Fatty acid control of nitric oxide production by macrophages

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Abstract Modulation of macrophage functions by fatty acids (FA) has been studied by several groups, but the effect of FA on nitric oxide production by macrophages has been poorly examined. In the present study the effect of palmitic, stearic, oleic, linoleic, arachidonic, docosahexaenoic and eicosapentaenoic acids on NF-κB activity and NO production in J774 cells (a murine macrophage cell line) was investigated. All FA tested stimulated NO production at low doses (1–10 μM) and inhibited it at high doses (50–200 μM). An increase of iNOS expression and activity in J774 cells treated with a low concentration of FA (5 μM) was observed. The activity of NF-κB was time-dependently enhanced by the FA treatment. The inhibitory effect of FA on NO production may be due to their cytotoxicity, as observed by loss of membrane integrity and/or increase of DNA fragmentation in cells treated for 48 h with high concentrations. The results indicate that, at low concentrations FA increase NO production by J774 cells, whereas at high concentrations they cause cell death.

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1. Introduction

NO produced by activated macrophages has been shown to regulate antimicrobial and antitumor activities. However, NO production in excess causes tissue damage that is associated with acute and chronic inflammation [1]. NO is synthesized from L-arginine by NO synthase (NOS) using NADPH and oxygen as cosubstrates [2]. Macrophages stimulated with lipopolysaccharide (LPS) and pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) [3,4] produce large amounts of NO through inducible NOS (iNOS) activity. The expression of iNOS is regulated by the nuclear factor kappa B (NF-κB) in several cell types, including macrophages [5,6]. NF-κB plays an important role in controlling inflammatory gene activation [7]. This transcription factor is usually found in the cytosol as a heterodimer complex with its inhibitory protein, IκB. When cells are stimulated with LPS, phorbol ester or inflammatory cytokines, IκB is phosphorylated by IκB kinase and degraded. IκB phosphorylation dissociates the dimmer and allows NF-κB to translocate to the nucleus, where it activates target genes, including iNOS [8].

Recent studies have shown that fatty acids (FA) can modulate NF-κB activation. In human monocytic THP-1 cells, linoleic (LA), α-linolenic (ALA) and docosahexaenoic (DHA) acids decreased NF-κB DNA-binding activity [9]. Palmitic acid (PA) increased NF-κB activity in 3T3-L1 adipocytes [10] and pericytes [11]. On the other hand, palmitic (PA), oleic (OL) and linoleic (LA) acids induced iKKα activation and decreased NF-κB activity in endothelial cells [12]. Eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids decreased LPS induced NF-κB activation in human kidney-2 (HK-2) cells [13].

Several authors have shown modulation of macrophage functions by FA [14–17]. The ω-6 polyunsaturated FA usually stimulate the inflammatory response, whereas ω-3 FA have been considered as anti-inflammatory agents. The effect of FA has been examined on cytokine, eicosanoids and reactive oxygen species production [18–20], and adhesion molecule expression [17,21]. However, the effect of FA on NO production and iNOS expression in macrophages has been poorly examined [22–25]. The studies have shown that ω-3 FA, especially DHA, markedly suppress NO production and iNOS expression in murine macrophages. An increase in NO production by macrophages from animals fed ω-3 FA rich diets has also been reported [26,27]. Up to now, however, there is no report comparing the effect of the more abundant FA in plasma, palmitic (saturated) and oleic acids (monounsaturated, ω-9), with ω-3 (DHA and EPA) and ω-6 (linoleic and arachidonic) FA.

As described above, NO production and iNOS expression are regulated by NF-κB activation, and the activity of this transcription factor can be modulated by FA. This information led us to investigate the effect of various FA on NF-κB activity and NO production in J774 cells (a murine macrophage cell line). The following FA were studied: palmitic (PA), stearic (SA), oleic (OA), linoleic (LA), arachidonic (AA), docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids.

