Mechanisms by which fatty acids regulate leucocyte function

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ABSTRACT

Fatty acids (FAs) have been shown to alter leucocyte function and thus to modulate inflammatory and immune responses. In this review, the effects of FAs on several aspects of lymphocyte, neutrophil and macrophage function are discussed. The mechanisms by which FAs modulate the production of lipid mediators, activity of intracellular signalling pathways, activity of lipid-raft-associated proteins, binding to TLRs (Toll-like receptors), control of gene expression, activation of transcription factors, induction of cell death and production of reactive oxygen and nitrogen species are described in this review. The rationale for the use of specific FAs to treat patients with impaired immune function is explained. Substantial improvement in the therapeutic usage of FAs or FA derivatives may be possible based on an improvement in the understanding of the precise molecular mechanisms of action with respect to the different leucocyte types and outcome with respect to the inflammatory responses.

INTRODUCTION

Most studies on FAs (fatty acids) as modulators of inflammation and immune responses are focused on two particular groups: n−3 (or omega-3) and n−6 (or omega-6) [1]. These FAs have potent immunomodulatory activities in various diseases (for example, rheumatoid arthritis, asthma, psoriasis, atherosclerosis and wound healing) [1]; however, the mechanisms of action still remain under extensive investigation. For instance, the use of n−3 FA supplementation in chronic and debilitating diseases has been used, but there is no consensus on the recommended dose and the type of the FA to be used. FAs have been reported to exert their effects by modulating immune cell (neutrophils, monocytes/macrophages and lymphocytes) function, resulting in stimulation and/or inhibition of the production of cytokines, chemokines, growth factors, reactive species, lipid mediators and antibodies.

Key words: apoptosis, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), fatty acid, immune response, inflammation, leucocyte.

Abbreviations: AA, arachidonic acid; COX-2, cyclo-oxygenase-2; DAG, diacylglycerol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERK, extracellular-signal-regulated kinase; FA, fatty acid; IMLP, N-formylmethionyl-leucylphenylalanine; Hsp, heat shock protein; IFN-γ, interferon-γ; IL, interleukin; iκB, inhibitor of NF-κB; JNK, c-Jun N-terminal kinase; LA, linoleic acid; CLA, conjugated LA; LAT, linker for activation of T-cells; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; NF-κB, nuclear factor κB; NOS, nitric oxide synthase; iNOS, inducible NOS; OA, oleic acid; PBMC, peripheral blood mononuclear cell; PG, prostaglandin; 15d-PGJ2, 15-deoxy-D12,14-PGJ2; PKC, protein kinase C; PLC, phospholipase C; PPAR, peroxisome-proliferator-activated receptor; PUFA, polyunsaturated FA; RNS, reactive nitrogen species; ROS, reactive oxygen species; RXX, 9-cis-retinoic acid receptor; TAC, triacylglycerol; TCR, T-cell receptor; TLR, Toll-like receptor; TNF, tumour necrosis factor; TNFR, TNF receptor.

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FAs regulate key metabolic and immune responses either directly as non-esterified FAs or through generation of biologically active metabolites (Figure 1). FAs can modulate intracellular signalling pathways indirectly by modulating cell-membrane fluidity, composition of lipid rafts and second messenger production. Alternatively, they can act directly on cell-membrane receptors, such as TLRs (Toll-like receptors), or nuclear receptors, such as PPARs (peroxisome-proliferator-activated receptors). FAs affect biological systems by stimulating the
production of eicosanoids, ROS (reactive oxygen species) and RNS (reactive nitrogen species) and inducing cell death.

In this review, the mechanisms underlying the effects of FAs on leucocyte function are discussed, including some of our findings on the regulation of gene expression in human leucocytes and leucocyte cell lines following treatment with AA (arachidonic acid), DHA (docosahexaenoic acid), EPA (eicosapentaenoic acid) and OA (oleic acid) [2–5]. Findings indicate that these FAs regulate the expression of particular genes, which is an important mechanism in the modulation of leucocyte function.

INTRACELLULAR SIGNALLING

PUFAs (polyunsaturated FAs) may control immune responses indirectly by modification of cell-membrane composition. n−3 FAs incorporated into cell phospholipids could alter the levels of phospholipid-derived second messengers, such as DAG (diacylglycerol) and ceramide. AA, DHA and EPA affect T-cell function in Jurkat cells (a human T-lymphocyte cell line) by incorporation into DAG and through the specific effects of their DAG metabolites on RasGRP (Ras guanyl-releasing protein) and, subsequently, on MAPK (mitogen-activated protein kinase) signalling [6].

