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Enrichment of RAW264.7 macrophages with essential 18-carbon fatty acids affects both respiratory burst and production of immune modulating cytokines

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Abstract

Macrophages play a vital role in the innate immune system. Thereby, production of both reactive oxygen intermediates and immune modulating cytokines is crucial for successful pathogen defense. Fatty acids may interfere with immune response in several ways. In this study, we investigated the influence of essential polyunsaturated fatty acids (PUFA) on key macrophage functions. RAW264.7 macrophages were cultured in a medium supplemented with 2 or 15 μmol/L of the n-6 PUFA linoleic acid (LA) or of the n-3 PUFA α-linolenic acid (LNA), respectively. Cells were tested for incorporation of fatty acids as well as NADPH oxidase activity. Furthermore, supernatants were collected for detection of NO and cytokine release (TNF-α, IL-6, IL-10). Exposure of RAW264.7 macrophages to LA or LNA resulted in incorporation of these fatty acids and their derivatives. Thereby, supplementation with both LA and LNA caused a significant increase in NADPH oxidase activity. In contrast, synthesis of NO was not affected by PUFA supplementation. Moreover, distinct effects could be seen in the release of immune modulating cytokines. Due to enhancement of NADPH oxidase activity, PUFA presumably promote the killing of pathogens crucial in host defense. In addition, the unsaturated fatty acids tested in our study were shown to modulate cytokine release by the macrophages, thus driving immune response into an anti-inflammatory direction. Of note, distinct differences between the n-6 PUFA LA and the n-3 PUFA LNA underline the impact of PUFA family on immune response.

Keywords: Fatty acids; Macrophages; Respiratory burst; Immune modulation

1. Introduction

Macrophages are of vital importance in immune response performing several functions in the innate and subsequently the adaptive immune system. Besides their phagocytic role, macrophages produce a number of reactive oxygen and nitrogen intermediates (respiratory burst) like the superoxide ion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) as well as nitric oxide (NO), which are sufficient to kill engulfed microorganisms [1]. Moreover, via secretion of immune modulating cytokines [1] macrophages make a considerable contribution both to local inflammation and to other induced innate responses underlining the relevance of the cells in orchestrating immune defense mechanisms. Thus, respiratory burst and production of immune modulating cytokines represent key elements in the macrophage response upon infection.

Fatty acids may impact the immune system by various ways. They influence the structural integrity of cell membranes [2,3]. Indeed, the length and degree of saturation of the fatty acids in the membrane phospholipids contribute to determine the membrane fluidity. Functionality of membrane proteins critically depends on membrane fluidity [2,3]. Thus, fatty acids might affect signaling mechanisms and membrane-associated enzymes necessary for respiratory burst. Moreover, many cell signaling pathways involve lipids or lipid-derived molecules [4,5]. Polyunsaturated fatty acids (PUFA) increasingly have been identified to affect cell signaling reactions at the cell membrane level [6–9] and the activation of transcription factors as peroxisome proliferator-activated receptors [10] and nuclear factor-kappaB [11]. In addition, some PUFA act as substrates for eicosanoid synthesis, thus probably interfering with cytokine release [12].

Proportion of fatty acids in dietary lipids may have an important impact on membrane fatty acid composition as well as on the efficiency of membrane-mediated processes [13–15]. Currently, the predominant PUFA in most diets (compromising over 95% in Western diets) are the two essential 18-carbon PUFA linoleic acid (18:2n6; LA) and alpha-linolenic acid (18:3n3; LNA) [16,17]. The ratio between these two fatty acids is of significance in determining cell function, whole body physiology and human health [18–20]. However, it is not well understood how enrichment of macrophage membranes with fatty acids from distinct families could differentially affect respiratory burst and cytokine release. Hence, given the interest in the relative effects of LA and LNA and regarding the importance of macrophage respiratory burst and cytokine release to

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Table 1
Fatty acid patterns (weight %) and MBI of RAW264.7 macrophages after 72 h of incubation in medium containing 2 μmol/L LA+2 μmol/L LNA, or 15 μmol/L LA+2 μmol/L LNA, or 2 μmol/L LA+15 μmol/L LNA

