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Signaling Pathways Involved in the Effects of Different Fatty Acids on Interleukin-2 Induced Human Lymphocyte Proliferation

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Abstract

The aim of the present study was to investigate the mechanisms involved in the modulation of human lymphocyte proliferation by different fatty acids. We evaluated the effects of palmitic (PA), stearic (SA), oleic (OA), linoleic (LA), docosahexaenoic (DHA), and eicosapentaenoic (EPA) acids on IL-2 signaling pathways in addition to the involvement of the *de novo* ceramide synthesis and PI3K pathway. PA, SA, DHA, and EPA decreased the JAK1/JAK3/STAT5 pathway and IL-2-induced Akt phosphorylation. OA and LA had no effect. The inhibitory effect of DHA and EPA on lymphocyte proliferation was abolished by fumonisin B1 (FB1, an inhibitor of the *de novo* ceramide synthesis), whereas the effect of the other fatty acids remained unchanged. ERK1/2 phosphorylation was increased by OA and LA but markedly decreased by the other fatty acids. PKC-ζ phosphorylation was increased by OA and LA only. These effects were abolished in the presence of wortmannin, a PI3K inhibitor. In conclusion, the findings reported herein contribute for understanding the mechanisms by which different fatty acids modulate lymphocyte proliferation. The inhibitory effect of PA, SA, DHA and EPA on lymphocyte proliferation was associated with a reduction in the IL-2-mediated activation of the JAK/STAT, ERK, and Akt pathways. Decreased lymphocyte proliferation promoted by DHA and EPA also involved *de novo* ceramide synthesis. The stimulatory effect of OA and LA on lymphocyte proliferation was associated with improved activity of the MAP kinase and PI3K pathways, as demonstrated by increased ERK1/2 and PKC-ζ phosphorylation.

Keywords: n-3 PUFA; Cell proliferation; IL-2 receptor; JAK; Ceramide; ERK; PKC

Abbreviations: ConA: Concanavalin A; DHA: Docosahexaenoic Acid; EPA: Eicosapentaenoic Acid; ERK: Extracellular signal-regulated Kinase; IL-2: Interleukin-2; IL-2R: Interleukin-2 Receptor; JAK: Janus Kinase; LA: Linoleic Acid; MAPK: Mitogen-Activated Protein Kinase; NF-κB: Nuclear Factor κB; OA: Oleic Acid; PA: Palmitic Acid; PI3K: Phosphatidylinositol 3-kinase; PKC: Protein Kinase C; PMA: Phorbol Myristate Acetate; PUFA: Polyunsaturated Fatty Acids; SA: Stearic Acid; STAT: Signal Transducer and Activator of Transcription

Introduction

The regulation of lymphocyte function is essential for maintaining immune system homeostasis under various physiological and pathological conditions. Disturbances in the lymphocyte proliferation has been associated to several autoimmune diseases, including type 1 diabetes mellitus, rheumatoid arthritis, lupus erythematosus, and Crohn's disease [1,2]. The lymphocyte activation begins with the antigen interaction, but the cell proliferation is mediated by the interaction between IL2 and its receptor (IL-2R) [3-8]. The high-affinity IL-2 receptor consists of three subunits: the α, β, and γ chains. IL-2 shares a common cytokine receptor γ chain with IL-4, IL-7, IL-9, IL-15, and IL-21 [9]. After mitogenic activation, human T cells show increased IL-2Rα expression resulting in high-affinity IL-2R formation [10]. IL-2 induces heterodimerization of the β and γ chains, resulting in the intermolecular transphosphorylation of their corresponding receptor-associated kinases, Janus kinase 1 (JAK1) and JAK3 [4,6]. JAK3 activates JAK1, which is then able to phosphorylate specific tyrosine residues in STAT5 molecules docked with the β subunit of the IL-2R. Therefore, STAT5 activation occurs when JAK1 and JAK3 are phosphorylated [4-6]. IL-2-induced STAT5 tyrosine phosphorylation allows Src homology 2 domain-mediated homodimerization or heterodimerization, leading to the induction of nuclear migration and sequence-specific DNA binding by STATs [7]. IL-2 also stimulates STAT5 serine phosphorylation that is required for the transcriptional activity of this factor [11]. Ras is activated through the binding of the adaptor Shc to the tyrosine-phosphorylated IL2R, which recruits the Grb2-Sos complex and activates the Raf-MEK-ERK pathway. The non-catalytic region of tyrosine kinase-1 (Nck-1) is also important for activation of this pathway. In fact, knockdown of this gene in Jurkat T cells decreased the ERK1/2 phosphorylation and T-cell activation [12]. IL-2 signal transduction also involves activation of other signaling pathways, such as phosphatidylinositol 3-kinase (PI3K)-Akt pathway. Activation of this pathway potentiates the proliferation associated with STAT5 [13]. PI3K may activate transcription factors that mediate downstream events of the G1 cell cycle checkpoint. Akt, a downstream protein of the PI3K pathway, phosphorylates several substrates including transcription factors and proteins directly involved in the regulation of gene transcription [14] and of T lymphocyte metabolism [15]. Activation of all these pathways is necessary for lymphocyte proliferative and regulatory function since IL2R activation is also related to generation and activity of regulatory T cells (Treg) [16]. Therefore, alterations on IL-2 pathways can modulate lymphocyte differentiation and activation.

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Fatty acids differently control lymphocyte proliferation, survival and death by various mechanisms [17-19]. N-3 polyunsaturated fatty acids (n-3 PUFAs) are demonstrated to have beneficial and protective effects on human health because of their immunosuppressive actions in some autoimmune and inflammatory diseases [20-22]. The n-3 PUFAs, eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), lead to a marked decrease in proliferation of lymphocytes from fish oil-fed mice in a Th1 model stimulated with ovalbumin in the footpad, and inhibition of IL-2-induced cell cycle progression in human lymphocytes cultured *in vitro* [17,23]. The saturated FAs palmitic acid (PA; 16:0) and stearic acid (SA; 18:0) also decrease human peripheral lymphocyte [17] and mouse T lymphocytes [24] proliferation. In contrast, n-6 PUFAs exhibit proinflammatory properties that increase intercellular adhesion molecule-1 expression [25], natural killer cell activity [26], and IgE production [27]. Linoleic acid (LA; C18:2 n-6) also potentiates the stimulatory effects of IL-2 on lymphocyte proliferation at a low concentration (25 μM) [17].

The effects of FAs on lymphocyte function have been extensively studied; however, not all mechanisms involved have been identified. EPA and DHA have been shown to inhibit the MAPK pathway in Jurkat T-cells during an antigenic challenge. Denys et al. [28-30] demonstrated that EPA and DHA decrease the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) that is induced by phorbol myristate acetate (PMA) and anti-CD3 antibodies in Jurkat T-cells. PMA is known to activate MAPKs via the protein kinase C (PKC) pathway [31]. DHA also attenuates ERK1/2 and Akt phosphorylation in regulatory T-cells that are activated by anti-CD3 and anti-CD28 antibodies [32]. In breast cancer cells, DHA impairs the ERK1/2 pathway and increases syndecan 1 expression, resulting in decreased proliferation and increased apoptosis, respectively [33]. These results suggest that these FAs may exert their inhibitory action on ERK1/2 activation by reducing PKC activity. Denys et al. [31] demonstrated that DHA and EPA inhibit the recruitment of PKC-α and PKC-ε to the membrane. These PKC isoforms are coupled to MAPK activation upstream of ERK1/2, which leads to a decrease in the nuclear translocation of nuclear factor κB (NF-κB), resulting in an inhibition of IL-2 gene expression and cell proliferation. Zeyda et al. [34] studied the effects of EPA on MAPK phosphorylation and the transcriptional activity of AP-1, NF-AT, and NF-κB in Jurkat and primary T-cells after 2 days of treatment and found that EPA promotes the highly selective inhibition of c-jun N-terminal kinase phosphorylation and activation but leaves the phosphorylation of other MAPKs, such as p38 MAPK, essentially unaltered. These studies suggest that PUFAs have a selective action on some intracellular proteins involved in T-cell activation and proliferation.

Some effects of DHA and EPA are associated with generation of specific lipid mediators. For example, DHA and EPA are precursors of resolvins, lipid mediators that were first identified by Serhan et al. [35]. DHA decreases M1 macrophage and increases M2 in adipose tissue through resolvin D1 generation. These effects are related to Th1 response attenuation [36]. Other mediators may be involved in the effects of n-3 fatty acids. Opreanu et al. [37] observed that DHA downregulates basal and cytokine-induced acid and neutral sphingomyelinase expression in endothelial cells from human retina. Inhibition of sphingomyelinases in endothelial cells prevents cytokine-induced inflammatory response. Ceramides play an essential role in sphingolipid synthesis pathways. These signaling molecules regulate several cell functions, including apoptosis, cell growth, differentiation, and inflammation [38,39]. In fact, ceramide-enriched microdomains appear to be a prerequisite for inflammatory cytokine signaling and apoptosis induction [37]. However, there are no studies about the involvement of ceramides in the effect of fatty acids on lymphocyte proliferation. Some studies have shown that the inhibitory effects of PA on lymphocytes are related to induction of apoptosis through *de novo* ceramide synthesis activation [19,40]. However, there are no studies investigating the effects of this fatty acid on proliferative pathways.

The effects of FAs on signaling pathways activated by T-cell receptor stimulation have been extensively studied [30,31,34,41]. However, the effect of FAs on cytokine signaling pathways, such as IL-2 signaling, remains unclear. Downstream proteins related to activation of the IL-2 receptor may be modulated by FAs, which potentially could explain some of their immunomodulatory effects. We have previously shown that DHA, EPA, palmitic acid (PA), and stearic acid (SA) decrease the stimulatory effects of IL-2 on lymphocyte proliferation, thereby increasing the percentage of cells in the G1 phase and decreasing the proportion of cells in the S and G2/M phases after 48 h of treatment; conversely, LA and oleic acid (OA) at a low concentration (25 μM) acted synergistically with IL-2, increasing cell proliferation [17].

In this study, we investigated the effects of administering 25 μM OA (C18:1 n-9) and LA (C18:2 n-6) and 50 μM PA (C16:0), SA (C18:0), DHA (C22:6 n-3), and EPA (C20:5 n-3) on the IL-2 signaling pathways in human lymphocytes. The mechanisms involved were evaluated by investigating the involvement of the *de novo* ceramide synthesis and PI3K pathway. The fatty acid concentrations used in this study were chosen based on a previous study, in which we observed that these FAs modulate lymphocyte proliferation without promoting cell death [17]. In addition, the fatty acids tested are abundant in occidental diet and in the lipid composition of clinical nutrition preparations.