2. Materials and methods

2.1. Reagents

RPMI-1640 medium, HEPES, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Fatty acids, LPS, sulfanilamide, naphthylene diamine dihydrochloride, sodium pyrophosphate and sodium orthovanadate were obtained from Sigma (St. Louis, MO, USA). Ethanol and phosphoric acid were purchased from Merck (Frankfurt, Germany). Sodium bicarbonate and sodium nitrite were purchased from Labsynth products (Diadema, SP, Brazil). DAF-DA was obtained from Molecular Probes (Eugene, OR, USA).
2.2. Culture conditions and fatty acid treatment

J774 cells were grown in RPMI-1640 medium containing 10% fetal calf serum (FCS). This medium was supplemented with glutamine (2 mM), HEPES (20 mM), streptomycin (10,000 μg/mL), penicillin (10,000 U/mL) and sodium bicarbonate (24 mM). Cells were grown in 75 cm² flasks containing 0.5·10⁶ cells per mL. The cells were kept in a humidified atmosphere containing 5% CO₂ at 37 °C.

Cells were treated with various concentrations (1–200 μM) of palmitic (PA), stearic (SA), oleic (OA), linoleic (LA), arachidonic (AA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids for different periods (from 3 up to 48 h). The fatty acids were dissolved in ethanol. The final concentration of ethanol in the culture medium did not exceed 0.5%. This concentration of ethanol is not toxic to the cells as reported by Siddiqui et al. [28].

2.3. Determination of nitric oxide

The content of nitrite was measured in the supernatant of cultured cells based on the method described by Ding et al. [29]. Cells (5·10⁶ per well) were seeded in 96 well plates and treated with 2.5 μg per mL LPS and different concentrations of FA for 48 h. At the end of the culture period, 50 μL of the supernatant were removed and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethenediamine dihydrochloride, 2.5% H₃PO₄) at room temperature for 10 min. The absorbance was determined at 550 nm. Nitrite concentration was determined by using sodium nitrite as standard.

2.4. Determination of iNOS protein expression

J774 cells were seeded in 25 cm² flasks and treated with 2.5 μg per mL LPS and 5 μM of the FA for 6, 12 and 24 h. At the end of the incubation period, cells were immediately homogenized in 150 μL extraction buffer (100 mM Trizma, pH 7.5; 10 mM EDTA; 100 mM sodium dodecyl sulfate (SDS); 100 mM NaF; 10 mM sodium pyrophosphate; 10 mM sodium orthovanadate) at 100 °C for 30 s. Samples were boiled for 5 min and centrifuged at 12000 rpm, for 40 min, at 4 °C. Aliquots of supernatants were used for the measurement of total protein content, as described by Bradford [30]. Equal amount of protein of each sample (70 μg) was separated using 5% SDS-polyacrylamide gel. Western blotting was carried out following the method described by Towbin et al. [31]. The proteins of the gel were transferred to a nitrocellulose membrane for 2.5 h at 100 V. After blocking for 1 h in a solution containing 5% defatted milk, at room temperature, the membranes were incubated with anti-iNOS antibody (Boster, CA, USA). After washing the membranes, they were incubated with anti-rabbit IgG antibody linked to horseradish peroxidase. Membranes were washed in basal solution three times for 10 min each and then incubated with 2·10⁻⁶ LPS and 5 μl of different concentrations of FA for 30 min. Films were then revealed in the conventional manner. Band intensities were analyzed using the ScionImage software (Scion Corporation, MD, USA).