MAPKs, such as ERK (extracellular-signal-regulated kinase) 1/2, are involved in cell proliferation and differentiation [7]. Denys et al. [8] have shown that EPA and DHA decreased the activity of ERK1/2 in PMA-stimulated Jurkat cells. These two FAs also inhibited the PMA-induced plasma-membrane translocation of PKC (protein kinase C)α and -ε and the nuclear translocation of NF-κB (nuclear factor κB) [9]. However, other studies have demonstrated that these effects are mediated by PKC-independent mechanisms [8]. Stuhl et al. [10] found that JNK (c-Jun N-terminal kinase) phosphorylation and activation was selectively inhibited in Jurkat cells and peripheral blood T-cells treated with EPA, whereas phosphorylation of p38 MAPK remained unaltered. Hii et al. [11] have shown that AA, as with EPA, did not enhance p38 MAPK phosphorylation in Jurkat cells, but increased ERK and JNK activity. An increase in ERK activity in Jurkat cells treated with DHA and EPA was also observed [11], which was in contrast with the study by Denys et al. [8]. These contradictory results may be partially explained by the concentrations and treatment periods used. For example, Denys et al. [8] investigated ERK activity after 5 min of treatment with DHA and EPA at concentrations up to 60 µmol/l, whereas Hii et al. [11] treated cells with up to 20 µmol/l of these FAs for 10 min. These studies indicate that PUFAs have a selective action on intracellular proteins involved in T-cell activation. In macrophages, n−3 FAs inhibited ERK1/2 and JNK phosphorylation, leading to a decrease in AP-1 activation and production of pro-inflammatory cytokines [12].

In human neutrophils, AA directly activates PKC and may thereby serve as a regulatory signal during cell stimulation [11]; however, O’Flaherty et al. [13] have shown that AA induced PKC translocation at concentrations 2–4 orders of magnitude below those activating the enzymes. These results suggest that AA is related more to the movement than the activation of PKC. AA has also been shown to stimulate TNFR (TNF (tumour necrosis factor) receptor) expression in human neutrophils, and this effect was dependent on PKC and ERK1/2 activation [14]. FAs also induced the secretion of GM-CSF (granulocyte/macrophage-colony stimulating factor) via PKC activation in THP-1 cells (a macrophage cell line) [15].

LIPID-RAFT-ASSOCIATED PROTEINS

Lipid rafts are specialized plasma-membrane microdomains that contain high concentrations of cholesterol and sphingolipids, and their polar lipids contain predominantly saturated fatty acyl residues [16]. Cytoplasmic and transmembrane proteins are targeted to lipid rafts mostly by acylation with saturated fatty acyl moieties (palmitoyl and myristoyl) that link these proteins to the cytoplasmic lipid leaflet of the microdomains [17]. Lipid rafts are found in large quantities in the plasma membranes of T-cells, and several proteins involved in T-cell signalling, such as Src-family protein tyrosine kinases and LAT (linker for activation of T-cells), are concentrated in rafts because of post-translational palmitoylation, indicating the role of lipid rafts in T-cell signalling [18].

PUFAs are incorporated into membrane phospholipids and, hence, modulate membrane structure and function. On the basis of the original fluid mosaic model of cellular membranes [19], incorporation of highly unsaturated FAs alters their general biophysical properties, such as microviscosity. However, PUFAs also affect the composition of lipid rafts. Fan et al. [20] observed that dietary n−3 PUFAs decreased lipid raft sphingolipid content and altered raft fatty acid composition.

There is strong evidence that lipid raft integrity is a prerequisite for optimized TCR (T-cell receptor) signal transduction and immune response [21]. The existence of MHC class II molecules in lipid rafts of DCs (dendritic cells) at the synapic interface is important for the activation of the interacting T-cell [22]. Polyclonal activation-induced co-localization of PKC-θ (a key mediator of cell proliferation) with lipid rafts was decreased in T-cells from n−3 PUFA-fed mice. With respect to the PKC-θ effector signalling pathway, activation of both AP-1 and NF-κB was inhibited by fish oil.
supplementation, decreasing IL (interleukin)-2 secretion and impairing lymphocyte proliferation [23].

PUFA treatment leads to the displacement of Src-family protein tyrosine kinases from lipid rafts in parallel with the inhibition of calcium responses [10]. Zeyda et al. [24] have shown that PUFA treatment of T-cells specifically blocked signal transduction at the level of LAT and PLC (phospholipase C)-γ1 by the partial displacement of LAT from lipid rafts. These results reveal that LAT displacement from lipid rafts is a crucial molecular mechanism for PUFA-mediated inhibition of T-cell signalling.