Materials and Methods

Chemicals and antibodies

RPMI-1640 medium, HEPES, fetal bovine serum, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Fatty acids, concanavalin A (ConA), Histopaque-1077, glutamine, DHA, EPA, PA, SA, OA, LA, Fumonisin B1 (FB1), wortmannin and recombinant IL-2 were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-phospho-JAK1 (Tyr 1022/1023), anti-phospho-JAK3 (Tyr 980), anti-phospho-STAT5 (Tyr 694), anti-phospho-STAT5 (Ser 726), anti-phospho-ERK1/2 (Tyr 204), anti-phospho-Akt (Ser 473), anti-JAK1, anti-JAK3, anti-STAT5, anti-ERK1/2, anti-rabbit and anti-mouse anti-bodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-PKC-ζ, anti-PKC-ζ, and anti-CD25 were obtained from Cell Signaling Technology (Beverly, MA). Anti-Akt was obtained from Invitrogen (Carlsbad, CA). The FITC-conjugated anti-CD25 antibody was purchased from Pharmingen-BD Biosciences (San Diego, CA). Salts used for buffer preparation were obtained from Labsynth (Diadema, Brazil).

Study design

This study was approved by the Ethical Committees of the Institute of Biomedical Sciences - University of Sao Paulo (ICB-USP), and of the Cruzeiro do Sul University. For the experiments preformed in the ICB-USP, the human blood for lymphocyte isolation was obtained from the Blood Bank of the Federal University of São Paulo. The blood was considered healthy after a routine laboratory analysis. Experiments were also carried out in the Institute of Physical Activity and Sports Science (ICAFE) of the Cruzeiro do Sul University using human blood obtained from healthy male volunteers that declared that they had no pathologies and took no medications or supplements during the experimental

timeframe. A statement of informed consent was obtained from all volunteers. The procedure used for blood collection was approved by the Ethical Committee of the Cruzeiro do Sul University.

Isolation of peripheral blood lymphocytes

Peripheral blood lymphocytes were isolated as previously described [17]. Blood was diluted in PBS (1:1) and the resulting suspension was layered onto Histopaque-1077 and centrifuged for 30 min at 400 g at room temperature. Peripheral blood mononuclear cells (a mixture of monocytes and lymphocytes) were collected from the interphase, and erythrocytes were lysed with 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 0.1 mM EDTA, pH 7.4, and washed once with PBS.

Peripheral blood mononuclear cells were maintained in RPMI-1640 medium to allow the monocytes to adhere to the plates in order to obtain a pure lymphocyte suspension (>98%).

Culture conditions

Cells were grown in culture flasks containing RPMI-1640 medium with 10% fetal bovine serum. This medium was supplemented with glutamine (2 mM), HEPES (20 mM), streptomycin (10 mg/mL), penicillin (10,000 IU/mL), and sodium bicarbonate (24 mM). The cells were grown in 25 mL flasks containing 1×10^6 cells/mL and maintained in a humidified atmosphere at 37°C containing 5% CO_2.

Fatty acid treatment

Assays used to evaluate the effects of FAs on IL-2-activated intracellular signaling were performed by incubating lymphocytes with 5 µg/mL ConA for 24 h to stimulate the expression of the α subunit of IL-2R (CD25). Afterward, the lymphocytes were washed with PBS and cultured with various FAs in the presence and absence of IL-2 (30 ng/mL) for 1 h to promote a maximal lymphocyte proliferation response.

The FAs used had been previously diluted in ethanol. The final concentration of ethanol in the experiment was 0.5% and was not toxic to the cells (data not shown), as also reported by Siddiqui et al. [42]. The concentrations of the FAs used (50 µM for EPA, DHA, PA, and SA and 25 µM for OA and LA) were not toxic but caused significant effects on the proliferation of human lymphocytes [17].

Western blotting

After the end of the incubation period, IL-2 stimulation was terminated by adding ice-cold PBS and the cells were pelleted by a short centrifugal run. Lymphocytes (1×10^7 cells) were homogenized in 120 µL of extraction buffer (100 mM Trizma, pH 7.5, 10 mM EDTA, 10% sodium dodecyl sulfate, 100 mM NaF, 10 mM sodium pyrophosphate, and 10 mM sodium orthovanadate) and immediately sonicated at 4°C for 60 s. The samples were boiled for 5 min and centrifuged at 13,000 *g* for 40 min at 4°C. Aliquots of the supernatants were used to measure the total protein content as described by Bradford [43]. Samples containing equal amounts of proteins (40 µg) were diluted in Laemmli buffer containing DL-dithiothreitol (1 M), heated in a boiling water bath for 3 min, and resolved in a 12% SDS-polyacrylamide gel [44]. Western blotting was performed according to the method described by Towbin, Staehelin, and Gordon [45]. The proteins in the gel were transferred to a nitrocellulose membrane at 120 V for 1 h. Nonspecific bonds were blocked by incubating the membranes with 5% non-fat milk in basal solution (10 mM Trizma, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) at room temperature for 2 h. Membranes were washed three times for 10 min each time in basal solution and then incubated in basal solution containing 3% defatted milk at room temperature for 3 h with the following antibodies: anti-phospho-JAK1 (1:1,000 dilution, Tyr 1022/1023), anti-JAK1 (1:1,000), anti-phospho-JAK3 (1:1,000, Tyr 980), anti-JAK3 (1:1,000), anti-phospho-STAT5 (1:500, Tyr 694), anti-phospho-STAT5 (1:400, Ser 726), anti-STAT5 (1:1,000), anti-phospho-ERK1/2 (1:500, Tyr 204), anti-phospho-Akt (1:500, Ser 473), anti-phospho-PKC-ζ (1:1,000), and anti-PKC-ζ (1:1,000) or anti-CD25 (1:1,000). Membranes were washed again (three times for 10 min each time) and incubated with the corresponding secondary antibody (1:10,000 dilution) linked to horseradish peroxidase in basal solution containing 1% defatted milk at room temperature for 1 h. After washing, the membranes were incubated with peroxidase substrate and chemiluminescence enhancer (ECL Western Blotting System Kit) for 1 min and immediately exposed to X-ray film. The resulting films were then developed in the conventional manner [45,46]. The blots were quantitatively analyzed using Image J software (NIH, USA).

Flow cytometric analysis

After treatment with FAs and IL-2 for 1 h, the lymphocytes (1×10^6 cells) were resuspended in PBS, labeled with FITC-conjugated anti-CD25 antibody (1:50) (Pharmingen-BD Biosciences), and incubated for 1 h at 4°C in the dark. Negative control cells were incubated with an isotype-matched nonreactive IgG1 antibody. After incubation, the cells were washed with PBS and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Juan, CA). Fluorescence was measured using the FL1-H channel (green fluorescence, 530/30 nm). Ten thousand events were analyzed per experiment. Cells exhibiting FITC fluorescence were evaluated using Cell Quest software (Becton Dickinson).

Effect of fatty acids in the presence of the inhibitors fumonisin B1 (FB1) and wortmannin Lymphocyte proliferation

To investigate the possible mechanisms involved with the effect of fatty acids on IL2 signaling pathways, we evaluated proliferation of lymphocytes treated with the different fatty acids in the presence or absence of fumonisin B1 (FB1), an inhibitor of ceramide *de novo* synthesis, or wortmannin (an inhibitor of PI3K).

Proliferation of lymphocytes was determined by the uptake of [2-^{14}C]-thymidine. Cells were cultured at a density of 2.5×10^5 cells per well in 96 well plates (200 µL). Cells were treated with 30 ng/mL IL2 and 50 µM of DHA, EPA, PA, SA and 25 µM of OA and LA in the presence and absence of 10 µM fumonisin or 100 nM of wortmannin. After 30 h, [2-^{14}C]-thymidine (1 mCi per mL) was added to the medium and the cells were incubated for a further period of 18 h. The radioactivity was analysed using a scintillation counter (Packard TRI-CARB 2100 TR counters; Downers Grove, IL, USA).

PKC-ζ phosphorylation

Studies already showed that PKC-ζ phosphorylation is activated by PI3K [47]. The involvement of PKC-ζ phosphorylation with the stimulatory effects of OA and LA on lymphocyte proliferation was confirmed by treating cells with the fatty acids in the presence of wortmannin (a PI3K inhibitor). Therefore, lymphocytes were treated with 30 ng/mL IL2 and 25 µM of OA and LA in the presence and absence of 100 nM of wortmannin for 60 minutes. PKC-ζ phosphorylation was determined by western blotting as described before.

Statistical analysis

Results are presented as mean ± SEM. Differences were assessed by Two-Way ANOVA and Bonferroni post-test using the GraphPad Prism

software (Graph Pad Software, Inc, San Diego, CA). The significance level was set for p<0.05.

Results

JAK1, JAK3, and STAT5 phosphorylation

The effects of IL-2 on JAK1 and JAK3 phosphorylation in human lymphocytes were evaluated for incubation periods of up to 90 min. The highest level of JAK1 and JAK3 phosphorylation promoted by IL-2 was observed at 60 min. DHA, EPA, SA, and PA (50 µM) caused marked decreases (49, 32, 54 and 56% respectively) in the level of JAK1 (Figure 1) and JAK3 phosphorylation (Figure 2) induced by IL-2. In contrast, 25 µM OA and LA did not affect the level of JAK1 and JAK3 phosphorylation. The total expression of the JAK1 and JAK3 proteins was unaltered by the tested fatty acids.

DHA, EPA, PA, and SA caused decreases (44, 32, 61 and 32%) in the level of STAT5 tyrosine residue phosphorylation induced by IL-2 (Figure 3). Serine phosphorylation was also decreased by treatment with EPA, DHA, PA, and SA (decrease of 64, 65, 57 and 52%) (Figure 3). Tyrosine and serine residues must both be phosphorylated to achieve the highest level of IL-2-induced STAT5 transcriptional activity [11].

