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The ethanolamide metabolite of DHA, docosahexaenoylethanolamine, shows immunomodulating effects in mouse peritoneal and RAW264.7 macrophages: evidence for a new link between fish oil and inflammation

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Abstract

Several mechanisms have been proposed for the positive health effects associated with dietary consumption of long-chain *n*-3 PUFA (*n*-3 LC-PUFA) including DHA (22 : 6*n*-3) and EPA (20 : 5*n*-3). After dietary intake, LC-PUFA are incorporated into membranes and can be converted to their corresponding *N*-acylethanolamines (NAE). However, little is known on the biological role of these metabolites. In the present study, we tested a series of unsaturated NAE on the lipopolysaccharide (LPS)-induced NO production in RAW264.7 macrophages. Among the compounds tested, docosahexaenoylethanolamine (DHEA), the ethanolamide of DHA, was found to be the most potent inhibitor, inducing a dose-dependent inhibition of NO release. Immune-modulating properties of DHEA were further studied in the same cell line, demonstrating that DHEA significantly suppressed the production of monocyte chemotactic protein-1 (MCP-1), a cytokine playing a pivotal role in chronic inflammation. In LPS-stimulated mouse peritoneal macrophages, DHEA also reduced MCP-1 and NO production. Furthermore, inhibition was also found to take place at a transcriptional level, as gene expression of MCP-1 and inducible NO synthase was inhibited by DHEA. To summarise, in the present study, we showed that DHEA, a DHA-derived NAE metabolite, modulates inflammation by reducing MCP-1 and NO production and expression. These results provide new leads in molecular mechanisms by which DHA can modulate inflammatory processes.

Key words: DHA: Docosahexaenoylethanolamine: Inflammation: Nitric oxide: Monocyte chemotactic protein-1: Macrophages

Fatty amides formed out of long-chain fatty acids and amino acids or ethanolamine widely occurr in nature. Increasing evidence suggests that many of these compounds are important signalling molecules in plants and animals, mediating a wide range of biological effects[1–3]. The most studied fatty amide so far is anandamide (arachidonoylethanolamine, AEA), which is one of the prototypical endogenous mediators of the endocannabinoid system[4,5]. Other fatty amides including *N*-palmitoylethanolamine and *N*-oleoylethanolamine have attracted great attention as lipid mediators involved in the anti-inflammatory processes[6,7] and in the regulation of food intake[8,9]. Also, *N*-stearoylethanolamine has been reported to have both anorexic[10] and anti-inflammatory properties[11]. Based on the ubiquitous presence of enzyme systems that are able to synthesise amides, the hypothesis has been raised that any available long-chain fatty acid can be amidated with any amino acid or related compound[5]. Indeed, the number of amides detected in tissues is rapidly increasing[5,12]. Probably several of these 'novel' fatty amides might possess bioactivity.

For several fatty amides, it has been demonstrated that they can be rapidly synthesised from their fatty acid precursors into membranes, released on demand and quickly broken down[5,13]. Therefore, the local availability of a specific type of fatty acid precursor may determine product formation and hence bioactivity. This suggests a link with dietary intake, since it is well known that incorporation of fatty acids into membranes can be modulated by their proportional abundance in the diet. From a dietary perspective, *n*-3 long-chain PUFA (*n*-3 LC-PUFA) such as DHA (22 : 6*n*-3) and EPA (20 : 5*n*-3) are of great interest because of their potential

Abbreviations: AEA, arachidonoylethanolamine; BCA, bicinchoninic acid; DEA, docosatetraenoylethanolamine; DHEA, docosahexaenoylethanolamine; EPEA, eicosapentaenoylethanolamine; iNOS, inducible NO synthase; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; *n*-3 LC-PUFA, *n*-3 long-chain PUFA; NAE, *N*-acylethanolamine; RPMI-1640, Roswell Park Memorial Institute 1640; ZTT, tetrazolium salt.

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health-related benefits including a reduction in inflammatory processes and risks for CVD, type 2 diabetes and, possibly, certain neurological disorders[14–18]. It is well known that these compounds present in high concentrations in fatty fish are incorporated into membranes[15] at the expense of that of arachidonic acid and other fatty acids. Although many mechanisms have been proposed, hardly anything is known about the possibility that conversion of *n*-3 LC-PUFA to fatty amides, thereby acting as bioactive lipid messengers, plays a role in these health-inducing effects. Evidence for the formation of *N*-acylethanolamines (NAE) from EPA (eicosapentaenoylethanolamine (EPEA)) and DHA (docosahexaenoylethanolamine (DHEA)) *in vivo* has been published[19–22]. Furthermore, a few publications reporting NAE formation reveal a direct link between dietary intake of *n*-3 LC-PUFA and elevated EPEA and DHEA concentrations in animal tissues[19,20]. Recently, we have demonstrated the formation of DHEA and EPEA by 3T3 adipocytes from their precursor fatty acids[23]. We have also found that DHEA was abundantly present in the human plasma of overnight fasted volunteers[23]. These observations raised further questions concerning the biological functions of DHEA, in particular, in relation to its immune-modulating effects.

First, a series of chemically closely related NAE, differing in the chain length and the number of double bonds, was evaluated for their ability to inhibit NO production by lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. NO, produced by inducible NO synthase (iNOS), is a late mediator induced during conditions of acute and chronic inflammation. Based on the results obtained, we further studied DHEA, demonstrating its action on monocyte chemotactic protein-1 (MCP-1), *in vitro* as well as in mouse peritoneal macrophages. Additionally, we studied whether DHEA-mediated effects are exerted at the gene expression level. Overall, the obtained results provide evidence for a novel molecular pathway mediating 'fish oil'/DHA-associated anti-inflammatory effects.

