.95 (0.04)	1.0 (0.02)	0.94 (0.02)	0.92* (0.02)	1.0 (0.07)	1.29* (0.03)	1.17* (0.05)	1.0 (0.05)	1.23* (0.01)	1.22* (0.01)
IKK	1.0 (0.03)	1.02 (0.03)	0.95 (0.03)	1.0 (0.11)	1.08 (0.03)	0.80 (0.08)	ND	ND	ND	1.0 (0.02)	0.81* (0.06)	0.77 (0.01)	ND	ND	ND

Results represent mean (SEM) of 3-4 immunoblot bands.
* $P \leq .05$ relative to DMSO.
† $P \leq .001$ relative to DMSO.

Table 2
Nuclear expression of p65 , IκBα and p50 from 0 to 5 h of LPS activation. Proteins were undetectable on 0.5 h and 2 h immunoblots

	0 h			1 h			5 h		
	DMSO	EPA	DHA	DMSO	EPA	DHA	DMSO	EPA	DHA
p65	1.0 (0.26)	1.56 (0.62)	2.49* (0.39)	1.0 (0.26)	1.09 (0.20)	0.36* (0.09)	1.0 (0.11)	0.97* (0.01)	0.97* (0.02)
IκBα	1.0 (0.30)	0.75 (0.24)	1.19 (0.37)	1.0 (0.03)	0.93 (0.04)	0.75* (0.03)	1.0 (0.04)	0.86* (0.03)	0.84* (0.02)
p50	1.0 (0.08)	0.90 (0.08)	0.81 (0.13)	1.0 (0.04)	0.85 (0.13)	0.90 (0.06)	1.0 (0.21)	0.61 (0.10)	0.56 (0.09)

Results represent mean (S.E.M.) of 3–4 immunoblot bands.
  * $P \leq .05$ relative to DMSO.

to DMSO. There were no significant differences in the effects of EPA and DHA on cytokine transcription.

3.3. Time course effects of EPA and DHA on NF-κB p65, p50, IκBα and IKK expression

Inflammatory responses are a dynamic reflection of the NF-κB transcriptional complex activation, which may be differentially regulated by EPA and DHA. Cytoplasmic and nuclear NF-κB p65, IκBα, p50 and IKK expression were determined within a 0–5 h time course in response to 0.1 μg/ml LPS in EPA-and DHA-treated cells. Results were normalised to expression in DMSO-treated cells (Tables 1 and 2, respectively).

p65 expression was differentially affected by fatty acid treatment. After 1 h of LPS activation, cytoplasmic p65 was significantly increased by both EPA and DHA treatment relative to DMSO. Significantly increased concentrations of p65 were maintained in the cytoplasm of DHA-treated cells at 2 and 5 h of LPS activation, but this effect was not observed in EPA-treated cells. At 5 h of LPS stimulation, cytoplasmic p65 was barely detectable in DMSO and EPA-treated cells (Table 1). In the nucleus, p65 expression was significantly increased by DHA treatment in unstimulated macrophages. At 1 h of LPS challenge, nuclear p65 levels were significantly reduced in the DHA- but not EPA-treated cells. At 5 h of LPS activation, nuclear p65 expression was significantly reduced in both EPA- and DHA-treated macrophages relative to DMSO (Table 2).

Cytoplasmic IκBα was significantly decreased in EPA- and DHA-treated macrophages relative to DMSO at 0.5 h LPS activation. At later time points, both EPA and DHA increased cytoplasmic IκBα expression relative to DMSO, significantly in the case of DHA-treated macrophages at 1 h LPS activation (Table 1). Nuclear IκBα expression was significantly decreased in DHA-treated macrophages relative to DMSO at 1 and 5 h of LPS activation. EPA significantly decreased nuclear IκBα expression only at the later time point of 5 h, relative to DMSO (Table 2).

Cytoplasmic p50 expression was significantly reduced after 1 h of LPS activation in DHA-treated cells. Later, both EPA and DHA increased cytoplasmic p50 relative to DMSO-treated cells at 2 and 5 h (Table 1). Nuclear p50 expression was not significantly affected by EPA or DHA treatment at 0, 1 and 5 h LPS activation (Table 2).

Cytoplasmic IKK expression was investigated at 0, 0.5, 1, 2 and 5 h of LPS activation, although IKK was not detectable in 1- and 5-h blots. DHA significantly reduced IKK expression relative to EPA, but not

DMSO at 0.5-h LPS challenge. At 2 h, both EPA- and DHA-treated macrophages expressed significantly less cytoplasmic IKK than DMSO-treated cells (Table 1). Representative blots are presented in Fig. 3.