ERK1/2 phosphorylation

IL-2 induced ERK1/2 phosphorylation was decreased by treatment with DHA and EPA (decrease of 65 and 78%, respectively) (Figure 4). DHA also caused a reduction in cells that had not been stimulated with IL-2. PA and SA also promoted decreases in the phosphorylation of these proteins (decrease of 39 and 46%) (Figure 4). Total ERK1/2 content was unchanged by these FAs. In contrast, OA and LA caused an increase of 34 and 51% in the levels of ERK1/2 phosphorylation induced by IL-2 (Figure 4).

Akt phosphorylation

DHA and EPA exerted inhibitory effects on IL-2-induced Akt phosphorylation (decreases of 60 and 69%, respectively) (Figure 5). PA and SA also reduced Akt phosphorylation in IL-2 stimulated cells (decreases of 64 and 50%, respectively). OA and LA did not alter Akt phosphorylation after 1 h of treatment (Figure 5). This observation suggests that these FAs most likely exert their stimulatory effects through the Ras/ERK1/2 pathway.

PKC-zeta phosphorylation

OA and LA stimulated PKC-ζ phosphorylation in the presence of IL-2 (increase of 19 and 40%, respectively). However, these FAs did not alter the total expression of this protein. PA, SA, DHA, and EPA did not alter PKC-ζ phosphorylation (Figure 6).

IL-2R α-chain expression

The IL-2R α-chain (CD25) assembles with β and γ subunits to form high-affinity IL-2R (27). Induction of CD25 expression is required for IL-2-induced signaling events. The histograms presented in Figure 7 show the fluorescence that corresponds to FITC emission. Inhibition of CD25 expression at the cell surface decreases the fluorescence signal. Only 7.5% of cells were CD25-positive prior to ConA stimulation.

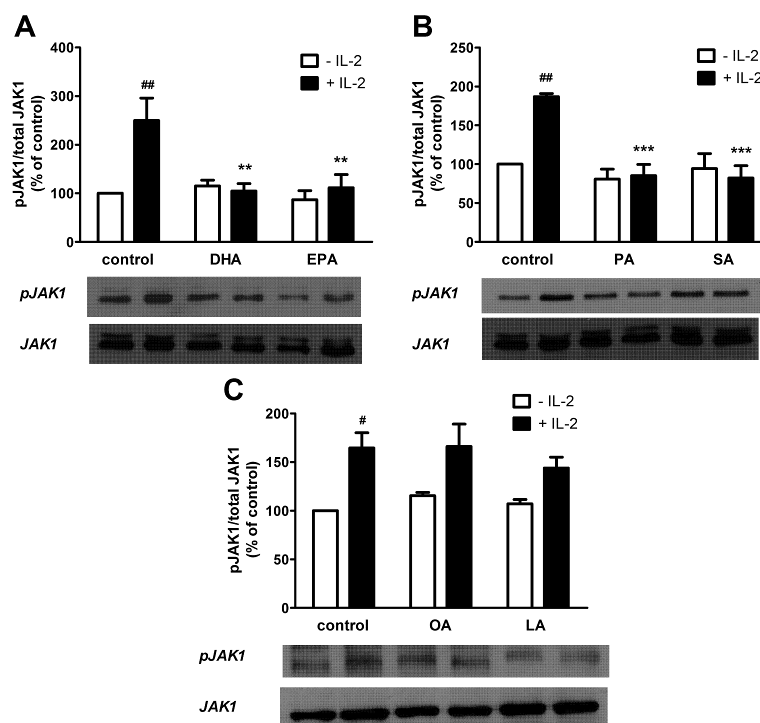

Figure 1: Effects of 50 µM docosahexaenoic (DHA), eicosapentaenoic (EPA), stearic (SA), and palmitic (PA) acids or 25 µM oleic (OA) and linoleic (LA) acids on IL-2-induced JAK1 phosphorylation. Lymphocytes were incubated with 5 µg/mL ConA for 24 h. Afterwards, lymphocytes were washed with PBS and cultured with the different fatty acids in the presence or absence of IL-2 (30 ng/mL) for 1 h. Total proteins were extracted from lymphocytes for western-blotting analysis. Blots were analyzed by densitometry and the results normalized to their respective controls, which were set to 100% in each experiment. The values are presented as the means ± SEM. #p<0.05 and ##p<0.01 for comparison with the control in the absence IL-2; **p<0.01 and ***p<0.001 for comparison with the control treated with IL-2.

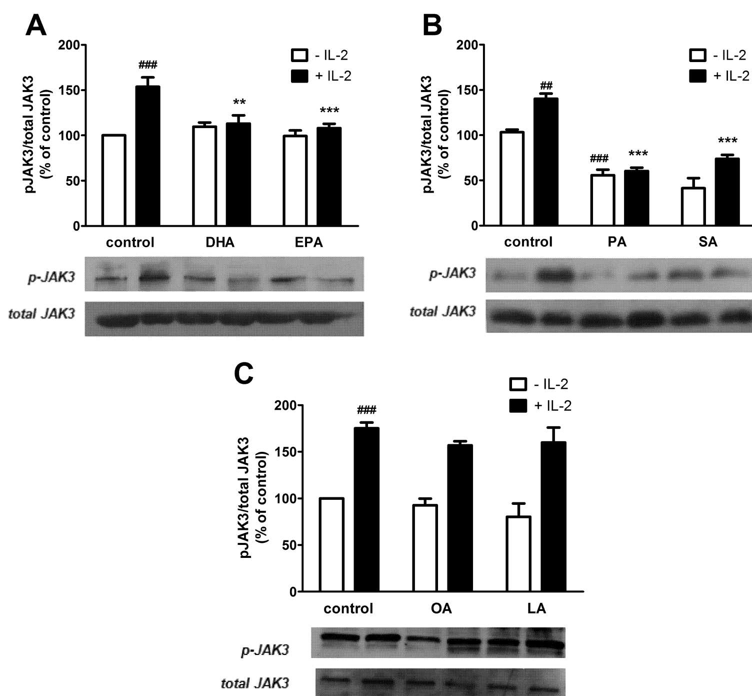

Figure 2: Effects of 50 μM DHA, EPA, SA, and PA or 25 μM OA and LA on IL-2-induced JAK3 phosphorylation. Lymphocytes were incubated with 5 μg/mL ConA for 24 h. Afterwards, lymphocytes were washed with PBS and cultured with the different fatty acids in the presence or absence of IL-2 (30 ng/mL) for 1 h. Total proteins were extracted from lymphocytes for western-blotting analysis. Blots were analyzed by densitometry and the results normalized to their respective controls, which were set to a value of 100% for each experiment. The values are presented as the means ± SEM. $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 for comparison with the control in the absence IL-2; **p<0.01 and p<0.001 for comparison the control treated with IL-2.

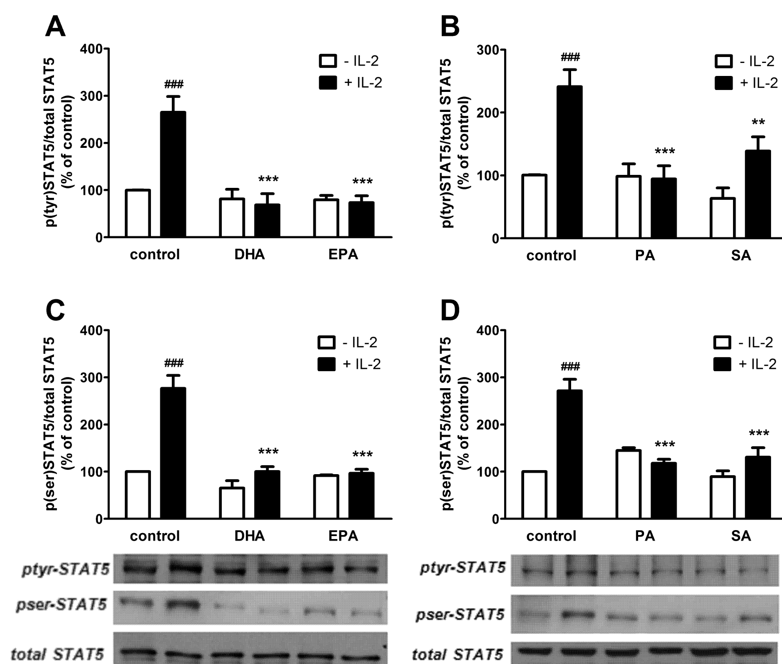

Figure 3: Effects of 50 μM DHA, EPA, SA, and PA on IL-2-induced signal transducer and activator of transcription (STAT) 5 tyrosine 694 and serine 726 phosphorylation. Lymphocytes were incubated with 5 μg/mL ConA for 24 h. Afterwards, lymphocytes were washed with PBS and cultured with the different fatty acids in the presence or absence of IL-2 (30 ng/mL) for 1 h. Total proteins were extracted from lymphocytes for western-blotting analysis. Blots were analyzed by densitometry and the results normalized to their respective controls, which were set to a value of 100% for each experiment. The values are presented as the means ± SEM. $^{\#\#\#}$p<0.001 for comparison with the control in the absence of IL-2); **p<0.01 and ***p<0.001 for comparison with the control treated with IL-2.

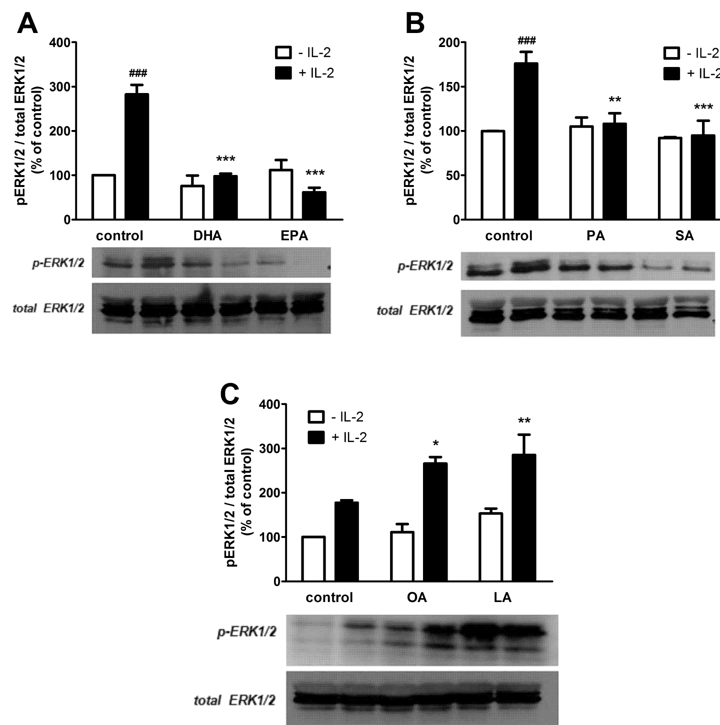

Figure 4: Effects of 50 μM DHA, EPA, SA, and PA or 25 μM OA and LA on IL-2-induced extracellular signal-regulated kinase (ERK) 1/2 phosphorylation. Lymphocytes were incubated with 5 μg/mL ConA for 24 h. Afterwards, lymphocytes were washed with PBS and cultured with the different fatty acids in the presence or absence of IL-2 (30 ng/mL) for 1 h. Total proteins were extracted from lymphocytes for western-blotting analysis. Blots were analyzed by densitometry and the results normalized to their respective controls, which were set to a value of 100% for each experiment. The values are presented as the means ± SEM. ###p<0.001 for comparison with the control in the absence IL-2); *p<0.05, **p<0.01, and ***p<0.001 for comparison with the control treated with IL-2.