2.5. Measurement of iNOS activity

iNOS activity was measured in J774 cells treated for 12, 24 and 48 h with 2.5 μg per mL LPS and 5 μM of FA. This assay is based on the biochemical conversion of L-arginine to L-citrulline by iNOS. Cells were homogenized in ice-cold Tris–HCl buffer (20 mM Tris–HCl, 10 mM EDTA, and 10 mM EGTA, pH 7.4) using a Teflon homogenizer. The homogenates were centrifuged at 12000 x g for 5 min at 4 °C. Supernatants were removed and iNOS activity assay was performed by incubating (37 °C for 20 min) 150 (20 μL) of protein in a final volume of 60 μL of assay mixture containing 50 mM Tris–HCl, 6 μM tetrahydrobiopterin, 2 μM FAD, 2 μM FMN, 10 mM NADPH, 100 μM L-arginine/L-[H³]-arginine (5 μCi/mL), 1 mM EDTA/EGTA. The reaction was stopped with 1 mL of ice-cold stop buffer (30 mM HEPES and 5 mM EDTA, pH 5.5) and 100 μL of cation-exchange resin (Dowex, Na⁺ form, equilibrated with 50 mM HEPES, pH 5.5) was added to each reaction mixture to remove the excess of L-[H³]-arginine. Aliquots were collected into vials, scintillation liquid (6 mL) was added and radioactivity was quantified in a scintillation counter (Packard TRI CARB 2100 TR Counters, Downers Grove, IL, USA). Protein concentrations in samples were determined as described by Bradford [30] with bovine serum albumin as standard.

2.6. Electrophoretic mobility shift assay

NFkB activation was evaluated after treatment of the cells for 3, 6, 12, 24 and 48 h with 5 μM FA.

Nuclear extract from J774 cells was obtained as previously described [32]. Double-stranded oligonucleotides containing the NF-kB (5′-AGTCTGGGACCTTCCAGGC-3′) consensus binding site [32] were end-labeled using T4 PNK and [γ-32P]ATP (Amersham Biosciences). Binding reactions of the probes (30000 cpm) were performed with 10 μg proteins from nuclear extract, at room temperature, for 20 min, in 20 μL of the binding buffer consisting of 20 mM HEPES, pH 7.6, 50 mM KCl, 10% glycerol, 0.2 mM EDTA, 1 mM DTT and 2 μg polyethyleneimino-deoxyectylic acid (poly[dI–dC]). Competitive binding assays were conducted under the same conditions with the addition of 2 pmol (100-fold molar excess) of unlabeled competitor oligonucleotides. The DNA–protein complexes were electrophoresed on 4% non-denaturing polyacrylamide gels, at 4 °C, in 45 mM Tris, 45 mM borate and 1 mM EDTA buffer. The gels were dried and subjected to autoradiography. The bands were analyzed by scanner densitometry (Image Master 1D, Amersham Biosciences) and the results of the binding activity were expressed as arbitrary units.

2.7. Determination of fatty acid cytotoxicity

Fatty acid cytotoxicity was assessed by flow cytometry after treatment of the cells for 48 h with high concentrations of fatty acids. At the end of the culture period, 0.5 mL of medium containing cells were used to evaluate the membrane integrity. In this assay, 50 μL of a propidium iodide (PI) solution (100 μg per mL in saline buffer) were added to the cells. Propidium iodide is a highly water-soluble fluorescent compound that cannot pass through intact membranes and is generally excluded from viable cells. It binds to DNA by intercalating between the bases with little or no sequence preference. After 5 min incubation at room temperature, the cells were evaluated in a FACScalibur flow cytometry equipment (Becton Dickinson, CA, USA) by using the Cell Quest software. Fluorescence was measured using the FL2 channel (Orange-red fluorescence – 585/42 nm). Ten thousand events were analyzed per experiment.

We also determined the percentage of cells with fragmented DNA after treatment of cells with fatty acids and different concentrations of propidium iodide. In this assay, cells were resuspended in a solution containing detergents that permeabilize the cells, which promptly incorporate the dye into DNA. Briefly, 0.5 mL of medium containing cells were centrifuged at 1000 x g, for 10 min, at 4 °C. The pellet was gently resuspended in 300 μL hypotonic solution containing 50 μg/mL propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. The cells were then incubated for 2 h at 4 °C. Fluorescence was measured and analyzed by flow cytometry as described above. Both the loss of membrane integrity and/or DNA fragmentation were considered signs of toxicity, regardless which one was first observed.