3.4. Quantification of p65 by confocal microscopy and p65 nuclear binding and activation

Confocal microscopy was used to visualise the effects of EPA and DHA on nuclear and cytoplasmic p65 expression after 0 and 2 h of LPS activation (Fig. 4A). Cytoplasmic p65 expression was significantly reduced by EPA and DHA treatment compared to DMSO control prior to LPS activation. At 2 h LPS activation, cytoplasmic expression of p65 was significantly reduced by EPA pretreatment and nonsignificantly by DHA pretreatment relative to DMSO control cells. Confocal showed no significant difference in nuclear p65 expression in EPA-, DHA- and DMSO-treated cells prior to LPS stimulation. At 2 h of LPS activation, EPA- and DHA-treated macrophages expressed significantly less nuclear p65 than DMSO-treated cells. These results are semiquantified in Fig. 4B.

To characterise the functional effect of altered levels of the NF-κB transcriptional complex, we assessed nuclear binding of the NF-κB p65 subunit using the ELISA-style TransAM kit (Active Motif, Rixensart, Belgium). EPA and DHA had no effect on nuclear binding of p65 relative to DMSO treatment in non-LPS stimulated cells. At 2 h LPS activation EPA and, to a greater extent, DHA treatment significantly reduced p65 nuclear binding relative to DMSO (Fig. 5).

4. Discussion

Fish oil supplementation is thought to have beneficial health effects on elements in the pathogenesis of the metabolic syndrome. Low-grade chronic inflammation, originating from adipose tissue macrophages and mediated by cytokines, is involved in the development of insulin resistance and may be an aetiological factor in metabolic syndrome [15]. Preliminary data published by our research group suggested important differential anti-inflammatory effects between EPA and DHA [39] and the present study explored this further. Our results confirm that DHA seems to be the more potent anti-inflammatory LC n-3 PUFA. We also focused on defining a "nutrient-sensitive window" during NF-κB signalling, by manipulating the exposure of macrophages to LPS, to investigate subtleties in the anti-inflammatory potential of LC n-3 PUFA.
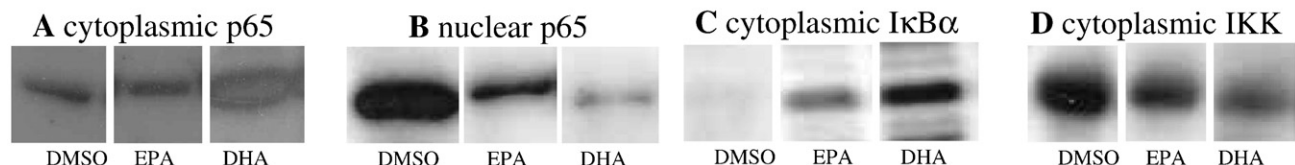

A cytoplasmic p65   **B** nuclear p65   **C** cytoplasmic IκBα   **D** cytoplasmic IKK