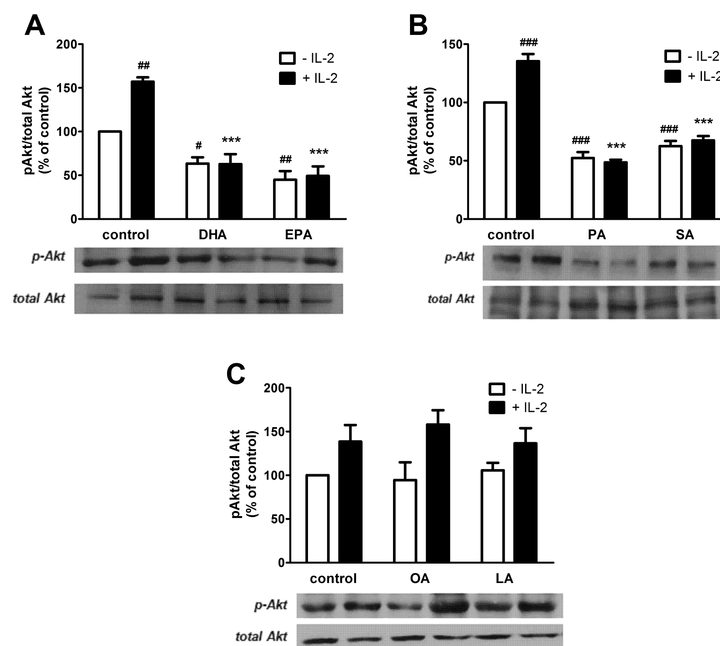

Figure 5: Effects of 50 μM DHA, EPA, SA, and PA or 25 μM OA and LA on IL-2-induced Akt phosphorylation. Lymphocytes were incubated with 5 μg/mL ConA for 24 h. Afterwards, lymphocytes were washed with PBS and cultured with the different fatty acids in the presence or absence of IL-2 (30 ng/mL) for 1 h. Total proteins were extracted from lymphocytes for western-blotting analysis. Blots were analyzed by densitometry and the results normalized to their respective controls, which were set to a value of 100% for each experiment. The values are presented as the means ± SEM. ##p<0.01 and ###p<0.001 for comparison with the control in the absence IL-2; ***p<0.001 for comparison with the control treated with IL-2.

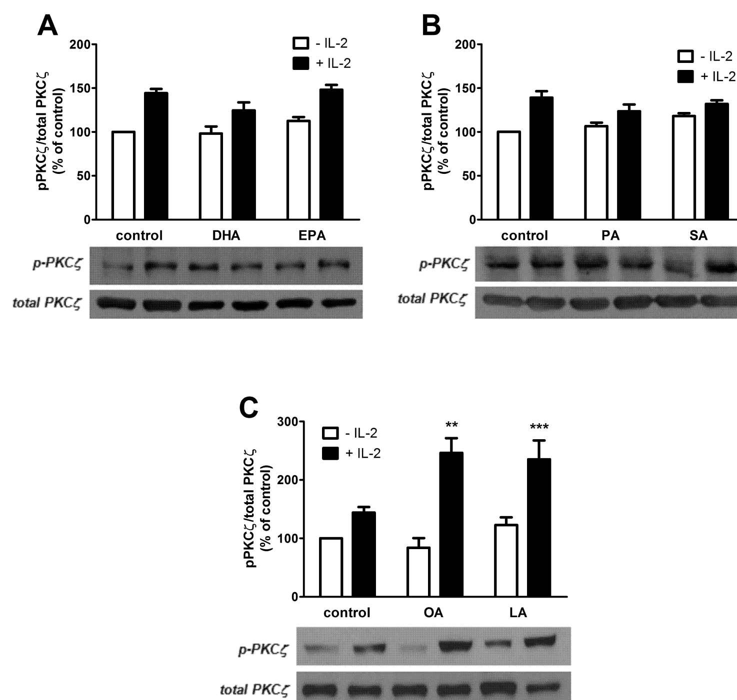

Figure 6: Effects of 50 µM DHA, EPA, SA, and PA or 25 µM OA and LA on IL-2-induced protein kinase C (PKC)-ζ phosphorylation. Lymphocytes were incubated with 5 µg/mL ConA for 24 h. Afterwards, lymphocytes were washed with PBS and cultured with the different fatty acids in the presence or absence of IL-2 (30 ng/mL) for 1 h. Total proteins were extracted from lymphocytes for western-blotting analysis. Blots were analyzed by densitometry and the results normalized to their respective controls, which were set to a value of 100% for each experiment. The values are presented as the means ± SEM. **p<0.01 and ***p<0.001 for comparison with the control treated with IL-2.

ConA-induced CD25 surface expression was unaltered by IL-2 treatment. A significant percentage of cells expressing CD25 was reduced by treatment with DHA, PA, and SA compared with the control (Figure 7) in the presence or absence of IL-2. The percentage of cells that were CD25-positive decreased from 36.5 ± 5.4% in cells treated with IL-2 to 12.6 ± 0.9%, 12.4 ± 1.6%, and 11.8 ± 1.1% (expressed as the mean ± SEM) in cells treated with DHA, SA, and PA, respectively. EPA, OA, and LA did not significantly affect CD25 surface expression after 1 h of treatment (Figure 7).

Effect of wortmannin on IL2-induced lymphocyte proliferation and PKCζ phosphorylation in the presence of oleic and linoleic acids

Wortmannin reduced the stimulatory effect of OA and LA on IL2-induced lymphocyte proliferation (decrease of 74% and 73%, in relation to IL2 plus OA or LA treatment, respectively, in the absence of wortmannin) (Figure 8). The PKCζ phosphorylation induced by OA and LA was also diminished in the presence of wortmannin (decrease of 58% and 63% in comparison with IL2 plus OA or LA treatment, respectively).

Effect of the fumonisin B1 on IL2-induced lymphocyte proliferation in the presence of DHA and EPA

The treatment with FB1 abolished the inhibition induced by DHA and EPA on proliferation of lymphocytes in the presence of IL-2. FB1 did not affect the inhibitory effects of PA or SA on IL2 induced lymphocyte proliferation neither the stimulatory effects of OA and LA (Figure 9).

Discussion

In this study, we demonstrated that some FAs acutely inhibit signaling pathways that are activated by IL-2 in human lymphocytes. We demonstrated that DHA, EPA, PA, and SA decrease IL-2-stimulated JAK1 and JAK3 phosphorylation, suggesting an important mechanism for the inhibition of lymphocyte proliferation induced by these FAs. The total JAK1 and 3 contents were unaltered by FA treatment, indicating that these FAs inhibit lymphocyte proliferation by altering the phosphorylated proteins. IL-2 is particularly important for the development, expansion, and survival of T-cells, and the inhibition of related signaling pathways results in severe immunodeficiency [48]. Thus, the inhibition of IL-2 production [49] and IL-2 action in lymphocytes markedly contributes to the potent suppressive effects of DHA and EPA on the function of these cells, as reported in previous studies [20,21,34,50].

Accordingly, the inhibition of JAK1 and 3 phosphorylation by treatment with PA, SA, EPA, and DHA was accompanied by a reduction in the level of STAT5 phosphorylation, suggesting that downregulation promoted by these fatty acids of JAK-STAT signaling by IL-2 is critically important for immune and inflammatory responses and T-cell differentiation and expansion. Li et al. [41] also demonstrated that DHA reduces IL-2 induced STAT5 activation in Jurkat T-cells. Inhibition of the signaling of this cytokine, as occurs in cases of genetic deficiency involving the γ subunit of JAK3, results in severe immunodeficiency. However, STAT5 is not the only protein involved in this process. In fact, deletion and mutation experiments that affect STAT5 DNA-binding activity or the prevention of STAT5 activation do not block IL-2R mitogenic signaling [48,51]. Although STAT5 does not appear to be