PUFA-rich lipid emulsions given to healthy volunteers by intravenous infusion promoted an alteration in lipid raft organization in lymphocyte membrane [25], which was associated with reduced T-cell proliferation. Tyrosine phosphorylation of LAT and activation of Akt/PKB (protein kinase B) in T-cells were also impaired. These results demonstrated that the enrichment of lipid raft phospholipids with unsaturated linoleyl chains from lipid emulsions may compromise the interaction of LAT with TCR and CD28 upon stimulation of T-cells, thereby preventing optimal intracellular signalling [25].

In T-lymphocytes, studies have shown that raft microdomains control the localization and function of many receptors that are important components of T-cell signalling. Recent studies have shown that DHA and EPA impair IL-2 signalling pathway activity by displacing IL-2RI (IL-2 receptor) from lipid rafts [26]. These functional modifications of the effect of IL-2 may explain some of the suppressive effects of n–3 FAs on lymphocyte function. In B-lymphocytes, FcγRIIa signalling is partially dependent upon palmitoylation of Cys239, which is critical for its localization on lipid rafts [27].

TLRs

TLRs are transmembrane receptors that play a critical role in the detection of microbial infection and in the induction of inflammatory and immune responses against conserved microbial structures, called pathogen-associated molecular patterns [28].

Eleven members of the TLR family have been identified in humans to date and they are ubiquitously expressed in human tissues [29]. Genetic and biochemical evidence have demonstrated that TLR4 confers the responsiveness to LPS (lipopolysaccharide) derived from Gram-negative bacteria [30,31]. Other agonists for TLR4 from non-microbial sources include Hsp (heat shock protein) 60, fibronectin, taxol, RSV (respiratory syncytial virus) coat protein and saturated FAs [32–37].

Rhee and Hwang [31] have demonstrated that the activation of TLR4 is sufficient to induce the activation of NF-κB and the expression of the mitogen-inducible COX-2 (cyclo-oxygenase-2) in murine macrophages. Furthermore, saturated FAs induce NF-κB activation, and the expression of COX-2 and other inflammatory markers in RAW264.7 cells (a murine monocyctic cell line). The inhibition of LPS-induced NF-κB activation and COX-2 expression by unsaturated FAs was mediated through suppression of TLR4-derived signalling pathways [36].

Saturated FAs can activate TLRs other than TLR4 [38]. They also activate TLR2 dimerization with TLR1 and TLR6, receptors in which natural bacterial ligands present acylated FAs in their molecules [36,39]. In contrast with saturated FAs, DHA suppressed NF-κB activation and COX-2 expression induced by various TLRs. This FA also inhibited NF-κB activation and COX-2 expression induced by an active TLR4 in RAW264.7 cells. LPS-induced COX-2 expression was decreased in peripheral blood monocytes following the consumption of food containing fish oil, a major source of n–3 PUFAs [39]. In fact, n–3 PUFAs are much more potent inhibitors of TLR4 and TLR2 activation compared with n–6 PUFAs or saturated FAs. This may explain why n–3 PUFAs are considered more effective anti-inflammatory compounds than n–6 PUFAs [40].

If the activation of TLRs and, consequently, inflammatory and immune responses are also differentially modulated by FAs in vivo, the risk of chronic inflammatory diseases and host defence against microbial infection may also be modified by dietary FAs.

GENE EXPRESSION IN LEUCOCYTES

Another mechanism for modulating immune responses by PUFAs occurs by changes in gene expression. Verlengia et al. [2] have shown, using a macroarray technique (in which 83 genes were evaluated using membranes from Clontech Laboratories), that the expression of genes related to signal transduction, cell survival, apoptosis and cytokine production was altered by the treatment of cultured Jurkat cells with 12.5 μmol/l of EPA or DHA for 24 h. Of the genes changed by the FAs, DHA induced the expression of 62 %, whereas EPA up-regulated 33 % [2]. DHA had a stimulatory effect on the expression of genes related to defence and repair. In contrast, EPA augmented the expression of other genes, such as the proto-oncogenes myc and c-jun, and p53-associated gene. These results indicate that the molecular mechanisms underlying the modulatory effect of these two n–3 FAs on T-lymphocytes are different.