Fig. 3. Significant representative blots: expression of 2h cytoplasmic and 5h nuclear NF-κB p65 (A and B, respectively), 1-h cytoplasmic IκBα (C) and 2-h cytoplasmic IKK (D) in DMSO, EPA- and DHA-treated macrophages activated with 0.1 μg/ml LPS.
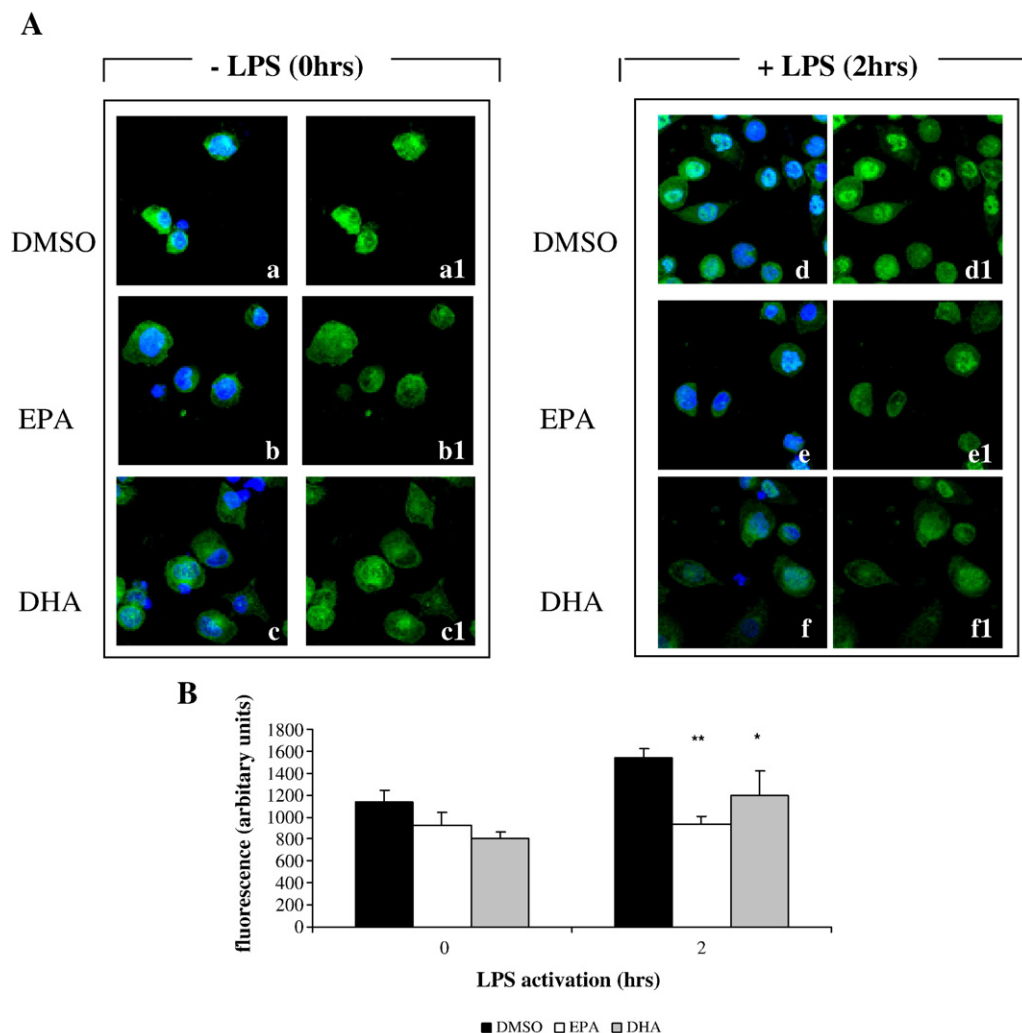
A

B

Fig. 4. A. Expression of NF-κB p65 (FITC, green) and nuclear staining (DAPI, blue) prior to (a–c) and following 2 h (d–f) of LPS activation. Macrophages were treated with DMSO (a and d), EPA (b and e) or DHA (c and f), immunostained and analysed by confocal microscopy. B. Semiquantitiation of NF-κB p65 nuclear expression during confocal microscopy at 0h and 2h of LPS activation. Results represent mean intensity of fluorescence+S.E.M. *P≤.05; **P<.001.

In this study, both EPA and DHA attenuated the inflammatory character of activated THP-1-derived macrophages over a range of LPS concentrations. As nutrients are less potent anti-inflammatory agents than pharmaceutical compounds, we focused on anti-inflammatory nutrient sensitivity at the lower levels of LPS stimulation. The intricacies of the LPS dose-response study were interesting. In brief, the anti-inflammatory effects exhibited by EPA and DHA were dependent upon the LPS dose and these effects may be missed by using less appropriate (generally higher) concentrations of stimulant. Our data suggest that DHA is more potent than EPA in reducing the secretion of IL-1β and IL-6, whereas EPA appeared to have more effect at modulating TNF-α. At the transcriptional level, these relative efficacies of EPA and DHA was also evident. Our results are supported by previous studies which have demonstrated the anti-inflammatory effects of LC n-3 PUFA on cytokine production by macrophages [33–37,39].

The nutrient-sensitive window that characterises the anti-inflammatory potential of the LC n-3 PUFA on the LPS-activated macrophage is time dependent. We studied the effects of LC n-3 PUFA on the NF-κB signalling system over 5 h of LPS activation. EPA and DHA modulated the NF-κB signalling elements p65, p50, IκBα and IKK. An interesting time course pattern was evident with respect to the cytoplasmic/