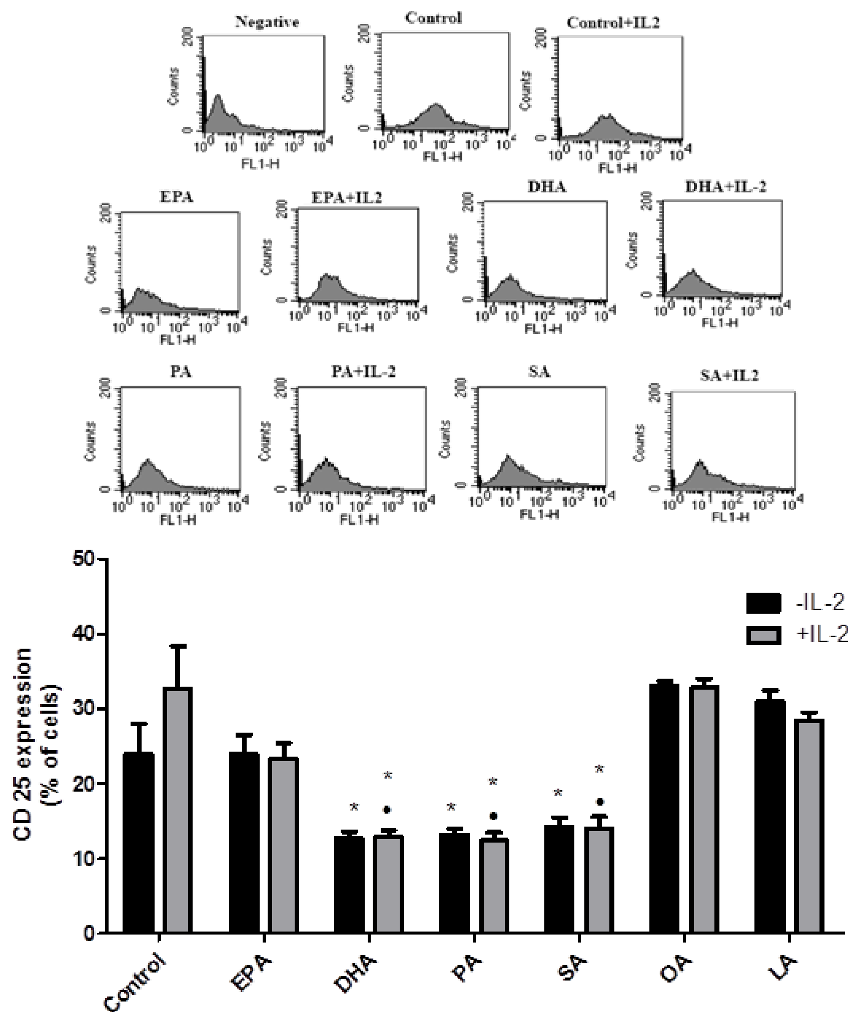

Figure 7: Effects of 50 µM DHA, EPA, SA, and PA or 25 µM OA and LA on CD25-alpha lymphocyte surface expression. Lymphocytes were stimulated with 5 µg/mL ConA for 24 h, washed, and incubated with 30 ng/mL IL-2 for 1 h. Cells were pelleted and labeled with an FITC-conjugated anti-CD25 antibody and analyzed using flow cytometry. Negative control cells were incubated with a labeled nonreactive control antibody. Histograms from 10,000 events were analyzed. Fluorescence was measured using the FL1-H channel (green fluorescence, 530/30 nm). A) Representative histograms obtained using flow cytometry. B) Percentage of cells expressing CD25. The values are presented as the means ± SEM of three determinations from four experiments. *$p<0.05$ for comparison between fatty acid treatments vs. the control (in the absence of FAs and IL-2); •$p<0.05$ for comparison between fatty acid treatments vs. the control treated with IL-2.

essential for mitogenesis, it participates in the induction of some genes involved in cell proliferation and survival [52,53].

Simultaneously, EPA and DHA promoted a decrease in the level of ERK1/2 phosphorylation stimulated by IL-2. This inhibitory effect was associated with a reduction in the levels of JAK1 and JAK3 phosphorylation that is promoted by these FAs, possibly leading to a decrease in ERK1/2 (or p42/p44 MAPK) activity. Activated ERK1/2 phosphorylates various substrates in cell compartments, playing an important role in cell growth and providing an integrative response, including increased nucleotide synthesis, transcriptional activation of various genes via transcription factors (e.g., Elk-1, Fos, AP-1, NF-AT, and c-myc), chromatin phosphorylation, stimulation of protein synthesis, and facilitation of the formation of an active cyclin D-CDK4 complex, which is a rate-limiting step for cell growth [54,55]. Khan et al. [56] also demonstrated that DHA arrests progression from the late G1 to S phase in the FM3A mouse mammary cancer cell cycle by decreasing ERK1/2 phosphorylation.

The inhibition of ERK1 and 2 activation by n-3 PUFAs has been demonstrated in previous studies using various cell lines [50] including Jurkat T-cells [28,29], T regulatory cells [32], macrophages [57], fibroblasts [30,58], endothelial cells [59], renal cells [60], and smooth muscle cells [61]. Furthermore, Denys et al. [29,30] demonstrated that EPA and DHA inhibit ERK1/2 phosphorylation by decreasing PKC-β activation in PMA-stimulated Jurkat cells. In our study, n-3 PUFAs also decreased the phosphorylation of JAK1 and JAK3, which are members of a signaling pathway that is stimulated by IL-2 and also results in ERK1/2 activation.

Several authors have investigated the effects of n-6 PUFAs on lymphocyte proliferation. Cury-Boaventura et al. [62] observed a decrease in the proliferation of lymphocytes from volunteers who received a LA-rich lipid emulsion. The authors concluded that this effect was related to LA toxicity, increasing cell death through apoptosis and necrosis. However, Thanasak et al. [63] demonstrated that the treatment of bovine lymphocytes with low concentrations of LA (<25

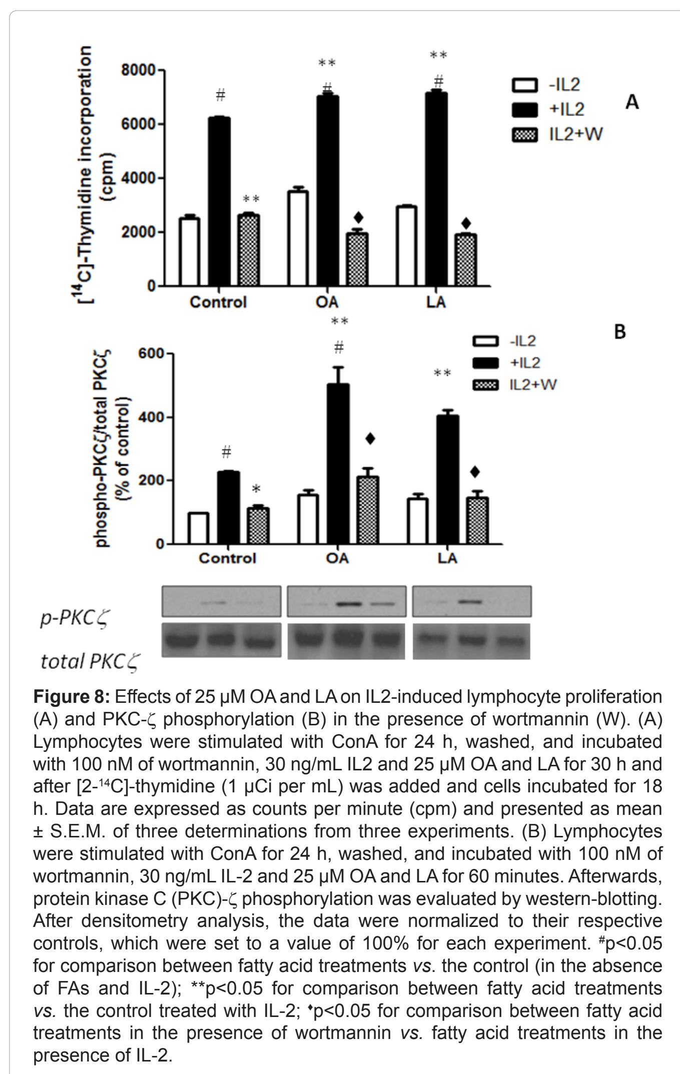

Figure 8: Effects of 25 µM OA and LA on IL2-induced lymphocyte proliferation (A) and PKC-ζ phosphorylation (B) in the presence of wortmannin (W). (A) Lymphocytes were stimulated with ConA for 24 h, washed, and incubated with 100 nM of wortmannin, 30 ng/mL IL2 and 25 µM OA and LA for 30 h and after [2-^{14}C]-thymidine (1 µCi per mL) was added and cells incubated for 18 h. Data are expressed as counts per minute (cpm) and presented as mean ± S.E.M. of three determinations from three experiments. (B) Lymphocytes were stimulated with ConA for 24 h, washed, and incubated with 100 nM of wortmannin, 30 ng/mL IL-2 and 25 µM OA and LA for 60 minutes. Afterwards, protein kinase C (PKC)-ζ phosphorylation was evaluated by western-blotting. After densitometry analysis, the data were normalized to their respective controls, which were set to a value of 100% for each experiment. #p<0.05 for comparison between fatty acid treatments *vs.* the control (in the absence of FAs and IL-2); **p<0.05 for comparison between fatty acid treatments *vs.* the control treated with IL-2; ♦p<0.05 for comparison between fatty acid treatments in the presence of wortmannin *vs.* fatty acid treatments in the presence of IL-2.

µM) increased lymphocyte proliferation, whereas higher concentrations of this fatty acid decreased it. Taken together, these results suggest that LA acts as a proinflammatory agent at low concentrations and stimulates lymphocyte proliferation. PKC activation is a key signaling event for cell growth and proliferation in response to several mitogens. Cis-unsaturated fatty acids (e.g., OA) can activate PKC and, in turn, ERKs as previously reported [64]. OA activates more potently and completely Ca^{2+}-independent and atypical PKC isoforms [65,66] in platelets. PKC-ζ has been shown to activate ERK and is associated with mitogenesis in various cell lines [67]. Other cis-fatty acids, such as LA, also fully activate PKC in the same manner [65]. Arachidonic acid, another n-6 PUFA, also has stimulatory effects on MAP kinases in various cell lines including hepatic cells [68], smooth muscle cells [69], monocytes [70], and renal epithelial cells [71]. In contrast, PKC-α, a Ca^{2+}-dependent isoform, is less potently stimulated by OA [66] and has been associated with the differentiation rather than proliferation of vascular smooth muscle cells [72]. Accordingly, in our study, OA and LA enhanced the effects of IL-2 on ERK1/2 phosphorylation, suggesting that this pathway may be involved in the stimulation of lymphocyte proliferation that is promoted by these FAs. This effect was associated with increased PKC-ζ phosphorylation after one hour of treatment. Because OA and LA did not alter JAK/STAT phosphorylation, the activation of novel and/or atypical PKC isoforms may explain, at least in part, the stimulatory effect of OA on IL-2-induced lymphocyte proliferation. However, these stimulatory effects were abolished in the presence of wortmannin. PI3K modulates atypical PKC activity such as PKC-ζ [47]. Studies have shown that PKC-ζ phosphorylation is decreased by the saturated palmitic acid in isolated islets as a result of PI3K activity inhibition [73]. On the other hand, in the present study, PA did not alter PKC-ζ phosphorylation in lymphocytes. However, we observed that only OA and LA stimulated PKC-ζ phosphorylation and that this effect was abolished in the presence of a PI3K inhibitor.