Methods

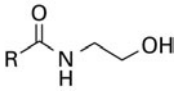
Chemicals

Oleoylethanolamine, DEA and AEA were purchased from Tocris (Bristol, UK). Novozym®435 was from Novozymes (A/S Bagsvaerd, Denmark). Dulbecco's modified Eagle's medium, Roswell Park Memorial Institute 1640 (RPMI-1640), fetal bovine serum, streptomycin and penicillin were acquired from Lonza (Verviers SPRL, Belgium). LPS was obtained from Sigma-Aldrich (Schnelldorf, Germany). Griess reagents and nitrite standard were purchased from Cayman Chemical (Ann Arbor, MI, USA). ELISA was performed using R&D Systems kits (Abingdon, UK). Bicinchoninic acid (BCA) protein assay kit was acquired from Pierce (Rockford, IL, USA), and Brewer thioglycollate was from BD Sciences (Franklin Lakes, NJ, USA).

N-acylethanolamine synthesis

NAE other than DEA, oleoylethanolamine and AEA (see Table 1 for the compounds tested) were prepared from ethanolamine and their corresponding fatty acids using an enzymatic procedure as described earlier[24]. Briefly, the method is based on a direct condensation reaction between ethanolamine and the fatty acid (molar ratio 1:1), carried out at 40°C in hexane, using Novozym®435 (consisting of immobilised *Candida antarctica* Lipase B) as the catalyst. Incubation times ranged between 8 and 15 h, and product yields obtained were in the range of 80 and 88 %. Authenticity of the products was verified by electrospray ionization-MS, ^{1}H NMR, ^{13}C NMR and Fourier transform infrared[24]. Purity of each compound was more than 98 % as determined by HPLC and NMR.

Table 1. Names of a series of *N*-acylethanolamines tested for their ability to inhibit nitric oxide production

	R	Acyl chain
Oleoylethanolamine	Oleic acid	18 : 1*n*-9
Linoleoylethanolamine	Linoleic acid	18 : 2*n*-6
Conjugated linoleoylethanolamine	Conjugated linoleic acid	18 : 2
Linolenoylethanolamine	Linolenic acid	18 : 3*n*-3
Arachidonoylethanolamine	Arachidonic acid	20 : 4*n*-6
Eicosapentaenoylethanolamine	EPA	20 : 5*n*-3
Docosatetraenoylethanolamine	Docosatetraenoic acid	22 : 4*n*-6
Docosahexaenoylethanolamine	DHA	22 : 6*n*-3

Cell culture

RAW264.7 macrophages (murine origin) were obtained from the American Type Culture Collection (Teddington, UK). RAW264.7 macrophages were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, streptomycin and penicillin at 37°C in a 5 % CO_2 humidified air atmosphere. Effects of the test compounds on the viability of the RAW264.7 cells were assessed using an XTT Cell Proliferation Kit II (Roche Applied Science, Almere, The Netherlands) according to the manufacturer's instructions. Briefly, cells were first incubated with the test compound(s). Thereafter, the tetrazolium salt (ZTT) assay was performed, where the cells' ability to metabolise XTT to formazan was a measure for cell viability. Conditions were considered toxic if metabolic activity to form formazan was decreased by >20 %. As a negative control, Triton X100 was added to the cells, yielding total cell lysis. For further details on XTT assays, see Lupinacci *et al.*[25].

Peritoneal macrophages

Male C57Bl/6 mice were injected intraperitoneally with a 4 % Brewer thioglycollate solution. After 3 d, mice were killed, and cells in the peritoneal cavity were collected by peritoneal lavage, using RPMI-1640 medium containing penicillin/streptomycin. Thereafter, peritoneal macrophages were collected by centrifugation for 5 min at 1250 rpm and 4°C, and pellets were subsequently treated with erythrocyte lysis

buffer (150 mM-NH_4Cl, 10 mM-$KHCO_3$ and 0·1 mM-EDTA) for 5–10 min on ice. Peritoneal macrophages were collected by centrifugation, and pellets were dissolved in RPMI-1640 containing 10 % heat-inactivated fetal bovine serum and penicillin/streptomycin. Cells were counted and plated in a density of approximately 750 000 cells/ml. After 2 h, adhering macrophages were washed with PBS and cultured for 72 h in RPMI-1640 medium (containing 10 % fetal bovine serum-heat-inactivated and penicillin/streptomycin) before stimulation.

For these experiments, the institutional and national guidelines for the care and use of animals were followed, and experiments were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University.

Effects of N-acylethanolamines on nitric oxide release, monocyte chemotactic protein-1 production and inducible nitric oxide synthase and monocyte chemotactic protein-1 gene expression

RAW264.7 macrophages ($2·5 \times 10^5$ cells/ml) were seeded into ninety-six-well cell culture plates for nitrite, ELISA and cell viability measurements, or into six-well plates (5×10^5 cells/ml) for the analysis of mRNA expression, and incubated overnight. Peritoneal macrophages were isolated and seeded as described earlier. Adherent cells were pre-incubated for 30 min with the test compound or vehicle in duplicate, after which LPS, 1 μg/ml for RAW264.7, was added in combination with the same test compound(s). For peritoneal macrophages, an optimal dose of 0·1 μg of LPS stimulation was assessed for the experimental conditions used. Therefore, peritoneal macrophages were activated with LPS (0·1 μg/ml) to measure MCP-1 release. However, because of nitrite detection limits, LPS (1 μg/ml) was used to measure NO production. Ethanol was used (final solvent concentration never exceeded 0·1 %, v/v) as the solvent. Incubation times were selected based on the specific properties of different inflammatory mediators. After incubating for 24 or 48 h, nitrite accumulated in the culture medium was measured as an indicator of NO production using the Griess method[26]. Briefly, 100 μl of the cell culture medium were mixed with 100 μl of Griess reagents and incubated at room temperature for 10 min. Absorbance was measured at 540 nm using an ELISA plate reader. Following 4 or 16 h incubation, MCP-1 levels were assessed by using ELISA.