In Raji cells (a human B-lymphocyte cell line) treated as described above, the up-regulation of the cell-cycle genes by EPA may be related to the pronounced increase in cell proliferation induced by this FA when compared with DHA [3]. In the same study, mRNA expression of IL-10, TNF-α and IFN-γ (interferon-γ) was found to be unaltered by treatment with both EPA and DHA, although their secretion was decreased. These findings...
indicate that the modulatory effects of EPA and DHA on cytokine production in Raji cells were not only due to changes in gene expression.

In another study from our group [41], ten healthy male volunteers were given a DHA-rich fish oil for 2 months. Fish oil supplementation altered the FA composition of lymphocytes, resulting in an increase of the $n$–3/n–6 ratio from 0.18 to 0.62. An overall suppression of gene expression (92% of the 588 genes evaluated by macroarray technique, using membranes purchased from Clontech Laboratories) in lymphocytes was observed. The genes most altered by the fish oil supplementation were related to signal transduction. Some of the genes down-regulated were those encoding PKA (cAMP-dependent protein kinase), PKC-δ, TYK2 (tyrosine kinase 2), MAPK8 and MAPKK1 (MAPK kinase 1). Evidence obtained in this study has also shown that $n$–3 FAs are involved in the regulation of genes related to cellular defence, with the expression of Hsp70 and Hsp90 in lymphocytes being increased by DHA-rich fish oil supplementation.

CLAs [conjugated LAs (linoleic acids)] are a mixture of positional and geometric isomers of LA. Zhao et al. [42] observed in pigs supplemented with diets containing 2% CLA or soya bean oil that dietary CLAs inhibited the expression of TNF-α mRNA in PBMCs (peripheral blood mononuclear cells). In the same study, both CLA isomers reduced LPS-stimulated TNF-α production and expression in PBMCs treated in vitro, which may be due partially to inhibition of the binding activity of NF-κB to the TNF-α promoter.

The effect of AA and OA on pleiotropic gene expression in Jurkat cells has also been examined by our group [4]. AA (5 µmol/l) treatment for 24 h markedly affected the expression of genes clustered as: (i) signal transduction pathways, (ii) transcription factors, (iii) cell cycle, (iv) defence and repair, (v) apoptosis, (vi) DNA synthesis, (vii) cell adhesion and (viii) cytoskeleton. AA increased the expression of 59% of the genes (83 genes were evaluated using membranes purchased from Clontech Laboratories), whereas OA (50 µmol/l for 24 h) decreased the expression of only two genes (Kruppel-related zinc finger protein and retinoblastoma-associated protein 1). These findings led us to postulate that the known pro-inflammatory and immunomodulatory effects of AA [43] may also result from activation and/or inhibition of gene expression in T-lymphocytes. It remains unknown whether these effects were caused by the FA itself or by eicosanoids produced through AA metabolism [44], although there is evidence that AA can affect lymphocyte function by eicosanoid-independent mechanisms [45]. This is an interesting issue to be investigated, as some authors postulate that lymphocytes are unable to produce eicosanoids [46].

Our group has also used the suppressive subtractive hybridization approach to identify genes with high or low expression after treatment of Jurkat cells with OA (50 µmol/l) for 24 h. OA was shown to regulate the expression of important genes, such as the translation elongation factor α1, ATP synthase 8, gp96 (human tumour rejection antigen gp96), Hsp60 and SPC4 (subtilisin-like protein 4) [47]. These results suggest that OA, at physiological plasma concentrations, can regulate the expression of important genes in maintaining the machinery that ensures normal cell function.

In Jurkat cells, we have also studied the effect of LA treatment (100 µmol/l) on pleiotropic gene expression by macroarray technique (558 genes were evaluated using membranes purchased from Clontech Laboratories). Cury-Boaventura et al. [48] observed a decrease in mRNA expression of 19 genes, most of them related to the cell cycle, and up-regulation in the expression of eight genes, related to apoptosis and intracellular modulators. These findings indicate that the modulatory effects of FAs on gene expression can be very specific.

**TRANSCRIPTION FACTORS**

As mentioned above, PUFAs have been shown to regulate the expression of several key genes required for leucocyte function. Studies on the effect of FAs on leucocyte gene expression have highlighted the activation of two main factors, namely PPARs and NF-κB.

**PPARs**

PPARs are members of the steroid/rexinoid nuclear receptor superfamily of ligand-activated transcription factors. Three isoforms of PPAR (PPAR-α/-β/-γ) have been described and all of them form heterodimers with another nuclear receptor of the same subgroup, RXR (9-cis-retinoic acid receptor) [49]. PPAR-γ is activated by PUFAs (i.e. γ-linolenic acid, AA and EPA), although these endogenous ligands are considered to be weak activators of this transcription factor [50]. PPAR-α can also be activated by PUFAs and can, in addition, be activated by some medium-chain saturated and monounsaturated FAs (i.e. myristic acid and OA) [50].

