Modulatory effect of fatty acids on fungicidal activity, respiratory burst and TNF-α and IL-6 production in J774 murine macrophages

Thais Martins de Lima-Salgado[1]*, Sandra Coccuzzo Sampaio[2], Maria Fernanda Cury-Boaventura[3] and Rui Curi[4]

[1]Emergency Medicine Division LIM 51, Faculdade de Medicina, University of São Paulo, Avenue Dr. Arnaldo, 455, Room 3189, CEP 01246-903, São Paulo, SP, Brazil
[2]Laboratory of Pathophysiology, Butantan Institute, São Paulo, SP, Brazil
[3]Post-Graduate Program in Human Movement Science, Biological Sciences and Health Center, Cruzeiro do Sul University, São Paulo, SP, Brazil
[4]Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil

Abstract

The reported effects of different families of fatty acids (FA; SFA, MUFA, n-3 and n-6 PUFA) on human health and the importance of macrophage respiratory burst and cytokine release to immune defence led us to examine the influence of palmitic acid (PA), oleic acid (OA), linoleic acid, arachidonic acid, EPA and DHA on macrophage function. We determined fungicidal activity, reactive oxygen species (ROS) and cytokine production after the treatment of J774 cells with non-toxic concentrations of the FA. PA had a late and discrete stimulating effect on ROS production, which may be associated with the reduced fungicidal activity of the cells after treatment with this FA. OA presented a sustained stimulatory effect on ROS production and increased fungicidal activity of the cells, suggesting that enrichment of diets with OA may be beneficial for pathogen elimination. The effects of PUFA on ROS production were time- and dose-dependently regulated, with no evident differences between n-3 and n-6 PUFA. It was worth noting that most changes induced after stimulation of the cells with lipopolysaccharide were suppressed by the FA. The present results suggest that supplementation of the diet with specific FA, not classes of FA, might enable an improvement in host defence mechanisms or a reduction in adverse immunological reactions.

Key words: J774 cells: Fatty acids: Fungicidal activity: Respiratory burst: TNF-α: IL-6

Monocytes and granulocytes are involved in the innate immune response, and their activation triggers the elimination of invading pathogens and repair of damaged tissue. These cells internalise microbes and lead to the formation of a phagosome into which reactive oxygen species (ROS) (such as superoxide and H_2O_2) and hydrolytic enzymes are secreted in order to kill the microbes. Moreover, via antigen presentation and secretion of immune-modulating cytokines, these cells make a considerable contribution to specific immune response activation[1].

Research conducted over the last 30 years has suggested that changing the nature of fatty acid (FA) nutrition can modify immune cell behaviour and the immune response, including its inflammatory component. Modifying the FA composition of the diet of animals leads to an altered FA composition of immune cells, typically with an increased appearance of the FA in which the diet has become enriched[2,3]. Changes in FA composition can alter the physical properties of the membrane such as membrane fluidity and raft structure, location and function of proteins, which might affect signalling mechanisms and membrane-associated enzymes necessary for the phagocytosis of microbes and the respiratory burst[4–7]. FA also regulate gene expression either through the effects on receptor activity, on intracellular signalling processes or through the effects on transcription factor activation[8–10]. These effects may lead to an altered production of important inflammatory mediators such as cytokines.

Recently, modulation of macrophage functions by FA has been reported. Yaqoob[11] demonstrated that diets rich in MUFA reduce the adhesion of peripheral monocytes, while Calder et al.[12] observed that SFA increase the adhesion of macrophages in culture when compared with PUFA. These observations may be related to altered expression of adhesion molecules. Monocytes treated with EPA have a low expression of inter-cellular adhesion

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molecule 1, while cells treated with DHA have an increased expression of adhesion molecules[13]. The production of ROS can also be modulated by FA. Our group showed that different concentrations of FA modulate inducible NO synthase expression and NF-κB activity[14]. Regarding cytokine production, most studies performed so far have shown that n-6 PUFA increase the production of pro-inflammatory cytokines, such as IL-1β, IL-8 and IL-6, while n-3 PUFA have an opposite effect[15,16].