Akt phosphorylation was decreased by DHA, EPA, PA, and SA. The effect of DHA was more pronounced than that of the other FAs tested, whereas OA and LA did not affect Akt protein phosphorylation. Mammalian target of rapamycin (mTOR) has been suggested as a key regulatory protein involved in T cell differentiation and proliferation induced by several factors, including IL-2. This cytokine activates mTOR through a PI3-K/Akt pathway, leading to important metabolic changes required for lymphocyte growth and proliferation mediated by T regulatory cells [74]. These latter cells play an important role in leukocyte activation [75]. Thus, it is possible that the inhibitory effect on Akt, JAK-STAT, and ERK1/2 pathways induced by DHA, EPA, PA, and SA is responsible for decreased lymphocyte proliferation [17].

Saturated fatty acids, particularly PA, interfere with various signaling pathways in several cell lines. PA decreases insulin-stimulated MAPK activity and phosphorylation in rat-1 fibroblasts [76] and rat skeletal muscle [46], and Akt phosphorylation and/or activity in perfused rat hearts and cardiac muscles, HL-1 cells [77], pmi28 myotubes [78], rat soleus muscles [46,79], C2C12 cells [80,81], and L6 muscle cells [82]. In these cells, insulin signaling pathway impairment by saturated fatty acids has been associated with the development of insulin resistance [83-85]. Herein, we demonstrated that PA and SA also decrease IL-2-stimulated ERK1/2 and Akt phosphorylation in human blood lymphocytes associated with decreased IL-2-induced lymphocyte proliferation. Thus, saturated fatty acids have anti-proliferative on human lymphocytes and impairing effects on insulin-sensitizing cells. On the other hand, n-3 PUFAs exhibit immunosuppressive effects but have been associated with improvement in insulin sensitivity by mechanisms that are currently under investigation such as increased insulin-stimulated p38 MAP kinase phosphorylation and activation of GPR120, PPAR-α and AMPK [86-91].

The α subunit of IL-2R (CD25-α) is undetectable in resting T-cells. The expression of this protein is triggered by antigens [92] that can be mimicked by lectins including ConA [93]. If a decrease in CD25-α externalization occurs, IL-2R-stimulated pathways are suppressed and lymphocyte proliferation is inhibited. DHA, PA, and SA decreased the CD25-α content on the plasma membrane surface in lymphocytes after one hour of treatment. CD25 membrane surface expression is necessary to form the high-affinity form of IL-2R that actually activates the intracellular signaling pathways (JAK-STAT, Ras-ERK1/2, and PI3K-Akt) leading to lymphocyte proliferation. It is likely that the inhibition of JAK1 and JAK3, ERK1/2, and Akt phosphorylation promoted by DHA, PA, and SA can be attributed to the decrease in CD25-α expression and content in the plasma membrane. Other authors have demonstrated that PUFAs alter IL-2 and epidermal growth factor receptors localization on the cell surface after 24 h of treatment [41,94]. However, a change in the content of this receptor subunit in the plasma membrane does not fully explain the effect of all FAs on IL-2 signaling. It is worth noting that both OA and LA stimulated ERK1/2 phosphorylation but did not alter CD25 expression. Measurements using a red fluorescent Alexa Fluor 594 conjugate of the cholera toxin subunit B demonstrated that, with

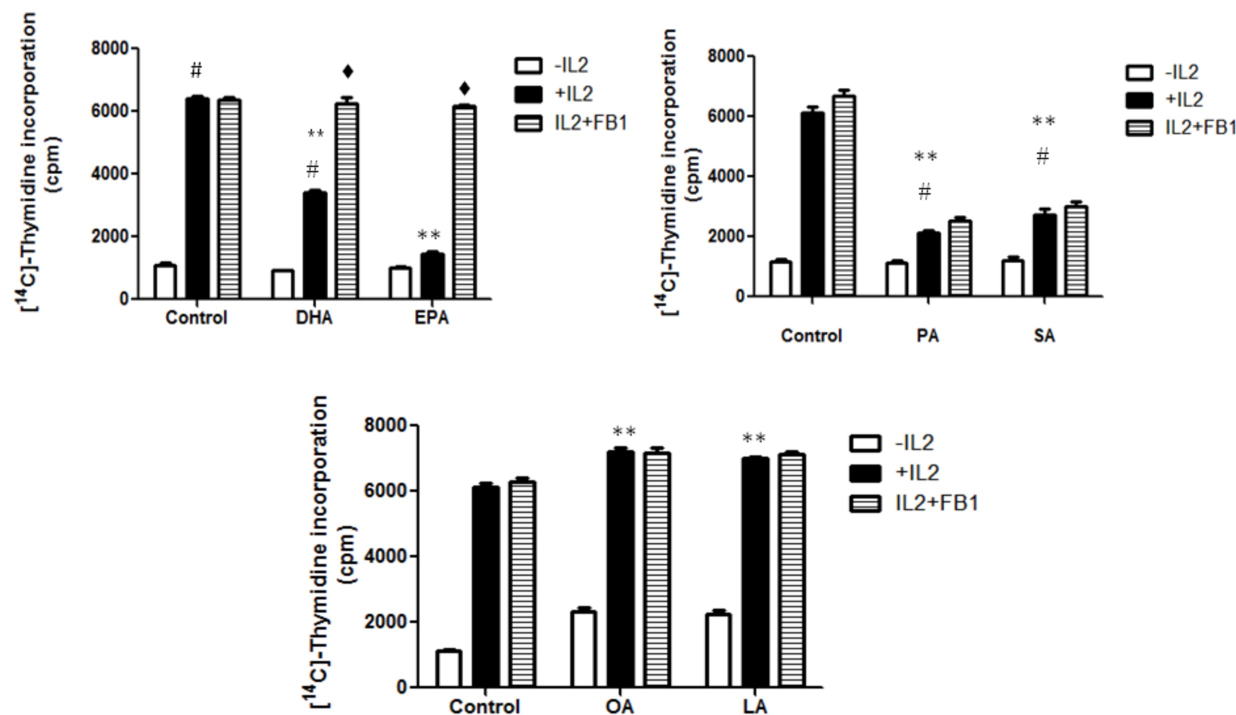

Figure 9: Effect of 50 μM DHA, EPA, SA, and PA and 25 μM OA and LA on IL2 induced lymphocyte proliferation in the presence and absence of fumonisin B1 (FB1). Lymphocytes were stimulated with ConA for 24 h, washed, and incubated with 10 μM FB1, 30 ng/mL IL-2 and 50 μM DHA, EPA, SA, and PA and 25 μM OA and LA for 48h. Data are expressed as counts per minute (cpm) and presented as mean ± S.E.M. of three determinations from three experiments. The values are presented as mean ± S.E.M. of four determinations from three experiments. #p<0.05 for comparison between fatty acid treatments vs. the control (in the absence of FAs and IL-2); **p<0.05 for comparison between fatty acid treatments vs. the control treated with IL-2; ♦p<0.05 for comparison between fatty acid treatments in the presence of wortmannin vs. fatty acid treatments in the presence of IL-2.

the exception of DHA, none of the FAs tested caused marked changes in lipid raft distribution under the conditions used in this study (data not shown). Therefore, acute treatment with EPA, PA, SA, OA and LA did not affect intracellular signaling through changes in lipid rafts.

The immunosuppressive and anti-inflammatory effects of n-3 PUFAs have been extensively studied and reported by several groups [20,21]. However, the precise mechanisms involved have not been completely described yet. In the present study, we observed that the inhibitory effect of DHA and EPA on lymphocyte proliferation is abolished in the presence of FB1, a *de novo* ceramide synthesis inhibitor. In fact, several studies have demonstrated that a decrease in intracellular ceramide content is related to uncontrolled cell proliferation [95] and an increase can lead to a cell cycle progression discontinuation [96]. Previous studies have demonstrated that ceramides and other sphingolipids modulate various processes, including inflammation, apoptosis, and cell proliferation [97]. Alpha-galactosylceramide (C20:2) a derivative of ceramide metabolism, enhances anti-inflammatory and Treg cell response in a model of type 1 *diabetes mellitus* [98]. C-2 ceramide exerts anti-inflammatory effects by inhibiting PI3K/Akt and JAK1/STAT1 pathways and promotes upregulation of protein kinase A in microglial cells [99]. In macrophages, the generation of ceramides via acid sphingomyelinase activation is a key step for regulating apoptosis, cytokine production, phagocytosis, reactive oxygen species generation, and cell survival and differentiation [100]. Chen et al. [101] also showed that the apoptotic effects of ceramides are related to p38 MAPK and JNK activation in a mouse T hybridome cell line. Some of these effects can be related to the presence of a linking site for ceramides in proteins, like atypical PKCs [102] or due to protein anchoring membrane microdomains such as lipid rafts [103]. The present study showed that a blockage of the ceramide synthesis pathway abolishes the effects of DHA and EPA on human lymphocyte proliferation. On the other hand, McMurray et al. [104] showed that dietary EPA and DHA decrease the production of intracellular ceramide in stimulated T lymphocytes. Further studies are necessary to clarify the full mechanisms involved in the synthesis of sphingolipids as well as the differences between DHA and EPA on cellular signaling pathways. The effects of PA, SA, OA and LA on lymphocyte proliferation were not affected by FB1, suggesting that ceramide synthesis is not involved in the effects of these fatty acids.

In conclusion, the findings reported herein contribute for understanding the mechanisms by which various FAs modulate lymphocyte proliferation. The inhibitory effects of PA, SA, DHA and EPA on lymphocyte proliferation are associated with a reduction in the IL-2-mediated activation of the JAK/STAT, ERK, and Akt pathways. The decrease in lymphocyte proliferation promoted by DHA and EPA also involves *de novo* ceramide synthesis. The stimulatory effects of OA and LA on lymphocyte proliferation are associated with improved activity of the MAP kinase and PI3K pathways, as demonstrated by increased ERK1/2 and PKC-ζ phosphorylation.

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Conflict of Interest

The authors declare no financial and commercial conflict of interest.