Fatty acid	LA (2)+LNA (2)		LA (15)+LNA (2)		LA (2)+LNA (15)	
	Weight %	MBI	Weight %	MBI	Weight %	MBI
Total saturated	38.12±0.17	–	38.64±0.35	–	40.34±0.18	–
Total n-3 PUFA	1.77±0.09[a]	4.87±0.26[a]	1.42±0.06[a]	2.70±0.22[a]	7.93±0.22[b]	17.82±0.67[b]
18:3n3	0.04±0.02[a]	0.08±0.04[a]	n.d.	–	0.35±0.01[b]	0.70±0.02[b]
20:5n3	0.86±0.03[a]	3.44±0.12[a]	0.35±0.03[b]	1.40±0.12[b]	3.72±0.10[c]	14.88±0.40[c]
Total n-6 PUFA	1.94±0.05[a]	3.31±0.19[a]	8.13±0.22[b]	11.13±0.31[b]	1.64±0.04[a]	2.83±0.10[a]
18:2n6	0.87±0.03[a]	0.87±0.03[a]	5.24±0.16[b]	5.24±0.16[b]	0.73±0.01[c]	0.73±0.01[c]
20:4n6	0.66±0.04[a]	1.98±0.12[a]	1.41±0.04[b]	4.23±0.12[b]	0.58±0.02[c]	1.74±0.06[c]
Total n-7 monounsaturated	39.94±0.40[a]	–	35.73±0.77[b]	–	35.15±0.30[b]	–
Total n-9 monounsaturated	18.23±0.12[a]	–	16.08±0.36[b]	–	14.94±0.14[b]	–
Total	100	8.18±0.45[a]	100	13.83±0.53[b]	100	20.65±0.77[c]

Data are mean±S.D. (n=6). Superscript letters across a row denote significant differences. n.d.=Below detection limit.

immune defense, we investigated the influence of LA and LNA on these key macrophage functions.

2. Materials and methods

2.1. Materials

All chemicals and reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany) unless noted otherwise. Cell culture flasks and plates were purchased from Greiner Bio-One (Frickenhausen, Germany), and HEPES (25 mmol/L)-buffered RPMI 1640 culture medium containing 300 mg/L L-glutamine was acquired from PAA Laboratories (Cölbe, Germany).

2.2. Cell culture

The permanent mouse monocyte/macrophage cell line RAW264.7 (ATCC number TIB-71) was used. RAW264.7 cells were cultured in RPMI 1640 medium containing 4.5 g/L glucose supplemented with 5 μmol/L α-tocopherol, 3% fetal calf serum and 7% serum replacement SerEx (all PAA Laboratories). The n-6 PUFA LA and the n-3 PUFA LNA (all Biotrend, Köln, Germany) were included in the culture medium at a concentration of 2 or 15 μmol/L using ethanol as a vehicle (0.2% v/v final ethanol concentration). Cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

2.3. Fatty acid composition of cultured RAW264.7 cells

RAW264.7 cells were seeded in 75-cm^2 cell culture flasks at a density of 1×10^6 cells/ml in medium containing 2 μmol/L of LA+LNA (control medium), or 15 μmol/L LA+2 μmol/L LNA, or 2 μmol/L LA+15 μmol/L LNA. Cells were harvested for measurement of fatty acid composition after 72 h. For this, 1×10^7 cells from one flask were washed in PBS, centrifuged, dried under nitrogen and stored at −25°C until analysis. Cell lipids were extracted, phospholipids isolated and the fatty acid compositions determined by gas chromatography as described elsewhere [21] using Di-C17-phosphatidylcholine as an internal standard. An aliquot of 1 μl was injected on-column on a Varian CP 3800 gas chromatograph (Varian, Darmstadt, Germany) equipped with an Omegawax 320 column (0.32 mm internal diameter, 30 m length) (Supelco, Bellefonte, PA, USA). The column temperature was 200°C. Based on the ratio of a fatty acid (weight %) detected by gas chromatography and the number of its bis-allyl-methylene positions the Methylene Bridge Index (MBI) was calculated [22].