The proportion of different classes of FA in the diet, especially the ratio between n-6 and n-3 PUFA, and their significance in determining cell function, whole-body physiology and human health are still a matter of debate[17]. Although the literature has some data concerning modulation of macrophage functions by FA, there is no study showing how treatment of macrophages with FA from distinct families (SFA, MUFA, n-3 and n-6 PUFA) could differentially affect the respiratory burst and cytokine release. Hence, given the interest in the effects of different families of FA on human health and regarding the importance of macrophage respiratory burst and cytokine release to immune defence, we compared the influence of palmitic acid (PA), oleic acid (OA), linoleic acid (LA), arachidonic acid (AA), EPA and DHA on these key macrophage functions. All experiments were performed in J774 murine macrophages.

Materials and methods

Reagents

Roswell Park Memorial Institute 1640 (RPMI-1640) culture medium, fetal calf serum, HEPES, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). FA, glutamine, HEPES, lipopolysaccharide (LPS) and phenol red were obtained from Sigma (St Louis, MO, USA). Ethanol was purchased from Merck (Frankfurter, Darmstadt, Germany), and sodium bicarbonate was from Labsynth Products (Diadema, SP, Brazil). Dihydroethidium was obtained from Molecular Probes (Eugene, OR, USA).

Culture conditions

J774 cells were grown in RPMI-1640 culture medium containing 10% fetal calf serum. This medium was supplemented with glutamine (2 mM), HEPES (20 mM), streptomycin (10 000 μg/ml), penicillin (10 000 UI/ml) and sodium bicarbonate (24 mM). Cells were grown in 75 ml flasks containing $0·5–1 \times 10^6$ cells/ml. The cells were kept in a humidified atmosphere containing 5% CO_2 at 37°C.

Fatty acid treatment

The cells were diluted to $2·5 \times 10^5$ cells/ml in a twenty-four-well plate. On the following day, when the number of cells reached 5×10^5 cells/ml, they were treated with various concentrations (25, 50 and 100 μM) of PA, OA, LA, AA, EPA and DHA for 1 or 24 h, in the presence or absence of LPS (2·5 μg/ml) to mimic an inflammatory condition. PA was not used at 100 μM due to its toxicity, as described previously by our group[18]. The FA were maintained in the dark at −20°C and freshly dissolved in ethanol before use. The final concentration of ethanol in the culture medium did not exceed 0·05%. This concentration of ethanol is not toxic to cells, as demonstrated previously[19].

Fungicidal activity of macrophages

Coverslips containing macrophages were incubated with 1 ml of RPMI-1640 culture medium containing opsonised *Candida albicans*, for 120 min, at 37°C. The number of particles per coverslip was of approximately 2×10^6 (ten times greater than the number of cells). The percentage of phagocytosis was determined after the coverslips were stained with a panchromatic dye and examined under light microscopy. In this method, live yeast was stained blue, whereas the dead cells were not stained. Fungicidal activity is determined as the score sum of 100 counted cells, as established by Herscowitz et al.[20] and described as follows:

Result	Score
No. of cells without dead *C. albicans*	× 0
No. of cells with one or two dead *C. albicans*	× 1
No. of cells with three or four dead *C. albicans*	× 2
No. of cells with more than four dead *C. albicans*	× 3

Intracellular production of reactive oxygen species

Dihydroethidium was used for the flow cytometric measurement of intracellular superoxide production. Dihydroethidium is rapidly oxidised to ethidium (a red fluorescent compound) by superoxide ($O_2^{·-}$). Ethidium is trapped in the nucleus by intercalating into DNA, leading to an increase of ethidium fluorescence. After FA treatment, the cells were stained with dihydroethidium (10 μM) by incubating for 30 min at room temperature in the dark and analysed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Fluorescence was measured using an FL-3 filter (670 nm). Cells with dihydroethidium fluorescence were then evaluated using CellQuest software (Becton Dickinson).