References

1. Datta S, Sarvetnick N (2009) Lymphocyte proliferation in immune-mediated diseases. Trends Immunol 30: 430-438.
2. Barcala Tabarrozzi AE, Castro CN, Dewey RA, Sogayar MC, Labriola L, et al. (2013) Cell-based interventions to halt autoimmunity in type 1 diabetes mellitus. Clin Exp Immunol 171: 135-146.
3. Smith KA (1988) The interleukin 2 receptor. Adv Immunol 42: 165-179.
4. Kirken RA, Rui H, Malabarba MG, Howard OM, Kawamura M, et al. (1995) Activation of JAK3, but not JAK1, is critical for IL-2-induced proliferation and STAT5 recruitment by a COOH-terminal region of the IL-2 receptor beta-chain. Cytokine 7: 689-700.
5. Beadling C, Guschin D, Witthuhn BA, Ziemiecki A, Ihle JN, et al. (1994) Activation of JAK kinases and STAT proteins by interleukin-2 and interferon alpha, but not the T cell antigen receptor, in human T lymphocytes. EMBO J 13: 5605-5615.
6. Russell SM, Johnston JA, Noguchi M, Kawamura M, Bacon CM, et al. (1994) Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. Science 266: 1042-1045.
7. Darnell JE Jr, Kerr IM, Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264: 1415-1421.
8. Popmihajlov Z, Xu D, Morgan H, Milligan Z, Smith KA (2012) Conditional IL-2 Gene Deletion: Consequences for T Cell Proliferation. Front Immunol 3: 102.
9. Liao W, Lin JX, Leonard WJ (2011) IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. Curr Opin Immunol 23: 598-604.
10. Kim HP, Imbert J, Leonard WJ (2006) Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. Cytokine Growth Factor Rev 17: 349-366.
11. Beadling C, Ng J, Babbage JW, Cantrell DA (1996) Interleukin-2 activation of STAT5 requires the convergent action of tyrosine kinases and a serine/threonine kinase pathway distinct from the Raf1/ERK2 MAP kinase pathway. EMBO J 15: 1902-1913.
12. Yiemwattana I, Ngoenkam J, Paensuwan P, Kriangkrai R, Chuenjitkuntaworn B, et al. (2012) Essential role of the adaptor protein Nck1 in Jurkat T cell activation and function. Clin Exp Immunol 167: 99-107.
13. Moon JJ, Nelson BH (2001) Phosphatidylinositol 3-kinase potentiates, but does not trigger, T cell proliferation mediated by the IL-2 receptor. J Immunol 167: 2714-2723.
14. Datta SR, Brunet A, Greenberg ME (1999) Cellular survival: a play in three Akts. Genes Dev 13: 2905-2927.
15. Zehn D, King C, Bevan MJ, Palmer E (2012) TCR signaling requirements for activating T cells and for generating memory. Cell Mol Life Sci 69: 1565-1575.
16. Malek TR, Bayer AL (2004) Tolerance, not immunity, crucially depends on IL-2. Nat Rev Immunol 4: 665-674.
17. Gorjão R, Cury-Boaventura MF, de Lima TM, Curi R (2007) Regulation of human lymphocyte proliferation by fatty acids. Cell Biochem Funct 25: 305-315.
18. Cury-Boaventura MF, Gorjão R, Martins de Lima T, Fiamoncini J, Godoy AB, et al. (2011) Effect of medium/ω-6 long chain triglyceride-based emulsion on leucocyte death and inflammatory gene expression. Clin Exp Immunol 165: 383-392.
19. Takahashi HK, Cambiaghi TD, Luchessi AD, Hirabara SM, Vinolo MA, et al. (2012) Activation of survival and apoptotic signaling pathways in lymphocytes exposed to palmitic acid. J Cell Physiol 227: 339-350.
20. Camuesco D, Galvez J, Nieto A, Comalada M, Rodriguez-Cabezas ME, et al. (2005) Dietary olive oil supplemented with fish oil, rich in EPA and DHA (n-3) polyunsaturated fatty acids, attenuates colonic inflammation in rats with DSS-induced colitis. J Nutr 135: 687-694.
21. Mills SC, Windsor AC, Knight SC (2005) The potential interactions between polyunsaturated fatty acids and colonic inflammatory processes. Clin Exp Immunol 142: 216-228.
22. Robinson DR, Xu LL, Tateno S, Guo M, Colvin RB (1993) Suppression of autoimmune disease by dietary n-3 fatty acids. J Lipid Res 34: 1435-1444.
23. Johansson S, Lonnqvist A, Ostman S, Sandberg AS, Wold AE (2010) Long-chain polyunsaturated fatty acids are consumed during allergic inflammation and affect T helper type 1 (Th1)- and Th2-mediated hypersensitivity differently. Clin Exp Immunol 160: 411-419.
24. Pourbohloul S, Mallett GS, Buttke TM (1985) Inhibition of lymphocyte proliferation by free fatty acids. III. Modulation of thymus-dependent immune responses. Immunology 56: 659-666.
25. Young VM, Toborek M, Yang F, McClain CJ, Hennig B (1998) Effect of linoleic acid on endothelial cell inflammatory mediators. Metabolism 47: 566-572.
26. Pompéia C, Lopes LR, Miyasaka CK, Procópio J, Sannomiya P, et al. (2000) Effect of fatty acids on leukocyte function. Braz J Med Biol Res 33: 1255-1268.
27. Harbige LS (1998) Dietary n-6 and n-3 fatty acids in immunity and autoimmune disease. Proc Nutr Soc 57: 555-562.
28. Denys A, Hichami A, Khan NA (2001) Eicosapentaenoic acid and docosahexaenoic acid modulate MAP kinase (ERK1/ERK2) signaling in human T cells. J Lipid Res 42: 2015-2020.
29. Denys A, Hichami A, Khan NA (2002) Eicosapentaenoic acid and docosahexaenoic acid modulate MAP kinase enzyme activity in human T-cells. Mol Cell Biochem 232: 143-148.
30. Denys A, Hichami A, Maume B, Khan NA (2001) Docosahexaenoic acid modulates phorbol ester-induced activation of extracellular signal-regulated kinases 1 and 2 in NIH/3T3 cells. Lipids 36: 813-818.
31. Denys A, Hichami A, Khan NA (2005) n-3 PUFAs modulate T-cell activation via protein kinase C-alpha and -epsilon and the NF-kappaB signaling pathway. J Lipid Res 46: 752-758.
32. Yessoufou A, Plé A, Moutairou K, Hichami A, Khan NA (2009) Docosahexaenoic acid reduces suppressive and migratory functions of CD4+CD25+ regulatory T-cells. J Lipid Res 50: 2377-2388.
33. Sun H, Hu Y, Gu Z, Owens RT, Chen YQ, et al. (2011) Omega-3 fatty acids induce apoptosis in human breast cancer cells and mouse mammary tissue through syndecan-1 inhibition of the MEK-Erk pathway. Carcinogenesis 32: 1518-1524.
34. Zeyda M, Szekeres AB, Säemann MD, Geyeregger R, Stockinger H, et al. (2003) Suppression of T cell signaling by polyunsaturated fatty acids: selectivity in inhibition of mitogen-activated protein kinase and nuclear factor activation. J Immunol 170: 6033-6039.
35. Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, et al. (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. J Exp Med 196: 1025-1037.
36. Titos E, Rius B, González-Périz A, López-Vicario C, Morán-Salvador E, et al. (2011) Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. J Immunol 187: 5408-5418.
37. Opreanu M, Lydic TA, Reid GE, McSorley KM, Esselman WJ, et al. (2010) Inhibition of cytokine signaling in human retinal endothelial cells through downregulation of sphingomyelinases by docosahexaenoic acid. Invest Ophthalmol Vis Sci 51: 3253-3263.
38. Kolesnick RN, Haimovitz-Friedman A, Fuks Z (1994) The sphingomyelin signal transduction pathway mediates apoptosis for tumor necrosis factor, Fas, and ionizing radiation. Biochem Cell Biol 72: 471-474.
39. Mencarelli C, Martinez-Martinez P (2013) Ceramide function in the brain: when a slight tilt is enough. Cell Mol Life Sci 70: 181-203.
40. Iturralde M, Gamen S, Pardo J, Bosque A, Piñeiro A, et al. (2003) Saturated free fatty acid release and intracellular ceramide generation during apoptosis induction are closely related processes. Biochim Biophys Acta 1634: 40-51.
41. Li Q, Wang M, Tan L, Wang C, Ma J, et al. (2005) Docosahexaenoic acid changes lipid composition and interleukin-2 receptor signaling in membrane rafts. J Lipid Res 46: 1904-1913.

42. Siddiqui RA, Jenski LJ, Neff K, Harvey K, Kovacs RJ, et al. (2001) Docosahexaenoic acid induces apoptosis in Jurkat cells by a protein phosphatase-mediated process. Biochim Biophys Acta 1499: 265-275.

43. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

44. Shapiro AL, Viñuela E, Maizel JV Jr (1967) Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem Biophys Res Commun 28: 815-820.

45. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350-4354.

46. Massao Hirabara S, de Oliveira Carvalho CR, Mendonça JR, Piltcher Haber E, Fernandes LC, et al. (2003) Palmitate acutely raises glycogen synthesis in rat soleus muscle by a mechanism that requires its metabolization (Randle cycle). FEBS Lett 541: 109-114.

47. Yaney GC, Korchak HM, Corkey BE (2000) Long-chain acyl CoA regulation of protein kinase C and fatty acid potentiation of glucose-stimulated insulin secretion in clonal beta-cells. Endocrinology 141: 1989-1998.

48. Gaffen SL, Lai SY, Xu W, Gouilleux F, Groner B, et al. (1995) Signaling through the interleukin 2 receptor beta chain activates a STAT-5-like DNA-binding activity. Proc Natl Acad Sci U S A 92: 7192-7196.

49. Terada S, Takizawa M, Yamamoto S, Ezaki O, Itakura H, et al. (2001) Suppressive mechanisms of EPA on human T cell proliferation. Microbiol Immunol 45: 473-481.

50. Kim W, Khan NA, McMurray DN, Prior IA, Wang N, et al. (2010) Regulatory activity of polyunsaturated fatty acids in T-cell signaling. Prog Lipid Res 49: 250-261.

51. Fujii H, Nakagawa Y, Schindler U, Kawahara A, Mori H, et al. (1995) Activation of Stat5 by interleukin 2 requires a carboxyl-terminal region of the interleukin 2 receptor beta chain but is not essential for the proliferative signal transmission. Proc Natl Acad Sci U S A 92: 5482-5486.

52. Lord JD, McIntosh BC, Greenberg PD, Nelson BH (2000) The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. J Immunol 164: 2533-2541.

53. Yu CR, Ortaldo JR, Curiel RE, Young HA, Anderson SK, et al. (1999) Role of a STAT binding site in the regulation of the human perforin promoter. J Immunol 162: 2785-2790.

54. Whitmarsh AJ, Davis RJ (2000) Regulation of transcription factor function by phosphorylation. Cell Mol Life Sci 57: 1172-1183.