There is evidence of PPAR activation by FAs in leucocytes, and most studies have shown the effect of eicosanoids and their precursors. PPAR activation by 15d-PGJ2 [15-deoxy-Δ12,14-PG (prostaglandin) J2], for example, is well characterized. Treatment of murine macrophages with 15d-PGJ2 or thiazolidinediones has been found to inhibit the secretion of several inflammatory mediators (including gelatinase B, IL-6, TNF-α and IL-1β) and also to reduce the expression of iNOS [inducible NOS (nitric oxide synthase)] [51,52]. Two naturally occurring PPAR-α ligands, LTB4 (leukotriene B4) and 8(S)-HETE (hydroxyeicosatetraenoic acid), have been shown to stimulate NOS activity [53]. In THP-1 and J774 cells (two macrophage cell lines), PPAR-γ activation by PUFAs decreased the production
of several pro-inflammatory mediators, such as PGE$_2$, TNF-α, IL-1, IL-6 and NO [54,55]. In addition, EPA markedly reduced leucocyte rolling and adhesion to venular endothelium in LPS-treated mice. This effect occurred via a PPAR-α-dependent mechanism, because EPA had no such effect in LPS-treated PPAR-α-deficient mice [56]. However, Zhang et al. [57] have observed that FA-mediated inhibition of IL-12 production by murine macrophages was independent of PPAR-γ activation.

There is evidence that PPAR activation may be related to the induction of leucocyte death by FAs. Treatment with AA, DHA and EPA induced apoptosis of human monocyte/macrophages in vitro and the results suggest that PPAR-γ activation was involved in this effect [58]. The findings reported by Cury-Boaventura et al. [48] have suggested that Jurkat cell death induced by OA and LA also involve PPAR-γ activation, and a previous study by our group [59] has shown that PPAR-γ activation by 15d-PGJ$_2$ induced Jurkat and Raji cell apoptosis. On the contrary, Martins de Lima et al. [60] did not observe PPAR activation after treatment of J774 cells (a macrophage cell line) with various FAs, and Nencioni et al. [61] reported that Jurkat cell death induced by PGs was not related to PPAR-γ activation. These differences may be explained by the different cell types, concentration ranges and periods of incubation used.

**NF-κB**

NF-κB plays an important role in controlling inflammatory gene activation. This transcription factor is usually found in the cytosol as a heterodimer complex with its inhibitory protein IκB (inhibitor of NF-κB). When cells are stimulated with LPS, phorbol ester or inflammatory cytokines, IκB is phosphorylated by IκB kinase and degraded. IκB phosphorylation dissociates the dimer and allows NF-κB to translocate to the nucleus, where it activates target genes, including iNOS [62].

A number of studies have shown that PUFAs can modulate NF-κB activation. Most of them have shown that FAs inhibit NF-κB DNA-binding activity in macrophages [53,63–65], and similar results have been observed by our group [66]. There is evidence, however, that saturated FAs induce NF-κB activation, and this effect may be related to the inflammatory changes observed in adipocyte and macrophage interactions [67].

**LIPID MEDIATORS**

AA serves as precursor of immune-active lipid mediators known as eicosanoids. Classes of eicosanoids include lipoxins, leukotrienes and PGs, and their effects on the immune system have been extensively explored and reviewed recently [68–70].

In addition to eicosanoids, important lipid mediators that modulate leucocyte functions include endocannabinoids, lysophospholipids, platelet-activating factor and resolvins [71,72].

Endocannabinoids serve as the endogenous agonists of the G$_i$-protein-coupled cannabinoid receptors [71]. They modulate IL-10 and IL-12 production, suppress CD4$^+$ T-cell proliferation and IFN-γ expression, and attenuate FMLP (N-formylmethionyl-leucylphenylalanine)-induced chemotaxis of murine macrophages [73]. Endocannabinoid production is required for the formation of T- and B-cell subsets involved in immune homeostasis, as mice lacking them are susceptible to immune dysregulation, notably developing inflammatory bowel disease [74].

Lysophospholipids exert a variety of effects on macrophages, neutrophils and lymphocytes, such as altering lymphocyte trafficking to secondary lymphoid organs [72] and cytokine production [75]. Ceramide, a sphingolipid metabolite, is an integral component of apoptotic cascades initiated through the binding of TNF-α and Fas ligand to their receptors [76], exhibiting an important role in macrophage and neutrophil apoptosis [77,78]. Ceramides also inhibit neutrophil phagocytic capacity, superoxide formation and calcium influx [79,80].