Extracellular production of reactive oxygen species

H_2O_2 production by J774 cells was measured using phenol red. This assay is based on horseradish peroxidase-dependent conversion of phenol red into a coloured compound by H_2O_2. After FA treatment, macrophages (final volume 0·1 ml) were incubated in the presence of glucose (5 mM), phenol red solution (0·56 mM) and horseradish peroxidase (8·5 UI/ml) in the dark for 1 h at 37°C. After this period, the

absorbance was measured at 620 nm on a plate reader. The concentration of H_2O_2 was determined from a standard curve prepared in parallel.

Measurement of cytokines

After FA treatment, the cell culture supernatant was harvested and the production of TNF-α and IL-6 was determined by ELISA using the OptEIA kit from Pharmingen (San Diego, CA, USA).

Statistical analysis

Data are presented as means with their standard errors of six to nine determinations from two to three experiments. Comparisons with the control were performed by ANOVA. Significant differences were analysed using the Tukey–Kramer test (GraphPad Prism 4; Graph Pad Software, Inc., San Diego, CA, USA). The level of significance was set at $P < 0.05$.

Results

Fungicidal activity of macrophages

Fungicidal activity was determined by counting the number of dead *C. albicans* inside J774 cells that had been previously treated with no toxic concentrations of the FA for 24 h (Fig. 1). PA reduced fungicidal activity in a dose-dependent manner (0·77 (SE 0·04) at 25 μM and 0·42 (SE 0·12) at 50 μM). OA presented a stimulatory effect especially at 50 μM (1·79 (SE 0·16)). LA presented no effect. AA and DHA stimulated the *C. albicans* killing activity of J774 cells at low concentrations but inhibited at 100 μM (0·66 (SE 0·14) and 0·48 (SE 0·35), respectively). EPA also presented an inhibitory effect at 100 μM (0·42 (SE 0·14)).
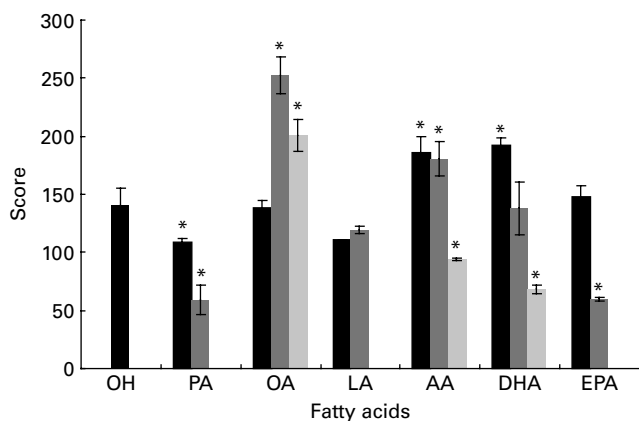
Fig. 1. Fungicidal activity of J774 cells treated with different concentrations of the fatty acids for 24 h. The amount of dead *Candida albicans* was determined after staining the coverslips with a panchromatic dye and examination under light microscopy. Values are means of six coverslips from two experiments, with standard errors represented by vertical bars. * $P < 0.05$ for comparison with control cells treated with ethanol (OH). PA, palmitic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid. ■, 25 μM; ■, 50 μM; □, 100 μM.
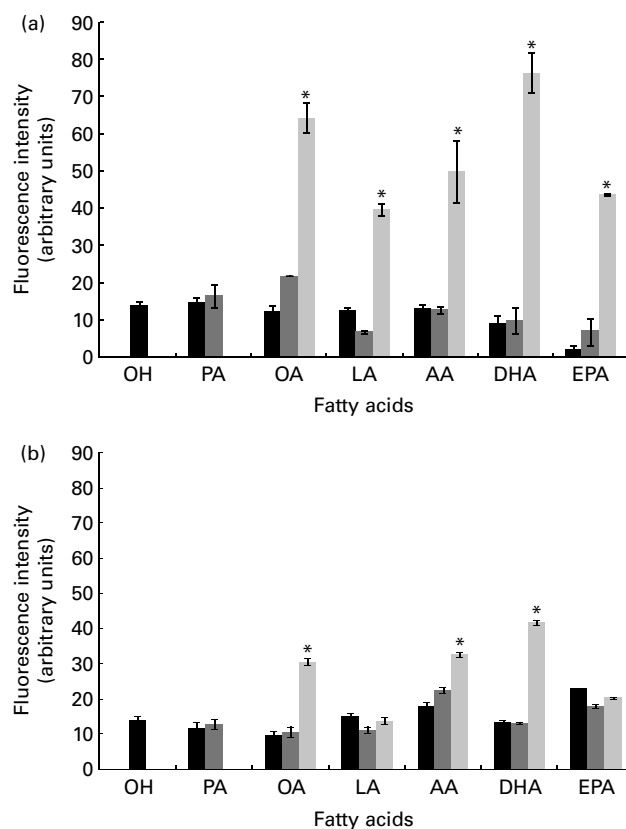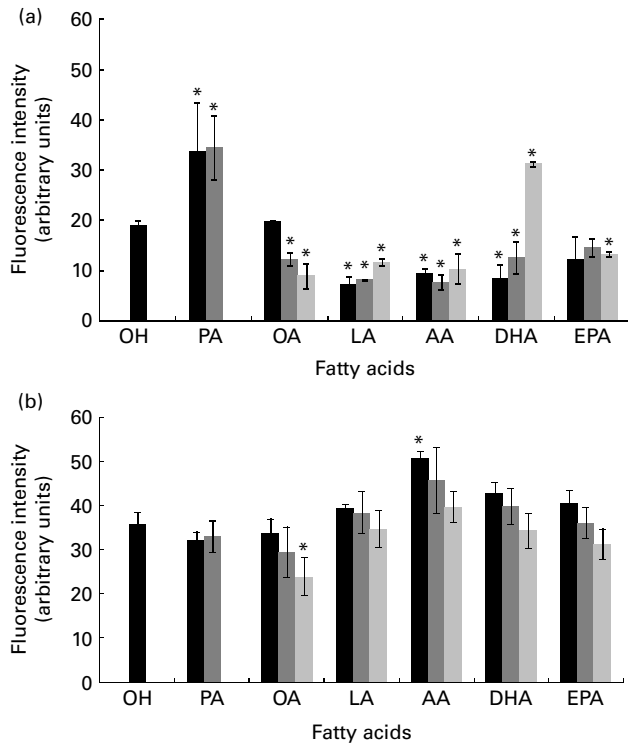