RNA purification and quantitative reverse transcription real-time PCR

Total RNA was extracted using TRIzol^R (Invitrogen, Breda, The Netherlands). RNA (1 μg/sample) was reverse transcribed to give complementary DNA using the reverse transcription system from Promega (Leiden, The Netherlands). Complementary DNA was amplified by PCR using platinum Taq DNA polymerase (Invitrogen) and SYBR green (Molecular Probes, Leiden, The Netherlands) on an iCycler apparatus (Bio-Rad, Veenendaal, The Netherlands). The following primer pairs were used for amplification of iNOS: 5′-GTT-CTC-AGC-CCA-ACA-ATA-CAA-GA-3′ (forward) and 5′-GTG-GAC-GGG-TCG-ATG-TCA-C-3′ (reverse); MCP-1: 5′-CCC-AAT-GAG-TAG-GCT-GGA-GA-3′ (forward) and 5′-TCT-GGA-CCC-ATT-CCT-TCT-TG-3′ (reverse). Samples were analysed in duplicate, and mRNA expression levels of the different genes were normalised to RPS27A2. Primer pairs for RPS27A2 were 5′-GGT-TGA-ACC-CTC-GGA-CAC-TA-3′ (forward) and 5′-GCC-ATC-TTC-CAG-CTG-CTT-AC-3′ (reverse).

Statistical analysis

All experiments in RAW264.7 macrophages were performed in duplicate, and those performed in the peritoneal macrophages were in triplicate in at least three independent experiments. Data from all experiments are expressed as percentage of the LPS-treated controls (set at 100 %). Data are presented as means and standard errors of the mean (see legends of the figures). Statistical differences between treatments and controls were evaluated by two-way ANOVA followed by Bonferroni's *post hoc* test. A P value $<0·05$ was considered as statistically significant. P values were assigned at three different levels, namely $P<0·05$, $<0·01$ and $<0·001$.

Results

Effect of N-acylethanolamines with different chain lengths and number of double bonds on lipopolysaccharide-induced nitric oxide production

Fatty acid-derived NAE, chemically closely related to DHEA, were compared for their immune-modulating properties. Therefore, a series of NAE differing in chain length (from C18 to C22) and number of double bonds (1n to 6n) were tested for their abilities to inhibit LPS-induced nitric oxide production in RAW264.7 macrophages. NO is a late inflammatory marker and can be determined in LPS-stimulated macrophages from 24 h onwards, with levels still increasing up to 48 h and possibly later. The effect on NO release was determined at two time points (24 and 48 h), with concentration dependencies assessed at 48 h. Table 1 shows the molecular structures of the NAE tested. Oleoylethanolamine and other compounds having a length of eighteen carbons did not inhibit NO production either at 24 h (Fig. 1) or at 48 h (Fig. 2). Among the C20 compounds, AEA did not affect NO levels at $t = 24$ h but inhibited NO release at $t = 48$ h in a slight and significant way. In contrast, EPEA (20 : 5) and DEA (22 : 4) significantly decreased NO release at $t = 24$ h (Fig. 1) and reduced NO production in a significant way at concentrations of 1 and 10 μM at $t = 48$ h (Fig. 2). At a concentration of 10 μM, DHEA (22 : 6) significantly suppressed NO production up to 40 % at $t = 24$ h (Fig. 1) and in a concentration-dependent way at $t = 48$ h (Fig. 2). The highest concentration of 10 μM gave a more than 60 % reduction at $t = 48$, while 1 μM decreased NO production by 20 %. Generally, the potency of NAE to reduce NO release was enhanced with their increasing chain length and number of double bonds. The precursor of DHEA, DHA, produced only little suppression of NO production.

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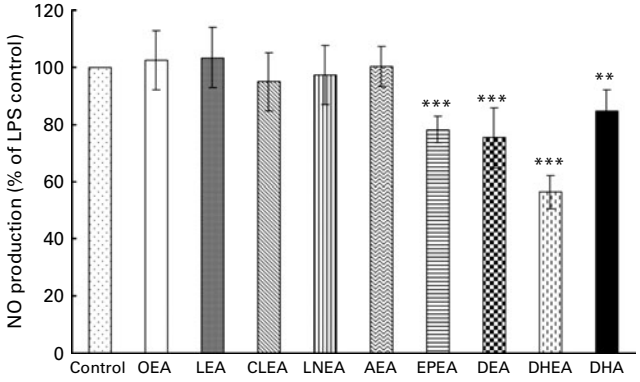

Fig. 1. Efficacy of a series of *N*-acylethanolamines to inhibit lipopolysaccharide (LPS)-induced nitric oxide release in RAW264.7 macrophages at 24 h. Efficacies were compared with nitric oxide-reducing effects of the precursor DHA. All compounds were tested at a dose of 10 μM. RAW264.7 macrophages were seeded at a density of 250 000 cells/ml and pre-incubated for 30 min with the respective ligands preceding a 24 h LPS (1 μg/ml) stimulation, in the presence of the particular ligand. Protein content of each well was determined by a bicinchoninic acid assay. Data are expressed as percentage, where LPS stimulation (containing vehicle) was set at 100 %. Average absolute value for nitrite production with LPS stimulation (control) after 24 h was approximately 16 μM. Values are means of four separate experiments (each done in duplicate), with standard errors of the mean represented by vertical bars. Mean values were significantly different from 100 %: $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$. OEA, oleoylethanolamine; LEA, linoleoylethanolamine; CLEA, conjugated linoleoylethanolamine; LNEA, linolenoylethanolamine; AEA, arachidonoylethanolamine; EPEA, eicosapentaenoylethanolamine; DEA, docosatetraenoylethanolamine; DHEA, docosahexaenoylethanolamine.