2.4. NADPH oxidase activity

RAW264.7 cells were seeded in 12-well cell culture plates at a density of 1×10^4 cells/ml for 72 h in the media described above. Activity of NADPH oxidase was determined using a modified colorimetric nitroblue tetrazolium (NBT) assay as previously described [23]. Cells were incubated with yellow-colored NBT solution (12 μM) for 40 min at 37°C and 5% CO_2. For stimulation of cells phorbol-12-myristate-13-acetate (PMA) solution (50 nM) and for negative control diphenyleneiodonium-chloride (DPI) solution (5 nM) were added. Absorbance was read on a UV/Vis spectrometer (Carry 50, Varian, Darmstadt, Germany) at 620 nm.

2.5. Production of NO

RAW264.7 cells were seeded in 75-cm^2 cell culture flasks at a density of 1×10^6 cells/ml in the media described above. On Day 1, cells were primed by adding IFN-γ (murine, 50 U/ml). On Day 2, media were exchanged by fresh control medium supplemented with LPS (from *E.coli* Serotype 0111:B4, 1 μg/ml) for stimulation of cells. Supernatants were collected after 24 h of stimulation [24]. Production of NO was detected using the Griess assay [25]. For this, 100 μl of the Griess reagent was added to 50 μl supernatant for 10 min at room temperature. Absorbance was read on a SpectraMax 340PC ELISA reader at 550 nm and analysis done using SoftMax Pro 3.1.2 software (all Molecular Device, München, Germany).

2.6. Cytokine production

RAW264.7 cells were seeded, primed and stimulated as described above. TNF-α, IL-6 and IL-10 were detected in supernatants using suitable murine ELISA kits (Preprotech, London, UK) according to the manufacturer's instructions. Absorbance was read on a SpectraMax 340PC ELISA reader at 450 nm and analysis done using SoftMax Pro 3.1.2 software (all Molecular Device, München, Germany).

2.7. Statistical analysis

Data are shown as means±S.D.. One-way analysis of variance followed by Bonferroni test was used to identify significant differences between means. The statistical analysis was carried out by means of the program SigmaStat 3.5 (Jandel Scientific, Erkrath, Germany). Values of P<.05 were considered to be significant.

3. Results

3.1. Fatty acid composition

Culture of RAW264.7 macrophages in medium containing 15 μmol/L LA resulted in a significant enrichment in the content of n-6 PUFA in the cells (LA and its metabolites C18:3n6, C20:3n6 and

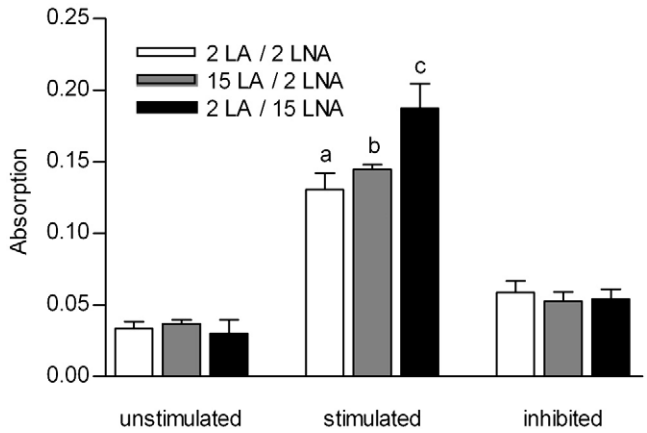

Fig. 1. NADPH oxidase activity (evaluated by NBT assay) of RAW264.7 macrophages after 72 h of incubation in medium containing 2 μmol/L LA+2 μmol/L LNA, or 15 μmol/L LA+2 μmol/L LNA, or 2 μmol/L LA+15 μmol/L LNA in the presence of (i) medium alone (unstimulated), (ii) PMA (stimulated) or (iii) DPI (inhibited). Data are mean±S.D. (n=6). Bars denoted by different letters are significantly different.