Fig. 2. Intracellular reactive oxygen species content in J774 cells treated with fatty acids (FA) for 1 h. Dihydroethidium was used for the flow cytometric measurement of intracellular superoxide content. (a) Cells treated with FA only. (b) Cells treated with FA and lipopolysaccharide (2·5 μg/ml). Values are means of three experiments in triplicate, with standard errors represented by vertical bars. * $P < 0.05$ for comparison with control cells treated with ethanol (OH). PA, palmitic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid. ■, 25 μM; ■, 50 μM; □, 100 μM.

Intracellular content of reactive oxygen species

Incubation with all FA at 100 μM for 1 h induced a large increase in the intracellular content of ROS in J774 cells (Fig. 2). The percentage of increase varied from 284 % for LA to 548 % for DHA. PA, which was not used in concentrations higher than 50 μM due to its toxicity, did not present any effect. In the presence of LPS, the stimulatory effect of all FA (100 μM) was significantly lower than the one observed without LPS. For example, the stimulatory effect of DHA (100 μM) decreased from 548 to 300 %. In fact, LA and EPA had no effect on intracellular ROS content (Fig. 2(b)).

Treatment of the cells for 24 h with the FA had different results. PA, which had no effect in 1 h treatment, stimulated ROS production by the cells by twofold. OA, LA, AA and EPA, on the other hand, diminished the intracellular ROS content. DHA presented a stimulatory effect at 100 μM only (1·6 (SE 0·33); Fig. 3(a)). LPS doubled ROS production in control cells (1·82 (SE 0·23)). The FA did not alter the LPS effect, with the exception of 100 μM-OA that had an inhibitory effect (0·66 (SE 0·08)) and 25 μM-AA that caused a small stimulatory effect (1·48 (SE 0·19); Fig. 3(b)).

Fig. 3. Intracellular reactive oxygen species content in J774 cells treated with fatty acids (FA) for 24 h. Dihydroethidium was used for the flow cytometric measurement of intracellular superoxide content. (a) Cells treated with FA only. (b) Cells treated with FA and lipopolysaccharide (2·5 μg/ml). Values are means of three experiments in triplicate, with standard errors represented by vertical bars. * $P < 0.05$ for comparison with control cells treated with ethanol (OH). PA, palmitic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid. ■, 25 μM; ■, 50 μM; □, 100 μM.

Extracellular reactive oxygen species concentration

H_2O_2 content was determined in the culture medium after 24 h treatment with the FA. PA, OA, LA and DHA stimulated H_2O_2 production in a dose-dependent manner. The stimulatory peak was 2·74 (SE 0·18) for PA, 5·83 (SE 0·16) for OA, 4·82 (SE 0·17) for LA and 2·58 (SE 0·13) for DHA. AA and EPA exerted a small stimulatory effect at 100 μM only (2·01 (SE 0·08) and 2·58 (SE 0·13), respectively). LPS treatment stimulated H_2O_2 production by the cells, but only OA and DHA increased the stimulatory effect of LPS (3·38 (SE 0·01) and 4·57 (SE 0·23), respectively; Fig. 4).

Production of TNF-α and IL-6

J774 cells treated with 50 μM-PA and -OA for 24 h produced a higher amount of TNF-α (Fig. 5). DHA and EPA had no effect, whatever the concentration tested. PA treatment for 24 h in the presence of LPS doubled TNF-α production by J774 cells when compared with cells treated with LPS only (2·85 (SE 0·31)). On the other hand, OA, LA and DHA reduced the LPS effect (0·53 (SE 0·01), 0·60 (SE 0·04) and 0·78 (SE 0·02), respectively at 100 μM).

IL-6 secretion was undetectable in non-stimulated J774 cells. However, in the presence of LPS, the cells produced a large amount of IL-6 (over 3000 pg/ml). PA and EPA further stimulated the LPS effect on IL-6 production by the cells (1·22 (SE 0·06) and 1·49 (SE 0·11), respectively). OA and DHA inhibited the LPS response (0·61 (SE 0·04) and 0·11 (SE 0·09), respectively).

Discussion

The ability of monocytes and macrophages to phagocytose micro-organisms and subsequently kill them is an important primary survival function of these cells as the first line of defence. Although some aspects of macrophage function have already been shown to be modulated by FA, in the present study, we compared the effect of various FA on macrophage killing activity, production of ROS and cytokines.