Resolvins are new mediators generated from EPA and DHA that were first identified by Serhan et al. [81] in resolving inflammatory exudates and in tissues enriched with DHA. The names resolvins (resolution phase interaction products) and docosatrienes were given because these bioactive compounds demonstrate potent anti-inflammatory and immunoregulatory actions. These mediators prevented neutrophil entry to inflammation sites and cytokine production and reduced exudates in rats with experimental peritonitis [81]. The compounds derived from EPA carrying potent biological actions (i.e., 1–10 nmol/l range) are named the E series, and are denoted as resolvins of the E series. Those synthesized from DHA are resolvins of the 17S-D series that have immunoregulatory [81] and neuroprotective actions [82]. These compounds are produced after acetylation of COX-2 by aspirin. In addition, aspirin treatment triggers the formation of 15-epimeric lipoxins, termed aspirin-triggered lipoxins, that also play a role in resolution of inflammation through inhibition of neutrophil tissue infiltration [83] and stimulation of macrophage phagocytosis of apoptotic neutrophils [84].

A novel approach in this field has been established by Ferrante and co-workers [46], who have developed chemically engineered FAs and have demonstrated specific structural-related effects. The engineered PUFAs containing an oxygen atom in the β-methylene group, β-oxa 21:3n−3, was found to be a very poor stimulator of the neutrophil respiratory burst, being more active in depressing T-lymphocyte function. This compound significantly inhibited T-lymphocyte proliferation and cytokine production in vitro when compared with DHA [46]. Other chemically engineered FAs, with either an
oxygen atom in the β position (β-oxa) or a sulfur atom in the β (β-thia) or γ (γ-thia) position, have also been synthesized and have specific effects on neutrophil and lymphocyte function [85,86]. These novel FAs may be useful as anti-inflammatory agents, as they exhibit increased biological activity compared with the natural long-chain PUFAs.

**INDUCTION OF CELL DEATH**

Apoptosis, also known as programmed cell death, occurs during both physiological and pathological processes, whereas necrosis is linked mostly to pathological conditions. Apoptosis is characterized by changes in cell morphology, including phosphatidylserine externalization, a decrease in nuclear size and DNA fragmentation. Necrosis is characterized by an early loss of membrane integrity and occurs when the production of ATP ceases abruptly [87].

There is increasing evidence that non-esterified FAs can cause cell death. Several cell types, including macrophages [60], neutrophils [88], murine splenic T-lymphocytes [89] and leukaemia cells [90–93] exhibit morphological features of apoptosis and necrosis after exposure to FAs at high doses. However, the toxic concentration varies according to the cell type and the FA tested. For example, our group has observed that Raji cells are more sensitive to the toxic effect of PUFAs than Jurkat cells [90]. Raji cells exhibit a loss of membrane integrity and DNA fragmentation after incubation for 24 h with 25 µmol/l AA, DHA and EPA, whereas Jurkat cells can tolerate these FAs up to 50 µmol/l [90]. Several studies have shown that PUFAs, such as LA, AA, DHA and EPA, can cause a more pronounced effect on cell death in lymphocytes and neutrophils than monounsaturated or saturated FAs [88,91–93].

Several mechanisms have been shown to be involved in FA-induced apoptosis: (i) DHA and OA lead to TAG (triacylglycerol) accumulation in HL-60 cells (a promyelocytic leukaemic cell line) [88]; (ii) pancreatic β-cells treated with palmitic acid have an increase in NO synthesis [94]; (iii) OA and LA enhance the generation of ROS in lymphocytes [91–93]; (iv) and AA increase the production of eicosanoids in B- and T-lymphocytes [95]. Phosphorylation and dephosphorylation of kinases and phosphatases are also involved, as Siddiqui et al. [96] have shown that DHA induced apoptosis in Jurkat cells via a protein-phosphatase-mediated process. PPAR activation is involved in T-cell apoptosis induced by LA [48], and a decrease in mitochondrial phospholipid cardiolipin synthesis was related to palmitic-acid-induced apoptosis of breast cancer cells [97]. Ceramide synthesis plays a role in the induction of apoptosis in several cell lines, such as pancreatic β-cells and skeletal myotubes, by palmitic acid [98,99]. Taken together, the mechanism by which FAs cause cell death varies markedly with cell type and FA species.