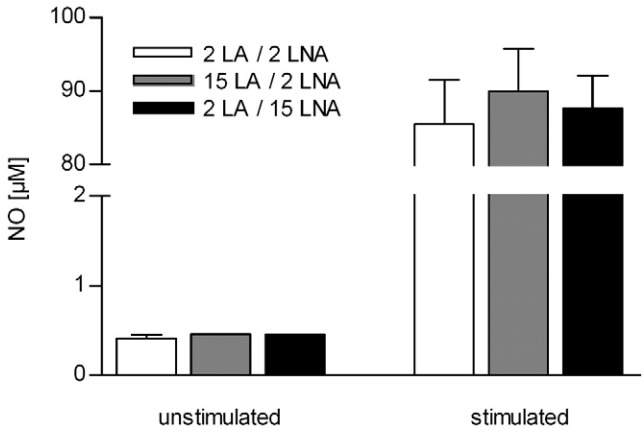

Fig. 2. NO concentration (evaluated by Griess assay) in supernatants of RAW264.7 macrophages after 72 h of incubation in medium containing 2 µmol/L LA+2 µmol/L LNA, or 15 µmol/L LA+2 µmol/L LNA, or 2 µmol/L LA+15 µmol/L LNA in the presence of (i) medium alone (unstimulated) or (ii) IFN-γ+LPS (stimulated). Data are mean±S.D. (n=6). Bars denoted by different letters are significantly different.

C20:4n6), while culture in medium containing 15 µmol/L LNA resulted in a significant enrichment in the content of n-3 PUFA (LNA and its metabolites C20:4n3 and C20:5n3) (Table 1). Cells cultured with 15 µmol/L LA had fourfold more n-6 fatty acids than those cultured with the control medium. Culture of cells with 15 µmol/L LNA caused a fourfold increase in total n-3 PUFA. In addition to the increase in n-6 or n-3 PUFA with culture in the presence of LA or LNA, there was a decrease in the content of n-7 and n-9 monounsaturated fatty acids (Table 1). Enrichment in PUFA content was connected with an increase in calculated MBI of the cells (Table 1). RAW264.7 macrophages cultured in medium containing 15 µmol/L of the n-3 fatty acid LNA thereby were calculated to have a significant higher MBI compared to the cells cultured in medium containing 15 µmol/L of the n-6 fatty acid LA (Table 1).

3.2. Oxidative burst

Stimulation of RAW264.7 cells with PMA resulted in a significant increase of NADPH oxidase activity up to sixfold (Fig. 1). For this, significant differences depending on PUFA supplementation could be seen. RAW264.7 cells cultured in medium containing 15 µmol/L of either LA or LNA showed significantly higher NADPH oxidase activity than those cultured in control medium (Fig. 1). LNA increased NADPH oxidase activity to a greater extent than LA did. Addition of the NADPH oxidase inhibitor DPI totally prevented production of superoxide radicals underlining specificity of the test system used (Fig. 1).

NO production by macrophages has been shown to depend on transcription and *de novo* synthesis of the gene for iNOS [26]. Treatment of RAW264.7 cells with IFN-γ and LPS induced increased iNOS mRNA levels as could be shown by RT-PCR (data not shown). Consistently to this observation, NO concentration in supernatants of RAW264.7 macrophages was significantly higher after stimulation with IFN-γ and LPS (Fig. 2). However, there was no difference in NO concentration regarding the culture medium used (Fig. 2).

3.3. Cytokine production

Distinct effects could be seen in release of immune modulating cytokines. Concentrations of the pro-inflammatory cytokines TNF-α as well as IL-6 in supernatants of stimulated RAW264.7 macrophages were decreased significantly following feeding with LA or LNA, respectively, compared to the control medium (Fig. 3). Moreover, incubation with the PUFA resulted in a significant increase of the anti-inflammatory cytokine IL-10 in supernatants of stimulated cells (Fig. 3). The n-6 PUFA LA thereby was more effective than the n-3 PUFA LNA at increasing IL-10.