2.8. Statistical analysis

Results are presented as means ± S.E.M. of 6–9 determinations from 2–3 experiments. Comparisons with control were performed by analysis of variance (2-way-ANOVA). Significant differences were analysed by the Bonferroni post-tests (Graph Pad Prism 4 – Graph Pad Software Inc., San Diego, CA, USA). The level of significance was set at P < 0.05.

3. Results

3.1. Effect of fatty acids on NO production

All FA tested stimulated NO production at low doses but showed inhibitory effect at high concentrations (Fig. 1). The...
stimulatory effect of the FA was more pronounced at concentrations between 2.5 and 5 μM (P < 0.001). At 5 μM, palmitic acid was the less effective, inducing an increase of 32% in NO production. Arachidonic acid, on the other hand, was the most potent, increasing in 119% the production of NO. The crescent order of stimulatory effect at 5 μM was: PA (32%), SA (60%), DHA (79%), LA (83%), EPA (89%), OA (94%), and AA (117%). PA and EPA inhibited NO production at 50 μM. SA suppressed NO production at 100 μM, AA and DHA at 150 μM, and OA and LA at 200 μM.

Fig. 1. Effects of fatty acids on NO production of J774 cells. The concentration (μM) of nitrite measured in the supernatant of cultured cells after treatment for 48 h with palmitic, stearic, oleic, linoleic, arachidonic, docosahexaenoic and eicosapentaenoic acids in the presence of 2.5 μg per mL LPS are shown. The supernatant was incubated with an equal volume of Griess reagent and the absorbance was determined at 550 nm. The values are presented as means ± S.E.M. of 16 determinations from four experiments. * P < 0.001 for comparison with control.
3.2. Effect of fatty acids on iNOS expression
In order to investigate the mechanisms involved in the regulation of NO production by FA, we determined iNOS protein expression in J774 cells treated for 6, 12 and 24 h with 5 µM FA in the presence of LPS (2.5 µg/mL). Cells treated with PA did not present changes in iNOS expression when compared with those treated with LPS and ethanol at any period of treatment. SA and OA treated cells presented higher iNOS expression after 12 h (P < 0.001). Cells treated with LA, AA, DHA and EPA showed increased iNOS expression after 6 h of treatment (P < 0.001) and this effect remained up to 24 h (Fig. 2).

3.3. Involvement of NFκB activation on modulation of iNOS expression by FA
Treatment of J774 cells with 5 µM FA altered NFκB activation as observed in Fig. 3. However, the period of incubation for the effect of FA to be observed varied considerably. LPS caused NFκB activation and presented the more potent effect after 3 h treatment (115%) but the addition of ethanol (vehicle) diminished NFκB activation by 16.6 ± 3.2% (means ± S.E.M. of five experiments). PA, DHA and EPA increased NFκB activation after 3 h treatment, when compared to cells treated with LPS and ethanol, by 51%, 52% and 62%, respectively. AA exerted its stimulatory effect after 6 h of treatment (36%) and reached its peak after 24 h (106%). OA increased NFκB activation after 12 h (39%). SA and LA did not affect NFκB activation at any period of treatment tested.

3.4. Effect of fatty acids on iNOS activity
Cells treated with AA for 12 h showed higher iNOS activity when compared with the correspondent control (ethanol 12 h). Exposure for 24 h to PA also led to an increased of iNOS activity. Cells treated with OA, LA, DHA and EPA presented higher iNOS activity after 48 h treatment. SA did not present significant effect on iNOS activity (Fig. 4).