Macrophage killing activity was distinctly affected by the different classes of FA. PA, a SFA, reduced fungicidal activity in a dose-dependent manner. OA, a MUFA, increased fungicidal activity and PUFA. AA, DHA and EPA stimulated the *C. albicans* killing activity of J774 cells at low concentrations but inhibited it at high doses. De Pablo *et al.*[21] observed an increase in phagocyte capacity in peritoneal macrophages from mouse supplemented with olive oil, rich in OA. The similar effects of *n*-3 and *n*-6
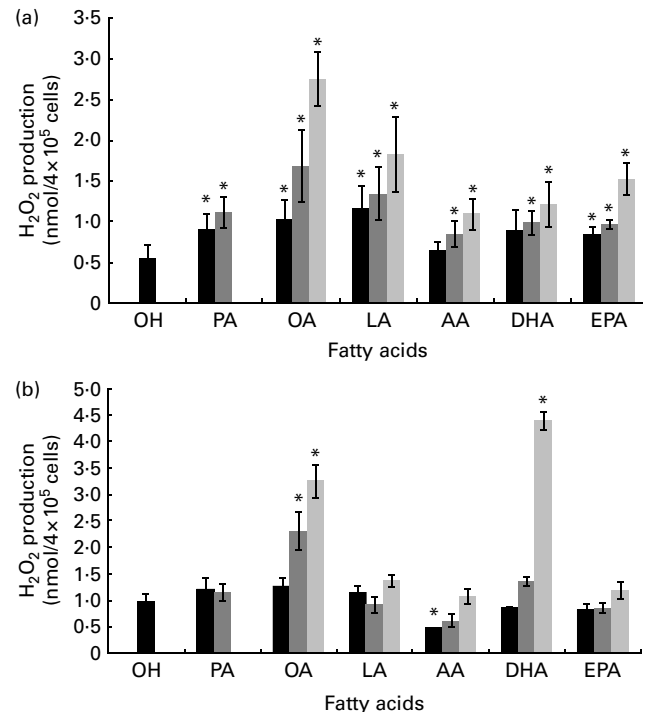
Fig. 4. Hydrogen peroxide production by J774 cells treated with fatty acids (FA) for 24 h. Phenol red was used for the measurement of hydrogen peroxide production by the cells. (a) Cells treated with FA only. (b) Cells treated with FA and lipopolysaccharide (2·5 μg/ml). Values are means of three experiments in triplicate, with standard errors represented by vertical bars. * $P < 0.05$ for comparison with control cells treated with ethanol (OH). PA, palmitic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid. ■, 25 μM; ■, 50 μM; □, 100 μM.
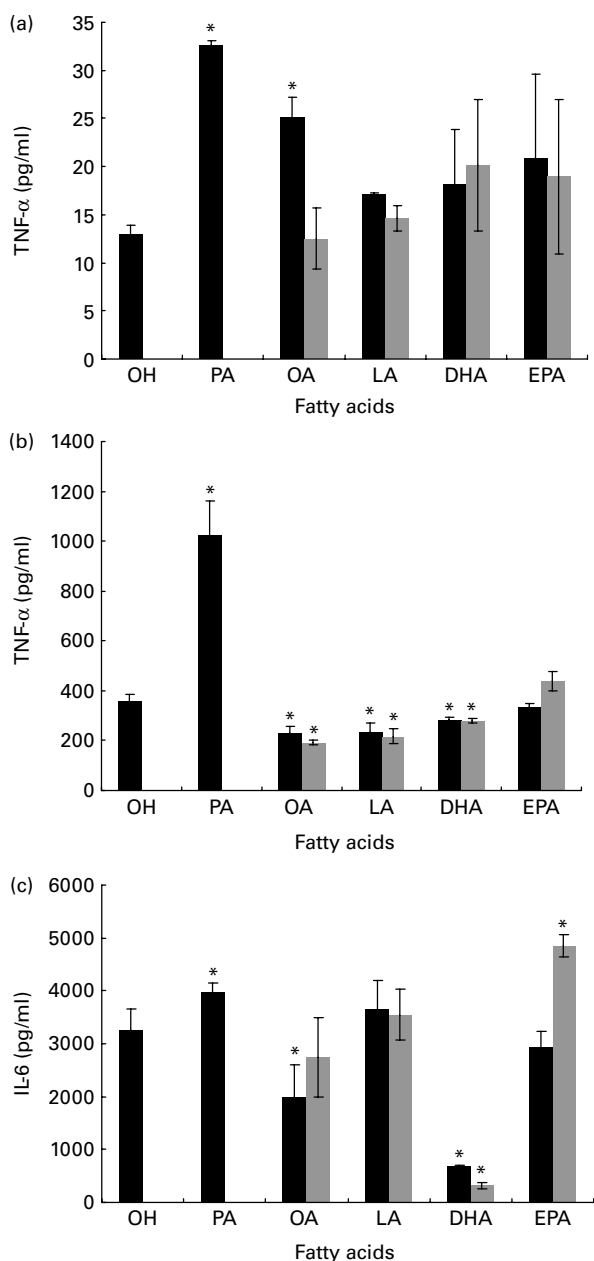
Fig. 5. Cytokine production by J774 cells treated with fatty acids (FA) for 24 h. TNF-α and IL-6 concentration was determined in the culture medium by ELISA. (a) TNF-α production by cells treated with FA only. (b) TNF-α production by cells treated with FA and lipopolysaccharide (LPS) (2·5 μg/ml). (c) IL-6 production by cells treated with FA and LPS (2·5 μg/ml). Values are means of three experiments in triplicate, with standard errors represented by vertical bars. * $P < 0.05$ for comparison with control cells treated with ethanol (OH). PA, palmitic acid; OA, oleic acid; LA, linoleic acid. ■, 50 μM; ■, 100 μM.