4. Discussion

Fatty acids participate in immune defense mechanisms based on a number of ways, i.e., cell membrane composition, cell signaling pathways as well as regulation of transcription factors [27–32]. Moreover, n-3 and n-6 PUFA have been referred to alter the type and concentration of immune-regulatory eicosanoids produced [28–31]. Thus, among others the amount and the type of fatty acids in a diet may have an impact on the course of inflammatory processes. To date, most studies have focused on the effects of long-chain, highly unsaturated PUFA such as arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid, which in most diets occur in only marginal amounts [17]. Hence, there is a need to identify the impact of the shorter-chain, less unsaturated and nutritional predominant [17] PUFA LA and LNA on immune cell function. In this study, we approach this issue highlighting the influence of LA and LNA on key macrophage activities.

Supplementation of the culture medium with LA and LNA, respectively resulted in a significant alteration in the fatty acid composition of RAW264.7 macrophages. Of note, both PUFA were incorporated to a similar degree into the membranes of cultured cells. The uptake of fatty acids into cellular phospholipids mainly depends on their chain length as could be shown before [33,34]. Since the PUFA tested in our study are both 18 carbons in length, the observations made here are in accordance with these earlier results. The fatty acids

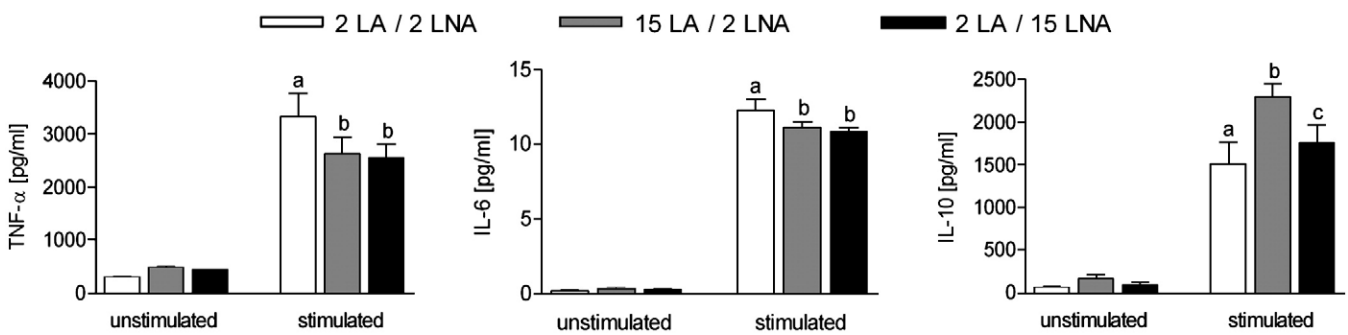

Fig. 3. Concentration of TNF-α, IL-6 and IL-10 (evaluated by ELISA) in supernatants of RAW264.7 macrophages after 72 h of incubation in medium containing 2 µmol/L LA+2 µmol/L LNA, or 15 µmol/L LA+2 µmol/L LNA, or 2 µmol/L LA+15 µmol/L LNA in the presence of (i) medium alone (unstimulated) or (ii) IFN-γ+LPS (stimulated). Data are mean±S.D. (n=6). Bars denoted by different letters are significantly different.

added to the culture medium were not only directly incorporated into the cell membranes but also were metabolized, thus resulting in a significant increase of their desaturation and elongation products mainly C20:4n6 and C20:5n3, respectively. The enhanced proportion of unsaturated fatty acids in the cell membrane thereby brought about a heightening of the MBI of the RAW264.7 macrophages. Of note, supplementation of culture medium with the n-3 fatty acid LNA resulted in a significantly higher MBI of cultured cells compared to the n-6 fatty acid LA. Cells with a heightened MBI are predicted to have an increased susceptibility against radical reactions [22]. Summing up, following supplementation there is a multitude of modifications in membrane configuration which very likely is closely connected to membrane-mediated events such as respiratory burst, cytokine release and other cellular processes [35,36].