3.5. Effect of fatty acids on cell viability and DNA fragmentation
In order to evaluate if the inhibitory effect of the FA on NO production at high concentrations was due to their cytotoxic-

![Graph](image-url)

Fig. 2. Effects of fatty acids on LPS induced iNOS expression. Cells were treated for 6, 12 and 24 h with 2.5 µg per mL LPS and 5 µM of the FA. Whole cell lysates were dissolved in a sample buffer and submitted to 8% SDS–PAGE. Western blotting was performed using mouse anti-iNOS polyclonal antibody. Band intensities were analysed using the ScionImage software (Scion Corporation) and are expressed as relative values compared to the respective control (LPS and ethanol). Controls received an arbitrary value of 1. The values are presented as means ± S.E.M. of three experiments. *P < 0.001, #P < 0.01 for comparison with control.
Fig. 3. Results of the electrophoretic mobility shift assay. (Right) Nuclear extracts prepared from J774 cells that had been treated with 2.5 μg per mL LPS and 5 μM of the FA for 3, 6, 12, 24 and 48 h were used for protein–DNA binding reactions in the presence of the radio-labeled probe (~30,000 cpm), as described in Section 2. A: control, B: ethanol, C: LPS 2.5 μg per mL, D: LPS 2.5 μg per mL + ethanol, E: PA 5 μM, F: SA 5 μM, G: OA 5 μM, H: LA 5 μM, I: AA 5 μM, J: DHA 5 μM, K: EPA 5 μM. (Left) Band intensities were analysed using the ScionImage software (Scion Corporation) and are expressed as relative values compared to the respective control. Controls received an arbitrary value of 1. The values are presented as means ± S.E.M. of three experiments. * P < 0.001, P < 0.05 for comparison with cells treated with LPS + ethanol.
ity, the percentage of cells with intact cell membrane and with fragmented DNA after the treatment for 48 h was determined (Fig. 5). All FA induced loss of membrane integrity, except AA. Cells treated with PA (50 μM), SA (100 μM), OA (200 μM), LA (200 μM), DHA (150 μM) and EPA (100 μM) presented a significant decrease in cell membrane integrity by 34%, 26%, 12%, 24%, 27% and 18%, respectively. The treatments also induced DNA fragmentation, except for LA. An increase in the percentage of cells with fragmented DNA was observed after treatment with PA (50 μM), SA (100 μM), OA (200 μM), AA (150 μM), DHA (150 μM) and EPA (100 μM) by 2-, 1.9-, 1.7-, 2.1-, 1.7- and 1.6-fold, respectively. Cells treated with ethanol, the vehicle used for FA preparation, did not present loss of membrane integrity or induction of DNA fragmentation, indicating that the concentration of ethanol used (0.5%) is not cytotoxic.

4. Discussion

The effect of different concentrations of various FA on nitric oxide production by J774 cells and the possible mechanisms involved were investigated in this study.

J774 cells cultivated for 48 h with FA and LPS showed high production of NO as compared to control cells, especially at low concentrations (1–10 μM). Cells treated with LA, AA, DHA and EPA showed a stimulatory effect up to 25 μM. On the other hand, higher concentration (50–200 μM) inhibited NO production. These results seem controversial but they may help to explain the discrepancy in the literature. Many studies observed a decrease in NO production by mice macrophages and cell lines after exposure to FA [22–25], whereas others found an increase [27,34]. In addition to cell type and period of stimulation, these controversial results may be also due to the different concentrations of the FA used. The inhibitory effect of FA on NO production may be due to their cytotoxicity, as observed by loss of membrane integrity and/or increase of DNA fragmentation in cells treated for 48 h with high concentrations. FA toxicity has been reported in several cell types, as a concentration and time-dependent effect of these metabolites [35,36].

The stimulatory effect of FA was also observed by fluorescence microscopy using DAF-DA. Cells treated for 12 and 24 h presented high intracellular NO content, which diminished after 48 h of exposure to FA. These results corroborate with the low level of NO in the medium of cells treated with FA for 24 h (data not shown).