A common feature of apoptosis is mitochondrial depolarization. Studies from our group have demonstrated that OA and LA cause significant mitochondrial depolarization in lymphocytes [91–93]. Permeabilization of the mitochondria results in the release of pro-apoptotic proteins into the cytosol, leading to subsequent activation of the mitochondria, which causes cell apoptosis [100]. Cury-Boaventura et al. [48] have observed in Jurkat cells that OA and LA activated caspases 3 and 6, but had no effect on caspase 8.

Depolarized and uncoupled mitochondria also may increase ROS generation [95,101]. There is evidence that oxidative stress may be involved in the triggering of cell death by FAs. FA treatment, mainly PUFAs, caused oxidative stress in macrophages [102], neutrophils [88] and human lymphocytes [91–93,103]. The treatment of neutrophils with AA, EPA and DHA increased oxidative stress, whereas saturated and monounsaturated FAs had no effect [88]. In addition, previous studies have shown that LA reduced peroxiredoxin, GST (glutathione S-transferase) and SOD (superoxide dismutase) expression, which diminished the antioxidant capacity of the cells [104].

Accumulation of excess FAs into TAG pools has been postulated to divert these molecules from pathways that lead to cytotoxic effects [91–93,105]. Cnop et al. [106] have shown that FA cytotoxicity is inversely related to cytoplasmic TAG accumulation. Other studies also observed that human lymphocytes treated with OA and LA increased neutral lipid accumulation, mainly in the form of TAG [91–93]. In a monocytic cell line, it has also been observed that FA accumulation in TAG protected them from cell death [60].

The information above indicates that some effects of FAs on immune function may be at least partially due to induction of leucocyte death.

**PRODUCTION OF ROS AND RNS**

ROS and RNS are well known to play a role as both deleterious and beneficial species. ROS and RNS are normally generated by NADPH oxidase isoforms and NOS [107] respectively, being efficiently modulated by FAs [108].

NADPH oxidase, a multicomponent superoxide-generating enzyme complex, is well known for its role in oxidative killing of micro-organisms by neutrophils. NADPH oxidase is formed by subunits found in intracellular granules and the plasma membrane. After activation, granules containing NADPH oxidase components fuse with phagocytic vacuoles and generate superoxide. Also, granules may migrate to the cell surface and release superoxide into the extracellular space [109,110].
Superoxide anion and H$_2$O$_2$ generated by NADPH oxidase give rise to other ROS that are strong cytolytic agents, such as hypochlorous acid [formed from H$_2$O$_2$ and chloride ions by the action of MPO (myeloperoxidase) released from neutrophil granules] and the hydroxyl radical [109–111]. Other sources of superoxide in the cells include the mitochondrial electron transport chain, xanthine/xanthine oxidase system and cytochrome P450 [112].

Excessive ROS production can damage cellular lipids, proteins and DNA, impairing cell function. In fact, oxidative stress has been implicated in a number of human diseases as well as in the aging process [107]. Research involving dietary supplementation with FAs, mainly PUFAs, have been shown to increase oxidative stress, as indicated by an increase in the generation of the products of oxidative metabolism in human plasma and urine [113,114]. This issue, however, is still under debate, owing to the fact that some studies were unable to detect increased oxidative stress and lipid peroxidation in patients and rats supplemented with PUFAs [115,116].

PUFAs have been shown to induce ROS production in murine and human macrophages [103,117], as well as rat and human neutrophils [118,119]. Also, accumulation of the superoxide anion following addition of EPA to the incubation medium of leukaemic cell lines [120] and human cervical carcinoma cells (HeLa cells) [121] has been reported. Treatment of HL-60 with AA, EPA and DHA increased the intracellular lipid peroxide content and oxidative respiratory burst activity, whereas saturated FAs, such as palmitic and arachidic acids, had no effect [88].

NADPH oxidase can be directly activated by AA, resulting in ROS production in thymocytes and HL-60, Jurkat and Raji cells [122]. Studies in which FA structure and function were correlated have demonstrated that, in general, as the number of double bonds in the FA molecule increases, so does its ability to stimulate an oxidative burst in unstimulated neutrophils [123,124]. According to Hardy et al. [125], exogenous long-chain FAs and very-long-chain FAs use the same signal transduction pathways to stimulate ROS production. The presence of medium-chain FAs in neutrophil culture increased the production of ROS, whereas in vivo treatment with these FAs reduced H$_2$O$_2$ production [126]. Sodium butyrate (a short-chain FA) up-regulated H$_2$O$_2$ generation but down-regulated the generation of MPO-mediated oxidants by human neutrophils [127].