Stimulation of RAW264.7 macrophages with PMA resulted in a significant increase of NADPH oxidase activity. This could further be heightened by culturing the cells in medium containing either LA or LNA. Thus, a key element in respiratory burst, the production of the superoxide ion ($O_2^{\cdot-}$) by NADPH oxidase, can be influenced via altering the amount of PUFA accessible to the cells. This is in accordance with previous experiments showing LA and LNA to affect membrane assembly of NADPH oxidase [37,38]. Of note, distinct differences depending on the PUFA tested could be seen. Culturing the cells in medium containing the n-3 fatty acid LNA increased NADPH oxidase activity to a much greater extent than culturing in the presence of the n-6 fatty acid LA did. Hence, cells detected to have a higher MBI are also shown to have the higher NADPH oxidase activity. The activity of NADPH oxidase therefore can be correlated to the MBI of cultured RAW264.7 macrophages. In summary, depending on PUFA family changing the pattern of dietary fatty acid intake is expected to affect immune defense by altering respiratory burst. The rise of oxidative stress of cells has already been implicated to PUFA by previous investigations. In an avian model, it was shown that diets rich in LA but deficient in vitamin E boost susceptibility to lipid peroxidation [39]. The pro-oxidative effect of n-6 fatty acids is reported by other authors, too. For instance, in men lipoproteins have been described to have an increased susceptibility to oxidation upon heightened intake of LA [40]. However, the results presented here underline the impact of PUFA especially of the n-3 fatty acid LNA in the NADPH oxidase-mediated respiratory burst. In contrast to the analyses concerning NADPH oxidase activity, no effect of PUFA supplementation on NO production of stimulated RAW264.7 cells could be ascertained. Hence, there is a complex action of the PUFA influencing respiratory burst in a divergent way.

Not only does PUFA supplementation affect respiratory burst of RAW264.7 macrophages, but also the synthesis and release of immune-regulatory cytokines by the cells are modulated. Interestingly, both PUFA tested caused a decrease in concentration of the pro-inflammatory cytokines TNF-α and IL-6 and an increase in concentration of the anti-inflammatory cytokine IL-10 in supernatants of stimulated cells. This effect was pronounced specially by the n-6 fatty acid LA boosting IL-10 synthesis to a significantly higher degree compared to the n-3 fatty acid LNA. Interaction of PUFA and cytokine production of immune cells has been studied several times. In agreement to our data, the fatty acids thereby are assigned to drive immune response into an anti-inflammatory direction [30,31]. However, most analyses are feeding studies limited to n-3 fatty acids and virtually no data about IL-10 exist. The results presented here link the fatty acids not only to arrest production of pro-inflammatory cytokines but also to promote synthesis of anti-inflammatory mediators thus extending our current knowledge. Furthermore, the distinct differences between the n-6 PUFA LA and the n-3 PUFA LNA underline the impact of PUFA family on immune response.

Focusing on the molecular mechanisms of observed effects, there are numerous possibilities. On the one hand, enrichment of cells with both LA and LNA brings about a change in eicosanoid pattern which in turn affects the activity of immune cells [28–31]. However, eicosanoid synthesis is a time-consuming process. So it is unlikely to be of importance at the conditions used in our experiments. On the other hand, changes in membrane fatty acid patterns themselves impact cell signaling pathways and membrane-associated enzymes via modulating membrane fluidity [27–32]. Moreover, a direct interaction between receptors, enzymes or transcription factors with the fatty acids released from membrane phospholipids upon stimulation would be conceivable [27–32]. Hence, to elucidate this complex interplay, future studies dealing with the effects of LA and LNA on cell signal transduction are necessary.

Summing up, using RAW264.7 macrophages as a model system, we identified the essential 18-carbon PUFA LA and LNA to affect macrophage function thereby influencing production of both superoxide radicals and immune modulating cytokines. The concentrations of free fatty acids tested in our study match physiological conditions [41,42], thus underlining the relevance of gained results. Since respiratory burst and cytokine production by macrophages represent early stages of the immune response which ultimately trigger the more specific acquired immune response, PUFA in this way may interact in immune defense mechanisms comprehensively. Hence, understanding the role of fatty acids in macrophage-mediated processes may help to identify new strategies in the battle against pathogen microorganisms.

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