nuclear distribution of NF-κB p65 subunit and IκBα levels. Throughout the 1 to 5 h LPS activation period, there was consistently more cytoplasmic p65 expression in the LC n-3 PUFA-treated cells, and this effect was more significant in DHA-treated cells. Conversely, nuclear p65 expression was less in cells treated with DHA at 1 and EPA and
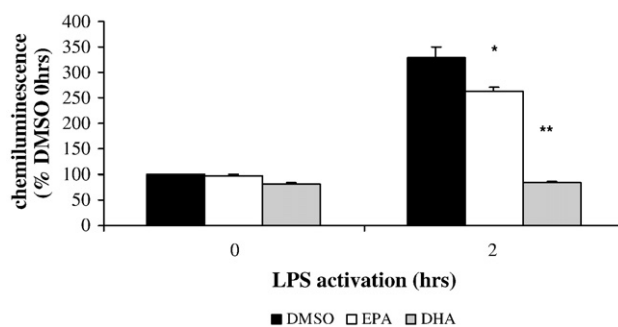

Fig. 5. Nuclear expression of NF-κB p65 binding activity at 0 and 2 h of LPS activation by consensus site (5′-GGGACTTTCC-3′) binding assay. Results represent mean chemiluminescence+S.E.M. expressed as a percentage of DMSO unstimulated control cells. *P≤.05; P=.001.

DHA at 5 h. This suggests that the localisation of NF-κB p65 subunit is differentially regulated by DHA particularly in the early LPS response and being retained within the cytoplasm. The IκBα data compliment that of p65, demonstrating an initial decline allowing p65 into the nucleus immediately after LPS stimulation, followed by greater levels in the DHA-treated cells at 1 h. Given the anti-inflammatory actions of LC n-3 PUFA the reduction in nuclear IκBα levels seem counter intuitive; however, this may reflect generalised decline of all components of the NF-κB system.

Confocal microscopy was also used to assess the localisation of NF-κB p65. This analysis showed that both LC n-3 PUFA reduced NF-κB p65 to an equivalent extent at 2 h LPS stimulation. Therefore, the apparently greater anti-inflammatory effect of DHA at 1 h LPS as assessed by Western blot seems to be a transient effect — only being relevant to the early response to LPS. Both EPA and DHA significantly decreased IKK expression in the cytoplasm at 2 h of LPS activation. IKK expression indicates enhanced potential for dissociation of p65 from the inhibitory cytoplasmic scaffold. Interestingly the ELISA-style NF-κB p65 nuclear binding assay clearly demonstrated that whilst both EPA and DHA attenuate NF-κB p65 DNA binding, this functional effect much greater for DHA. These results are supported in the literature [33,34,36,39], although tracing the response of a multitude of NF-κB elements over time is, to the best of our knowledge, unique to the present study.

Whilst our study demonstrates that EPA and DHA mediate their anti-inflammatory via the NF-κB signalling system, further research is required to characterise whether the potential differential effects of LC n-3 PUFA occur upstream of NF-κB and particular upstream of IKK. Of interest are the effects of EPA and DHA on expression of kinase and accessory molecules linking LPS, TNF and IL-1 membrane binding to IκBα phosphorylation by IKK. It has been demonstrated that [^{14}C]DHA becomes incorporated into the phosphotidylethanolamine pool found in the inner plasma membrane, a strategic position from which to alter intracellular signal transduction pathways [38]. Pretreatment of THP-1 monocytes with EPA or DHA reduced FITC-conjugated LPS binding by approximately 70% and significantly reduced CD14 up-regulation [37]. Unsaturated fatty acids have been demonstrated to inhibit toll-like receptor (TLR) 4, the LPS receptor [41]. DHA inhibited the activation of all TLRs tested in a macrophage study [42]. More recently, it has been shown that cytosolic phospholipase A$_2$, an enzyme involved in the production of reactive oxygen species from arachidonic acid, may be activated through TLR9 in macrophages [43], highlighting the interaction between fatty acids, inflammation and pattern recognition receptors. In conclusion, our data confirms the anti-inflammatory potential of the LC n-3 PUFA in activated THP-1-derived macrophages and suggests that, in terms of NF-κB signalling, DHA may be the more potent anti-inflammatory LC n-3 PUFA. DHA appeared to induce an earlier and more potent reduction of nuclear NF-κB p65 levels, a more sustained maintenance of p65 in the cytoplasm, and a stronger inhibition of p65 nuclear binding, than EPA. Both EPA and DHA reduced cytoplasmic IKK expression, suggesting that the LC n-3 PUFA may moderate NF-κB signalling closer to the cell surface. Further work is underway to determine whether LC n-3 PUFA modified macrophages can alter the expression of molecular markers of insulin resistance in adipocytes — to further characterise the relevance of LC n-3 PUFA in inflammation, adipose tissue biology and insulin resistance.

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