On the other hand, other studies have shown inhibitory effects of FAs on ROS production. Neutrophils from patients fed diets containing fish oil (n–3 FAs) had a decreased production of ROS after stimulation [128,129].

Although the effect of FAs on ROS production has been studied extensively, several points remain to be fully established, as some authors have found an increase [130,131], whereas others have found a decrease [132,133], in ROS production by neutrophils in response to FA treatment. Recently, we have investigated the effect of OA, LA and γ-linolenic acid on intra- and extra-cellular content of ROS by unstimulated and PMA-stimulated neutrophils by using five techniques: luminol-amplified chemiluminescence, lucigenin-amplified chemiluminescence, cytochrome c reduction, hydroethidine and phenol red reduction [134]. All FAs tested stimulated ROS production by neutrophils; however, measurements using luminol-amplified chemiluminescence and cytochrome c reduction required further analysis. In the luminol technique, peroxidase activity is required in the reaction of luminol with ROS for light generation, but OA, LA and γ-linolenic acid inhibited MPO activity in stimulated neutrophils. Thus these FAs jeopardize the results of ROS content determined using the luminol technique. OA, LA and γ-linolenic acid led to cytochrome c reduction, indicating that this method is not appropriate for measuring ROS production induced by FAs [125,134]. The discrepancy found in the literature may be partially due to the method used.

Some FAs are unable to stimulate neutrophil oxygen consumption by themselves, but cause an increment in the maximal rate of oxygen consumption when a second stimulus occurs. Recently, we have demonstrated that OA, LA and γ-linolenic acid exert a priming effect on the oxidative burst in neutrophils [134]. In fact, addition of PMA to the assay medium caused an additive effect on superoxide and H$_2$O$_2$ production induced by these FAs. The priming effect of FAs has also been observed for AA, EPA and DHA. These FAs were poor inducers of oxygen-dependent respiratory activity in human monocytes and macrophages, but markedly enhanced the response to the tripeptide fMLP [135]. The priming effect of these FAs on monocytes and macrophages, causing increased/synergistic oxidative respiratory burst activity to other stimuli, has been shown to be dependent on PKC translocation and activation [135].

NO produced by activated macrophages and neutrophils regulate antimicrobial and antitumor activities. However, excess NO production causes tissue damage that is associated with acute and chronic inflammation [136]. NO is synthesized from l-arginine by NOS using NADPH and oxygen as co-substrates [137]. Macrophages and neutrophils stimulated with LPS and pro-inflammatory cytokines, such as IFN-γ and TNF-α, produce large amounts of NO through iNOS activity [138,139].

The findings of in vivo studies analysing changes in NO production in macrophages from rats fed FA-enriched diets are controversial. Pizato et al. [140] did not observe a significant difference in NO production in macrophages from non-tumour-bearing and tumour-bearing rats fed with diets rich in n–6 and n–3 FAs. In addition, Babu et al. [141] did not observe any difference in rats fed a flaxseed-oil-rich meal. On the other
hand, Yaqoob and Calder [102] have observed that LPS-stimulated NO production was greater in macrophages from olive-oil-, safflower-oil- or fish-oil-fed mice than from those fed with low-fat or hydrogenated coconut fat diets. Similar effects have been observed by others [142–144]. The reasons for these discrepancies remain unclear, but it may be related to the differences in the experimental protocols.

In vitro studies have shown a decrease in NO production by mice macrophages and cell lines after exposure to FAs, especially from the n-3 class [145,146]. A recent study from our group [66] has found that palmitic acid, stearic acid, OA, LA, AA, DHA and EPA stimulated NO production at low doses (1–10 µmol/l) but inhibited it at high doses (50–200 µmol/l). The stimulatory effect of most of the FAs on NO production was time-dependent, involved NF-κB activation and increased iNOS expression and activity [66]. The inhibitory effect on NO production was therefore due to FA toxicity at high concentrations. These results are indicative that the concentration of the FA used can determine if their effects will be inhibitory or stimulatory.

CONCLUDING REMARKS

FAs can affect leucocyte function by different mechanisms of action, which are summarized in Figure 1. This review presents evidence that many mechanisms by which FAs regulate leucocyte function have been elucidated and supports the proposition that these molecules might have important and specific roles in controlling leucocyte function. FAs may act through modulating the activity of intracellular signalling pathways and lipid-raft-associated proteins, binding to TLRs, regulating gene expression, activating transcription factors, inducing cell death and producing eicosanoids, ROS and RNS. This precise mechanism still remains to be completely elucidated and continues to be a stimulating field of research.

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