Only the highest concentration tested elicited a significant NO decrease both after 24 and 48 h.

Cell viability was verified using an XTT cell proliferation kit assay. Neither LPS alone nor LPS in combination with the highest concentration (10 μM) of different NAE affected the viability of cells after 24 h. At 48 h, cell viability slightly dropped to a range of 83–87 % but only in the LPS-treated cells. Cell viability was not further decreased by adding the tested components, indicating that the observed effect was caused by LPS. Conditions are regarded toxic if cell viability lowers more than 20 %. As all the tested compounds were within the viability range of 83–100 %, experimental conditions were considered non-toxic.

Docosahexaenoylethanolamine elicits dose-dependent suppression of monocyte chemotactic protein-1

Out of the NAE series, DHEA turned out to be the most effective component suppressing LPS-induced NO release. These results prompted us to further focus on DHEA and to investigate its effects on MCP-1, a cytokine playing a pivotal role in inflammatory processes. In contrast to the late inflammatory marker nitric oxide, MCP-1 is a relatively early marker of the LPS-induced inflammatory cascade. MCP-1 production induced by LPS stimulation in RAW264.7 macrophages is already observed at 4 h, with levels further increasing to reach maximum levels at 16 h to stay high until later time points (J Meijerink, unpublished results). In order to assess whether DHEA reduces cytokine production also at earlier time points during the inflammatory cascade, MCP-1 levels were determined both at 4 h and at 16 h when MCP-1 has reached maximum levels. Pre-incubation with DHEA dose-dependently reduced LPS-induced MCP-1 production in RAW264.7 macrophages both at 4 h (Fig. 3(a)) and at 16 h (Fig. 3(b)). At 16 h, a significant inhibition of 53 % ($P<0{\cdot}001$) was elicited by 10 μM-DHEA, while 100 nM still significantly evoked a reduction of 18 % ($P<0{\cdot}05$). The dose–response curve at 4 h shows an almost identical curve compared with the 16 h time point, with the only difference being the lack of statistical significance for the MCP-1 reduction elicited by 100 nM-DHEA at 4 h. The lowest dose for DHEA-induced reduction in MCP-1, observed at 16 h, was approximately ten times lower than the dose needed to reduce NO levels.

Anti-inflammatory effects of docosahexaenoylethanolamine on mouse peritoneal macrophages

To study whether DHEA exerts similar anti-inflammatory effects under *ex vivo* conditions as *in vitro,* thioglycollate-elicited peritoneal macrophages were exposed to different concentrations of DHEA and subsequently activated by LPS. Pre-incubation with DHEA resulted in diminished levels of both NO (Fig. 4(a)) and MCP-1 (Fig. 4(b)). A dose-dependent effect was found for both markers. Due to the limited lifespan of the primary peritoneal macrophages, NO measurements were performed at 24 h rather than at 48 h when maximal levels of production were reached. At the 24 h time point, 10 μM-DHEA significantly inhibited NO release up to 47 % (Fig. 4(a)). Effects are comparable with those exerted in RAW264.7 cells (Fig. 1). Also, a DHEA-elicited concentration-dependent effect was found for MCP-1 at 16 h, with the lowest dose of 1 μM being still significant. Effects were less pronounced as those assessed in RAW264.7 macrophages. Cell viability of peritoneal macrophages during experimental conditions was determined using an XTT assay and considered non-toxic.

Docosahexaenoylethanolamine suppresses lipopolysaccharide-induced mRNA expression of inducible nitric oxide synthase and monocyte chemotactic protein-1

In order to investigate whether NO and MCP-1 inhibition elicited by DHEA is regulated at the gene expression level, we used quantitative RT-PCR to determine iNOS (Fig. 5(a)) and MCP-1 (Fig. 5(b)) mRNA expression. A concentration of 10 μM-DHEA significantly suppressed LPS-induced iNOS mRNA gene expression at 24 h up to 52 %. A similar dose–response relationship was found for MCP-1 gene expression, where the highest dose of DHEA tested resulted in a reduction of 51 %. Lowest doses eliciting significant reduction were considerably higher at the gene expression level than at the production level.