High production of NO is achieved when the expression of inducible NOS is stimulated [3]. J774 cells treated with LPS and FA (5 μM) presented higher expression of iNOS protein than cells treated with LPS and ethanol (vehicle). The period of incubations where they exerted their effects varied, and may be related to the activity of the nuclear factor kappa B (NF-κB). Activation of NF-κB can trigger inflammatory responses by transcriptional induction of several pro-inflammatory proteins and enzymes that generate mediators of inflammation (e.g. iNOS) [37,38]. J774 cells presented significant NF-κB activity in basal conditions (absence of LPS and FA) that was increased by two fold after LPS addition. The FA tested, with exception of SA and LA, stimulated NF-κB activation, and this effect occurred prior to or at the same incubation period where an increase in iNOS protein level was observed.

Several studies have shown a decrease in NF-κB activation by DHA and EPA [39–44]. However, Maziere et al. [45] observed an increase of NFκB activation in fibroblasts treated with DHA and EPA and Camandola et al. [46] stated that EPA (45 μM) exerts no effect on the nuclear translocation of NF-κB in the human promonocytic cell line U937. In the present study, DHA and EPA stimulated NO production in J774 cells at low concentrations (1–25 μM). Concomitantly, an increase of NF-κB activation was observed at 5 μM, indicating that this effect on NF-κB may raise iNOS protein level and, consequently, NO production.

Stimulatory effect of PA, AA and OA on the nuclear translocation of NF-κB has also been observed in different cells.
types, such as L6 myotubes, mouse C2C12 myoblasts, skeletal muscle cells, human promonocytic cell line U937, human endothelial cells and mouse macrophages [46–51]. On the other hand, no effect on NF-κB activation and iNOS expression was observed in insulinoma (INS)-1E cells, human fibroblasts and human endothelial cells treated with OA [52,53].

In the periods of incubation studied, LA and SA did not affect NF-κB activation but increased iNOS expression after 6 and 12 h, respectively. Other transcriptional factors are involved in iNOS expression, as the octamer factor (Oct) [54,55], signal transducer and activator of transcription-1a (STAT-1a) [56–58], cAMP-induced transcription factors such as cAMP-responsive element binding protein (CREB) and CCAAT-enhancer box binding protein (C/EBP) [59,60], and activating protein-1 (AP-1) [61]. SA and LA may have activated one of these transcriptional factors and increased iNOS expression.

Three proteins that interact with iNOS and regulate its activity have been recently identified. In the central nervous system, the protein kalirin appears to inhibit iNOS by preventing enzyme dimerization [62]. In murine macrophages, a 110-kDa protein (named NAP110) has been identified that directly interacts with the amino-terminus of iNOS, thereby preventing dimer formation and inhibiting NOS activity [63]. Stable overexpression of Rac2 in RAW 264.7 cells increased LPS-induced nitrite generation and iNOS activity without measurably affecting iNOS protein abundance [64]. Our results suggest that FA are also modulators of iNOS activity in J774 cells.

The results presented herein demonstrate that FA stimulate NO production by J774 cells at low concentrations (1–10 μM) and inhibit it at high doses (50–200 μM). The stimulatory effect of most FA on NO production is time dependent, involves NF-κB activation, and increase of iNOS expression and activ-

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**Fig. 5.** Effects of fatty acids on cell membrane integrity and DNA fragmentation. The percentage of cells with intact membrane (A) and fragmented DNA (B) after treatment for 48 h with palmitic (PA), stearic (SA), oleic (OA), linoleic (LA), arachidonic (AA), docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids are shown. Cells were stained with a saline buffer containing propidium iodide to assess membrane integrity. A buffer containing citrate, triton X-100 and propidium iodide was used to assess DNA fragmentation. The values are presented as means ± S.E.M. of 9 determinations from three experiments. *P < 0.001 for comparison with control (ethanol – OH).
ity. The inhibitory effect of FA on NO production by J774 cells is mainly due to their cytotoxicity.

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