PUFA on fungicidal activity is a noteworthy result due to the fact that these classes of FA usually have distinct effects on immune function[22]. However, recent studies have shown that the so-called anti-inflammatory n-3 PUFA can actually have pro-inflammatory effects depending on the experimental model and the concentration used[23,24]. Bonatto et al.[23] compared the effects of supplementation of the diet of pregnant and lactating rats and subsequent supplementation of the offspring with coconut fat or fish

oil on macrophage activities involved in anti-tumour defence. Fish oil supplementation, rich in n-3 FA, was able to induce an increase in phagocytosis, in $O_2^{.-}$, H_2O_2, NO and TNF-α production by macrophages from non-tumour-bearing rats. Dietary fish oil also increased the secretion of the pro-inflammatory cytokine, TNF, but decreased the secretion of the anti-inflammatory cytokine, IL-10, in peritoneal macrophages from mice fed for 4 weeks[24].

An additional major characteristic of activated macrophages is their high capacity to release superoxide and H_2O_2. ROS participate in the regulation of numerous cellular functions, e.g. cellular differentiation, intracellular signalling, gene expression regulation and cell apoptosis[25]. Production of ROS is also associated with phagocytosis and microparticle digestion. In monocytes/macrophages, the mitochondrial electron transport chain, cyclo-oxygenases, lipo-oxygenases, cytochrome P-450 and nicotinamide adenosine dinucleotide phosphate oxidase are sources of ROS[26].

At first, we evaluated the acute effect of FA. We observed an increase in superoxide ($O_2^{.-}$) content in J774 cells treated with a high concentration of all unsaturated FA (100 μM) for 1 h, regardless of their family. Modulation of ROS production by FA has been observed in other cell types such as lymphocytes and fibroblasts[27,28]. Most studies with phagocytes have shown a decrease in ROS production by FA treatment[29,30]. However, Hatanaka et al.[31] showed that acute treatment with OA, LA and γ-linolenic acids (10 min) increases ROS production by neutrophils, corroborating with the present results. These effects of FA may be due to an increase in the activity of NADPH oxidase resulting from protein kinase C activation by FA. Indeed, protein kinase C activation by PUFA is well documented[32].

Under inflammatory condition by LPS administration, the acute stimulatory effect of all FA on the production of $O_2^{.-}$ was diminished. The effect of LA and EPA was completely abolished. This reduction in $O_2^{.-}$ content inside the cells may be due to the release to the extracellular medium or degradation by antioxidant enzymes. The superoxide anion is converted to H_2O_2 by superoxide dismutase, and this latter reactive species is more stable and has more potent microbicidal potential[33].

Dietary supplementation with different FA has been shown to modulate ROS production by leucocytes. The study of Yaqoob et al.[34] showed that macrophages from mouse fed diets rich in fish oil produce more $O_2^{.-}$ and H_2O_2 than the cells from animals fed diets rich in coconut oil, olive oil or sunflower oil. The production of $O_2^{.-}$ and H_2O_2 was also increased in neutrophils from animals supplemented with fish and sunflower oils[35].