Discussion

Despite the high abundance of nutritionally important *n*-3 LC-PUFA in brain and other tissues[27–30], remarkably little has been described so far on their conjugation with amines

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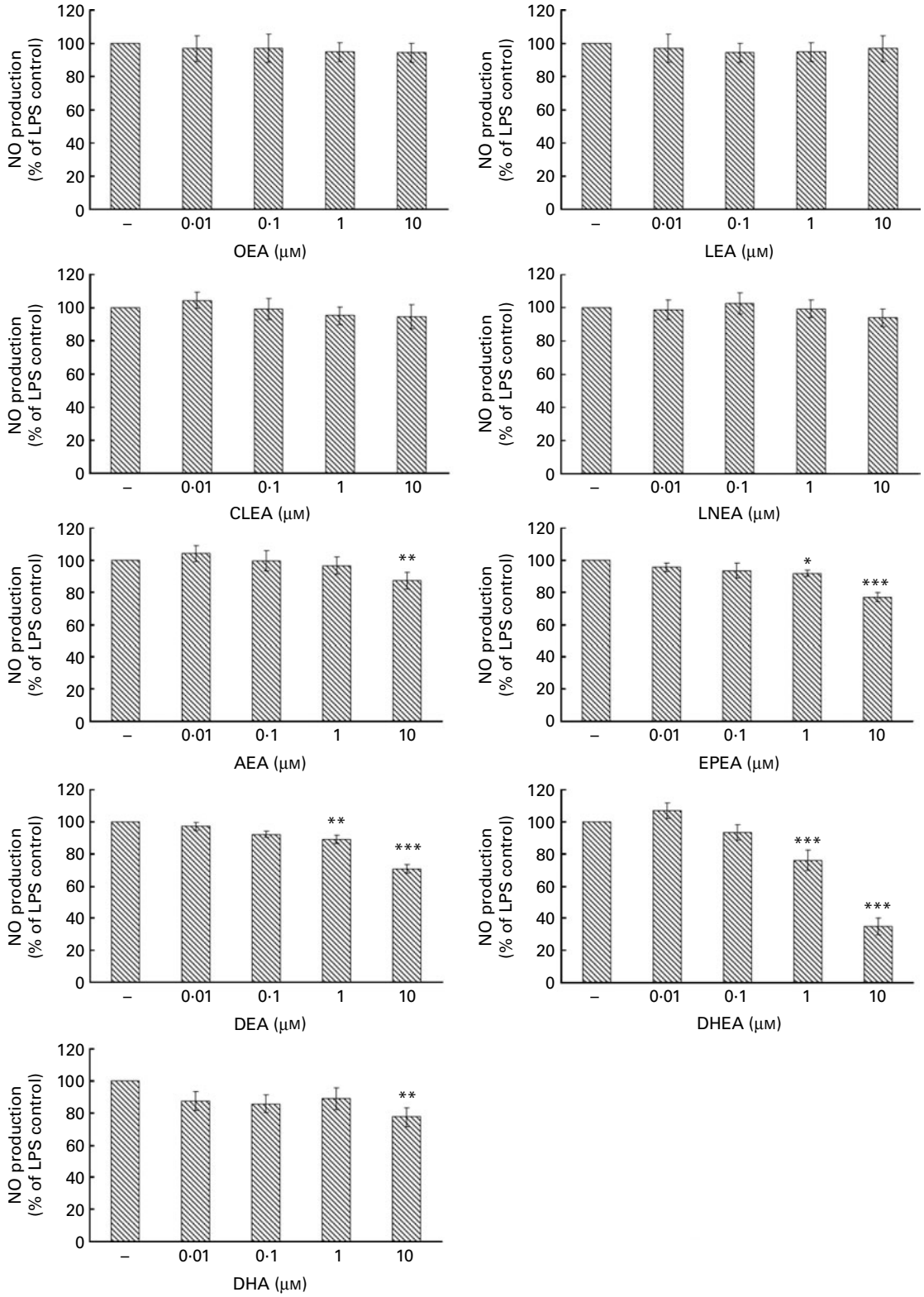

Fig. 2. Dose–response graphs of lipopolysaccharide (LPS)-induced nitric oxide reduction elicited by a series of *N*-acylethanolamines and DHA in RAW264.7 macrophages. Compounds were tested in a concentration range of 0·01–10 μM. RAW264.7 macrophages were seeded at a density of 250 000 cells/ml and pre-incubated for 30 min with the respective ligands before a 48 h LPS (1 μg/ml) stimulation, in the presence of the particular ligand. The protein content of each well was determined by a bicinchoninic acid assay. Data are expressed as percentage, where LPS stimulation (containing vehicle) was set at 100 %. Average absolute value for nitrite production with LPS stimulation (control) after 48 h was approximately 45 μM. Values are means of four separate experiments (each done in duplicate), with standard errors of the mean represented by vertical bars. Mean values were significantly different from the control: $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$. OEA, oleoylethanolamine; LEA, linoleoylethanolamine; CLEA, conjugated linoleoylethanolamine; LNEA, linolenoylethanolamine; AEA, arachidonoylethanolamine; EPEA, eicosapentaenoylethanolamine; DEA, docosatetraenoylethanolamine; DHEA, docosahexaenoylethanolamine.

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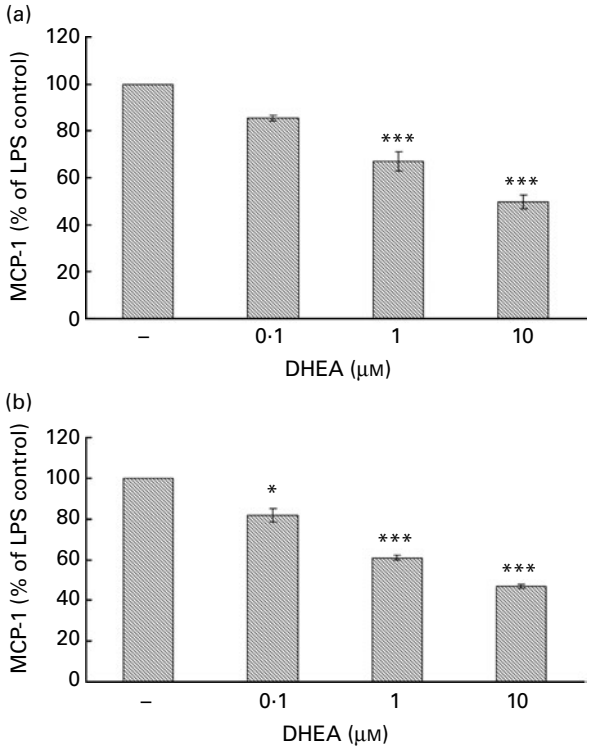

Fig. 3. Effect of docosahexaenoylethanolamine (DHEA) on monocyte chemotactic protein-1 (MCP-1) cytokine production in RAW264.7 macrophages at different time points. RAW264.7 cells were seeded at a density of 250 000 cells/ml and pre-incubated with a concentration series of DHEA for 30 min before (a) a 4 h lipopolysaccharide (LPS, 1 μg/ml) stimulation in the presence of DHEA and (b) a 16 h LPS (1 μg/ml) stimulation in the presence of DHEA. The supernatant of the cells was analysed for MCP-1 production by ELISA. The protein content of each well was determined by a bicinchoninic acid assay. Values for MCP-1 production were corrected for the amount of protein for each well separately. Data are expressed as percentage, where LPS stimulation (containing vehicle) was set at 100 %. Values are means of three separate experiments (each done in duplicate), with standard errors of the mean represented by vertical bars. Mean values were significantly different from the control: $^{*}P<0{\cdot}05$, $^{***}P<0{\cdot}001$.

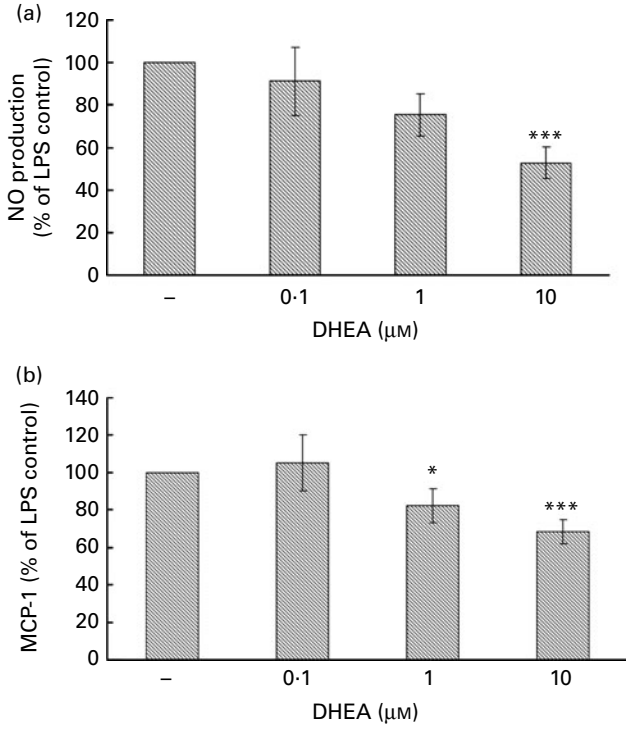

Fig. 4. Effect of docosahexaenoylethanolamine (DHEA) on monocyte chemotactic protein-1 (MCP-1) cytokine and nitric oxide production in peritoneal macrophages. Cells were seeded at a density of approximately 750 000 cells/ml and pre-incubated with a concentration series of DHEA 30 min before (a) a 24 h lipopolysaccharide (LPS, 1 μg/ml) stimulation in the presence of DHEA for nitric oxide determination and (b) a 16 h LPS (0·1 μg/ml) stimulation in the presence of DHEA for MCP-1 determination. The supernatant of the cells was analysed for nitric oxide using the Griess assay and for MCP-1 by ELISA. The protein content of each well was measured by a bicinchoninic acid assay. Values for MCP-1 and nitric oxide production were corrected for the amount of protein for each well separately. Data are expressed as percentage, where LPS stimulation (containing vehicle) was set at 100 %. Data for nitric oxide represent means of cells isolated from five different mice (with each biological sample performed in duplicate or triplicate), except for the doses of 0·1 μM-DHEA that was performed in three mice, with standard errors of the mean represented by vertical bars. For MCP-1 analysis, four mice were used (with each biological sample performed in duplicate or triplicate). Mean values were significantly different from the control: $^{*}P<0{\cdot}05$, $^{***}P<0{\cdot}001$.

into fatty amides. In addition, hardly anything is known about the potential biological significance of these amides. About 10 years ago, the presence of DHEA in bovine retina was demonstrated by Bisogno *et al.*[21]. A few years later, Berger *et al.*[20] showed that brain levels of EPEA and DHEA were highly increased after feeding newborn piglets a milk formula rich in EPA and DHA. More recently, increased levels of DHEA and EPEA were demonstrated in the liver and jejunum of rats that had been fed a high-fish oil diet[19]. In a recently published study, we showed that DHA can be rapidly and efficiently converted to its ethanolamide, DHEA, by 3T3-L1 adipocytes[23]. This is in accordance with the findings of Eckardt *et al.*[22] who demonstrated the presence of DHEA among other NAE in the culture medium of human primary pre-adipocytes. In the present study, we investigated the potential biological implications of these findings and established that DHEA, a 'novel' bioactive member of the group of NAE, possesses immune-modulating properties in macrophages.

Effects on nitric oxide release

NO produced by iNOS has been shown to be a key inflammatory mediator in several diseases associated with the metabolic syndrome. Disruption of iNOS, by using knockout mice models, was found to diminish diet-induced atherosclerosis and improve insulin signalling and glucose tolerance in obese mice[31–33]. NO is a signalling molecule produced in the oxidative deamination of L-arginine catalysed by a NOS. During an inflammatory process, the inducible isoform of this enzyme (iNOS or NOS2), which is not expressed in non-pathological situations, is up-regulated[34,35]. This up-regulation can be initiated by microbial products such as LPS, inflammatory cytokines or interferon-γ.