For this reason, the effects of FA on $O_2^{.-}$ production by macrophages after a longer period of incubation (24 h) were also evaluated. Prolonged treatment of J774 cells with FA had distinct effects. PA did not present any effect

in 1 h treatment but increased intracellular $O_2^{\cdot-}$ content and H_2O_2 concentration in the medium after 24 h. Seifert & Schultz[36] also observed that SFA and *trans*-FA increase the activity of NADPH oxidase in neutrophils. Protein kinase C translocation is also stimulated by PA in COS-7 cells, which may lead to the activation of subsequent NADPH oxidase[37]. The remaining FA tested, with the exception of DHA at 100 μM, decreased intracellular $O_2^{\cdot-}$ content after 24 h of treatment. This effect was concomitant with an increase in H_2O_2 concentration in cell-cultured medium, as observed in Fig. 4. This result suggests that $O_2^{\cdot-}$ produced after the stimulation of J774 cells with these FA was converted to H_2O_2, increasing its production by the cells after prolonged periods of exposure to FA. Padma & Das[38] observed an increase in the activity of superoxide dismutase in the macrophage cell line AK-5 treated with various FA for 24 h. The ingestion of diets rich in *n*-3, *n*-6 and *n*-9 PUFA also led to an augment of superoxide dismutase and glutathione peroxidase activities[39,40].

LPS treatment for 24 h raised $O_2^{\cdot-}$ production by twofold in control cells (treated with ethanol). FA did not alter the LPS effect, with the exception of OA at 100 μM, that had an inhibitory effect on LPS-induced $O_2^{\cdot-}$ production but increased H_2O_2 concentration in the medium. This sustained effect of OA on ROS production may be related to the increased fungicidal activity observed in cells treated with this FA, suggesting that enrichment of diets with OA may be beneficial to patients suffering from diseases that require pathogen elimination.

FA treatment not only affected the respiratory burst of J774 macrophages but also modulated the synthesis and release of immune-regulatory cytokines. PA and OA (50 μM) stimulated TNF-α production by the cells, regardless of their activation by LPS. Interestingly, both *n*-3 and *n*-6 PUFA did not alter the production of this pro-inflammatory cytokine. When cells were stimulated with LPS, PA caused a further increase in the release of TNF-α and IL-6 by the cells. This effect may be related to the known activation of toll-like receptor 4 by this FA[41]. In fact, SFA induce NF-κB activation and expression of inflammatory markers in a murine monocyte cell line, RAW264.7, due to toll-like receptor 4 signalling. The inhibition of LPS-induced NF-κB activation and expression of inflammatory mediators by unsaturated FA were mediated through the suppression of toll-like receptor 4-derived signalling pathways[42]. This may be related to the observed decrease in cytokine production, particularly TNF-α, by OA, LA and DHA.

Although ROS and cytokine production are intrinsically related due to NF-κB activation[43], we did not find a cause–consequence relationship between modulation of ROS production and modulation of cytokine release by FA. We observed that most FA stimulated ROS production in the absence of LPS, but only PA and OA stimulated TNF-α production in the same condition. In the presence of LPS, most FA had no effect on ROS production, but most of them inhibited TNF-α production by J774 cells.

In summary, the present results show that PA, a SFA, has a late effect on stimulating ROS production by J774 cells, which may be related to the reduced fungicidal activity of the cells after the treatment with this FA. The effects of unsaturated FA on ROS production were time- and dose-dependently regulated. OA presented a sustained stimulatory effect on ROS production and increased fungicidal activity of the cells, suggesting that enrichment of diets with OA may be beneficial for pathogen elimination. Evidence that the so-called difference between *n*-3 and *n*-6 PUFA is not completely true in this model was also shown as ROS production, and TNF-α and IL-6 release were similarly modulated by these classes of FA. However, it was worth noting that most effects were diminished after stimulation of the cells with LPS. The present results suggest that supplementation of the diet with specific FA, not classes of FA, might enable either an improvement in host defence mechanisms, thus reducing the incidence and severity of infectious diseases, or a reduction in adverse immunological reactions, thus decreasing the severity and burden of autoimmune, allergic and inflammatory conditions.

Acknowledgements

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