Of the NAE tested in the present study, we found that DHEA had the highest potency to inhibit NO formation in LPS-stimulated RAW264.7 macrophages. For the C18 chain-length components, the number and position of double bonds did

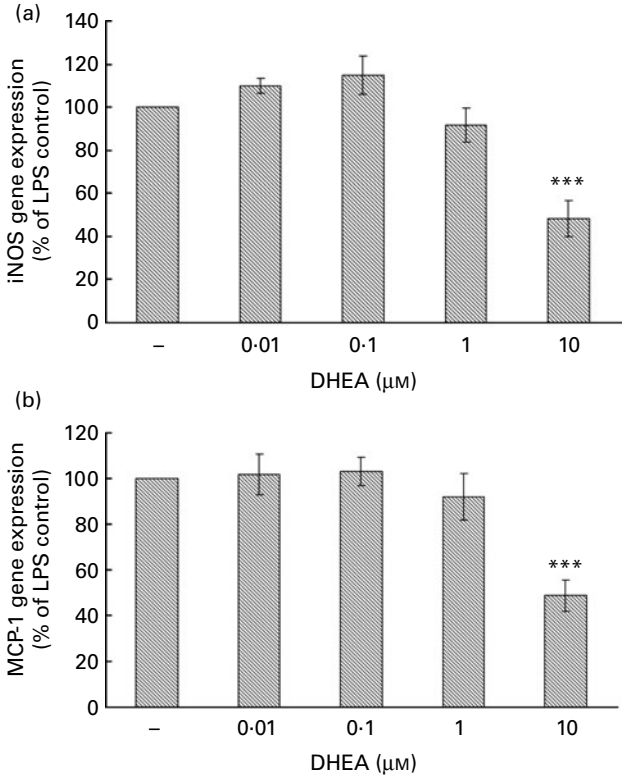

Fig. 5. Effect of docosahexaenoylethanolamine (DHEA) on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) and monocyte chemotactic protein-1 (MCP-1) mRNA expression at 24 h. RAW264.7 cells were seeded at a density of 500 000 cells/ml and pre-incubated with a concentration series of DHEA for 30 min before a 24 h LPS stimulation in the presence of DHEA. Total RNA was isolated and reverse transcribed to give complementary DNA before performing quantitative real-time PCR. Details are described in the Methods section. iNOS and MCP-1 fold increase normalised to RPS27A2 is expressed as percentage, where LPS stimulation (without DHEA) was set at 100 %. Values are means of three separate experiments performed in duplicate, with standard errors represented by vertical bars. *** Mean value was significantly different from that of the control ($P<0{\cdot}001$).

not influence the activity of the fatty amides; none of them being effective in inhibiting NO release up to a concentration of 10 μM. Increasing the chain length demonstrated that AEA (20 : 4; anandamide), a well-known endocannabinoid, inhibited NO release at $t = 48$ h in a slight and significant way. Further increase in the number of double bonds (20 : 5) and/or chain length (22 : 4, 22 : 6) led to an increased capacity to inhibit nitric oxide production, with that of DHEA (22 : 6*n*-3) being detectable at a concentration of 0·1 μM. Its precursor DHA only had a small effect in our assay. This might be in line with results reported by Weldon *et al.*[36] who needed relatively high concentrations (100 μM) of DHA and a pre-incubation period to achieve inhibition of the release of inflammatory mediators in LPS-stimulated THP-1 (human leukaemic monocyte cell line) macrophages. It also indicates that the effects of DHEA found are not due to its hydrolysis to DHA, which could occur via fatty acid amide hydrolase[37]. Control studies performed in our group using LC-MS to assess the stability of DHEA in the experimental set-up has shown that DHEA concentrations slightly declined with a 10 % reduction at 2 h to reach an approximately 75 % decrease after 48 h (M Balvers, unpublished results). This shows that doses of DHEA were in the active range (> 1 μM) during the entire experiment. Moreover, although NO has been measured at 48 h, data represent DHEA-elicited effects of a late marker of the inflammatory cascade. It is, however, very likely that DHEA already exerts its effects early during the initiation of the cascade. Data for the 4 h time point support this theory, as DHEA significantly reduced levels of the relatively early marker MCP-1 at 4 h. Taken together, all data indicate that the DHEA-elicited immunomodulatory effects can be explained from the activity of DHEA itself.

Effects of docosahexaenoylethanolamine on monocyte chemotactic protein-1 protein and mRNA levels

Similar to NO, MCP-1 has been shown to be a key inflammatory mediator in metabolism-related disorders. The pro-inflammatory chemokine MCP-1 functions as a leucocyte chemoattractor and is predominantly produced by macrophages or endothelial cells. Blocking expression of MCP-1 or its receptor under conditions leading to artherosclerosis in mice resulted in diminished formation of atherosclerotic lesions and lipid deposition in their aortas[38,39]. Furthermore, in obesity, a casual relationship for MCP-1 and macrophage infiltration in adipose tissue, insulin resistance and hepatic steatosis has been established[40], and only recently, another study has shown that high levels of circulating MCP-1 in the plasma are crucial for inducing insulin resistance in mice[41].

We found that DHEA dose-dependently reduced MCP-1 production in both RAW264.7 and thioglycollate-elicited mouse peritoneal macrophages. The lowest dose at which DHEA inhibited MCP-1 release was at 100 nM at 16 h in RAW264.7 cells. Minimum doses eliciting significant reduction were a factor 100–1000 lower than those reported in the literature for anti-inflammatory effects mediated by its precursor DHA or for the *n*-3 PUFA, EPA[42,43]. Interestingly, Shi & Pestka[44] reported that in peritoneal macrophages of mice, which were fed a DHA-enriched diet for several weeks, anti-inflammatory pathways were induced, which were not found when naive peritoneal macrophages were directly exposed to DHA. The authors suggest that DHA-derived metabolites might be responsible for the immune-modulating effects *in vivo* rather than DHA itself.

Our data showed that DHEA elicited a suppression of both MCP-1 and iNOS mRNA. These results suggest that DHEA-induced inhibition is primarily regulated at the mRNA level. However, although anti-inflammatory effects at a dose of 10 μM reflect inhibition as determined at the protein level, dose-dependent effects in mRNA expression were only slightly visible, with 1 μM being the lowest dose showing a small non-significant reduction. Consequently, it cannot be ruled out that DHEA-elicited effects are regulated by different mechanisms, with lower doses regulated at the protein level, while suppressive effects at higher doses are regulated at the expression level. Alternatively, this discrepancy might also be caused by differences in relative sensitivity of the techniques used.

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n-3 Long-chain PUFA and inflammation: alternative mechanisms of action

It is well known that eating a diet rich in *n*-3 LC-PUFA, for example fatty fish, leads to a gradual incorporation of *n*-3 LC-PUFA in cellular membranes of different tissues at the expense of other fatty acids including arachidonic acid[29,45]. This has also been described for cells of the immune system, causing changes in eicosanoid production patterns and cellular functionality[14]. The fatty acid composition and positional distribution of dietary TAG have also consequences for the relative amounts of 2-acylglycerols formed. This may affect CB1/CB2 receptor binding, either directly (via *sn*-2-arachidonoylglycerol) or indirectly, for example via an 'entourage' effect. EPA and DHA also serve as precursors for resolvins, another class of inflammatory mediators[14,15,46]. Resolvins or other DHA eicosanoid products can form novel *N*-linked ethanolamines that may be biologically active[4,27]. The formation of amides from *n*-3 LC-PUFA, as shown in the present study, might represent a new branch on the tree of pathways involved in the complex regulation of inflammation. Endocannabinoid and eicosanoid pathways are known to share a number of common biotransformation pathways, regulatory enzymes and, possibly, receptors[47]. Local availability of precursors can play a role in the inflammatory balance, as has been suggested for *n*-3 *v.* *n*-6 in the diet[45,48]. However, it is also clear that there is not a direct stoichiometric relationship between dietary intake of precursors and production of inflammatory mediators. Instead, a well-regulated homeostatic equilibrium exists that fluctuates between rather narrow boundaries[18,45,46]. However, in the case of endocannabinoids, dietary studies have clearly shown that this equilibrium is subject to gradual changes. Although there are only few studies showing direct formation of NAE from *n*-3 LC-PUFA precursors, several other studies have shown that dietary intervention with *n*-3 fatty acids also indirectly affects the endocannabinoid balance. For example, Watanabe *et al.*[49] have demonstrated that *n*-3 LC-PUFA deficiency elevates and *n*-3 LC-PUFA administration reduces brain *sn*-2-arachidonoylglycerol levels in mice. Batetta *et al.*[50] have recently shown that diets rich in *n*-3 LC-PUFA decreased AEA and *sn*-2-arachidonoylglycerol levels in the visceral fat of obese Zucker rats. In the study of Artmann *et al.*[19], decreased levels of all NAE measured were found in the livers of rats fed with *n*-3 LC-PUFA for 1 week, except for DHEA and EPEA. However, in the brain, these effects were not observed, which may be due to the short treatment period. Taken together, it appears that NAE patterns tend to follow the relative abundance of lipids in the diet. However, a few weeks of intake are needed to achieve significant changes. In addition to stimulating the formation of DHEA by increasing dietary supply, inhibition of its breakdown by fatty acid amide hydrolase could further elevate local concentrations[37]. Some NAE, including arachidonoyl serotonin, are known to have fatty acid amide hydrolase-inhibiting properties[51,52]. Hence, NAE that do not bind to CB1 or CB2 can still exert biological action in an indirect way.

Is there a role for docosahexaenoylethanolamine as an immune modulator?

In relation to the reported health effects of fish oil and *n*-3 LC-PUFA, our findings might be of interest for several applications and areas. One such area is obesity/metabolic syndrome, where an inflammatory process plays a role[53–56]. A biochemical connection between fish oil intake and the pathology of obesity has recently been suggested by Batetta *et al.*[50], showing that *n*-3 LC-PUFA supplementation inhibited the inflammatory process and ectopic fat deposition while reducing AEA in obese Zucker rats. Several studies have demonstrated the importance of NO in obesity and the metabolic syndrome[57–60]. Fish oil has also shown potential beneficial activity in inflammatory diseases of the intestinal tract[61], and high incorporation of DHEA and EPEA was found in gut tissue after a fish oil diet[19]. In conclusion, our data provide evidence for a novel mechanism by which DHA exerts immune-modulating effects, namely as a precursor of DHEA. Combined with findings from previous studies, our data provide new viewpoints on the relationship between dietary intake of DHA and local inflammatory processes. It is tempting to speculate that DHEA might possibly play a significant role in inflammatory processes *in vivo*, thereby functioning as an endogenous 'natural' ligand. Further studies should elucidate the relevance of this 'fish oil messenger' *in vivo* and the relationship to other inflammatory pathways in which DHA is involved.

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