55. Lawan A, Shi H, Gatzke F, Bennett AM (2013) Diversity and specificity of the mitogen-activated protein kinase phosphatase-1 functions. Cell Mol Life Sci 70: 223-237.

56. Khan NA, Nishimura K, Aires V, Yamashita T, Oaxaca-Castillo D, et al. (2006) Docosahexaenoic acid inhibits cancer cell growth via p27Kip1, CDK2, ERK1/ERK2, and retinoblastoma phosphorylation. J Lipid Res 47: 2306-2313.

57. Lo CJ, Chiu KC, Fu M, Chu A, Helton S (2000) Fish oil modulates macrophage P44/P42 mitogen-activated protein kinase activity induced by lipopolysaccharide. JPEN J Parenter Enteral Nutr 24: 159-163.

58. Kim HH, Shin CM, Park CH, Kim KH, Cho KH, et al. (2005) Eicosapentaenoic acid inhibits UV-induced MMP-1 expression in human dermal fibroblasts. J Lipid Res 46: 1712-1720.

59. Massaro M, Habib A, Lubrano L, Del Turco S, Lazzerini G, et al. (2006) The omega-3 fatty acid docosahexaenoate attenuates endothelial cyclooxygenase-2 induction through both NADP(H) oxidase and PKC epsilon inhibition. Proc Natl Acad Sci U S A 103: 15184-15189.

60. Hagiwara S, Makita Y, Gu L, Tanimoto M, Zhang M, et al. (2006) Eicosapentaenoic acid ameliorates diabetic nephropathy of type 2 diabetic KKAy/Ta mice: involvement of MCP-1 suppression and decreased ERK1/2 and p38 phosphorylation. Nephrol Dial Transplant 21: 605-615.

61. Bousserouel S, Brouillet A, Béréziat G, Raymondjean M, Andréani M (2003) Different effects of n-6 and n-3 polyunsaturated fatty acids on the activation of rat smooth muscle cells by interleukin-1 beta. J Lipid Res 44: 601-611.

62. Cury-Boaventura MF, Gorjão R, de Lima TM, Piva TM, Peres CM, et al. (2006) Toxicity of a soybean oil emulsion on human lymphocytes and neutrophils. JPEN J Parenter Enteral Nutr 30: 115-123.

63. Thanasak J, Müller KE, Dieleman SJ, Hoek A, Noordhuizen JP, et al. (2005) Effects of polyunsaturated fatty acids on the proliferation of mitogen stimulated bovine peripheral blood mononuclear cells. Vet Immunol Immunopathol 104: 289-295.

64. Lu G, Morinelli TA, Meier KE, Rosenzweig SA, Egan BM (1996) Oleic acid-induced mitogenic signaling in vascular smooth muscle cells. A role for protein kinase C. Circ Res 79: 611-618.

65. Murakami K, Chan SY, Routtenberg A (1986) Protein kinase C activation by cis-fatty acid in the absence of Ca2+ and phospholipids. J Biol Chem 261: 15424-15429.

66. Khan WA, Blobe G, Halpern A, Taylor W, Wetsel WC, et al. (1993) Selective regulation of protein kinase C isoenzymes by oleic acid in human platelets. J Biol Chem 268: 5063-5068.

67. Berra E, Diaz-Meco MT, Dominguez I, Municio MM, Sanz L, et al. (1993) Protein kinase C zeta isoform is critical for mitogenic signal transduction. Cell 74: 555-563.

68. Hii CS, Ferrante A, Edwards YS, Huang ZH, Hartfield PJ, et al. (1995) Activation of mitogen-activated protein kinase by arachidonic acid in rat liver epithelial WB cells by a protein kinase C-dependent mechanism. J Biol Chem 270: 4201-4204.

69. Rao GN, Baas AS, Glasgow WC, Eling TE, Runge MS, et al. (1994) Activation of mitogen-activated protein kinases by arachidonic acid and its metabolites in vascular smooth muscle cells. J Biol Chem 269: 32586-32591.

70. Dulin NO, Sorokin A, Douglas JG (1998) Arachidonate-induced tyrosine phosphorylation of epidermal growth factor receptor and Shc-Grb2-Sos association. Hypertension 32: 1089-1093.

71. Barry OP, Kazanietz MG, Pratico D, FitzGerald GA (1999) Arachidonic acid in platelet microparticles up-regulates cyclooxygenase-2-dependent prostaglandin formation via a protein kinase C/mitogen-activated protein kinase-dependent pathway. J Biol Chem 274: 7545-7556.

72. Haller H, Lindschau C, Quass P, Distler A, Luft FC (1995) Differentiation of vascular smooth muscle cells and the regulation of protein kinase C-alpha. Circ Res 76: 21-29.

73. Nogueira TC, Anhê GF, Carvalho CR, Curi R, Bordin S, et al. (2008) Involvement of phosphatidylinositol-3 kinase/AKT/PKCzeta/lambda pathway in the effect of palmitate on glucose-induced insulin secretion. Pancreas 37: 309-315.

74. Procaccini C, Matarese G (2012) Regulatory T cells, mTOR kinase, and metabolic activity. Cell Mol Life Sci.

75. Tsai S, Clemente-Casares X, Santamaria P (2011) CD8(+) Tregs in autoimmunity: learning “self”-control from experience. Cell Mol Life Sci 68: 3781-3795.

76. Usui I, Takata Y, Imamura T, Morioka H, Sasaoka T, et al. (1997) Fatty acid induced insulin resistance in rat-1 fibroblasts overexpressing human insulin receptors: impaired insulin-stimulated mitogen-activated protein kinase activity. Diabetologia 40: 894-901.

77. Soltys CL, Buchholz L, Gandhi M, Clanachan AS, Walsh K, et al. (2002) Phosphorylation of cardiac protein kinase B is regulated by palmitate. Am J Physiol Heart Circ Physiol 283: H1056-H1064.

78. Storz P, Döppler H, Wernig A, Pfizenmaier K, Müller G (1999) Cross-talk mechanisms in the development of insulin resistance of skeletal muscle cells palmitate rather than tumour necrosis factor inhibits insulin-dependent protein kinase B (PKB)/Akt stimulation and glucose uptake. Eur J Biochem 266: 17-25.

79. Thompson AL, Lim-Fraser MY, Kraegen EW, Cooney GJ (2000) Effects of individual fatty acids on glucose uptake and glycogen synthesis in soleus muscle in vitro. Am J Physiol Endocrinol Metab 279: E577-E584.

80. Cazzolli R, Carpenter L, Biden TJ, Schmitz-Peiffer C (2001) A role for protein phosphatase 2A-like activity, but not atypical protein kinase Czeta, in the inhibition of protein kinase B/Akt and glycogen synthesis by palmitate. Diabetes 50: 2210-2218.

81. Hirabara SM, Curi R, Maechler P (2010) Saturated fatty acid-induced insulin resistance is associated with mitochondrial dysfunction in skeletal muscle cells. J Cell Physiol 222: 187-194.

82. Hajduch E, Balendran A, Batty IH, Litherland GJ, Blair AS, et al. (2001)

Ceramide impairs the insulin-dependent membrane recruitment of protein kinase B leading to a loss in downstream signalling in L6 skeletal muscle cells. Diabetologia 44: 173-183.

83. Hirabara SM, Silveira LR, Abdulkader F, Carvalho CR, Procopio J, et al. (2007) Time-dependent effects of fatty acids on skeletal muscle metabolism. J Cell Physiol 210: 7-15.

84. Hirabara SM, Gorjão R, Vinolo MA, Rodrigues AC, Nachbar RT, et al. (2012) Molecular targets related to inflammation and insulin resistance and potential interventions. J Biomed Biotechnol 2012: 379024.

85. Martins AR, Nachbar RT, Gorjao R, Vinolo MA, Festuccia WT, et al. (2012) Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. Lipids Health Dis 11: 30.

86. Storlien LH, Kraegen EW, Chisholm DJ, Ford GL, Bruce DG, et al. (1987) Fish oil prevents insulin resistance induced by high-fat feeding in rats. Science 237: 885-888.

87. Akinkuolie AO, Ngwa JS, Meigs JB, Djoussé L (2011) Omega-3 polyunsaturated fatty acid and insulin sensitivity: a meta-analysis of randomized controlled trials. Clin Nutr 30: 702-707.

88. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, et al. (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142: 687-698.

89. Hirabara SM, Folador A, Fiamoncini J, Lambertucci RH, Rodrigues CF Jr, et al. (2013) Fish oil supplementation for two generations increases insulin sensitivity in rats. J Nutr Biochem 24: 1136-1145.

90. Neschen S, Moore I, Regittnig W, Yu CL, Wang Y, et al. (2002) Contrasting effects of fish oil and safflower oil on hepatic peroxisomal and tissue lipid content. Am J Physiol Endocrinol Metab 282: E395-E401.

91. Suchankova G, Tekle M, Saha AK, Ruderman NB, Clarke SD, et al. (2005) Dietary polyunsaturated fatty acids enhance hepatic AMP-activated protein kinase activity in rats. Biochem Biophys Res Commun 326: 851-858.

92. Robb RJ, Smith KA (1981) Heterogeneity of human T-cell growth factor(s) due to variable glycosylation. Mol Immunol 18: 1087-1094.

93. Kimura GM, Miller ME, Leake RD, Raghunathan R, Cheung AT (1981) Reduced concanavalin A capping of neonatal polymorphonuclear leukocytes (PMNs). Pediatr Res 15: 1271-1273.

94. Schley PD, Brindley DN, Field CJ (2007) (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. J Nutr 137: 548-553.

95. Cuvillier O, Ader I, Bouquerel P, Brizuela L, Malavaud B, et al. (2010) Activation of sphingosine kinase-1 in cancer: implications for therapeutic targeting. Curr Mol Pharmacol 3: 53-65.

96. Siddiqui RA, Jenski LJ, Harvey KA, Wiesehan JD, Stillwell W, et al. (2003) Cell-cycle arrest in Jurkat leukaemic cells: a possible role for docosahexaenoic acid. Biochem J 371: 621-629.

97. Bikman BT (2012) A role for sphingolipids in the pathophysiology of obesity-induced inflammation. Cell Mol Life Sci 69: 2135-2146.

98. Ly D, Tohn R, Rubin B, Blumenfeld H, Besra GS, et al. (2010) An alpha-galactosylceramide C20:2 N-acyl variant enhances anti-inflammatory and regulatory T cell-independent responses that prevent type 1 diabetes. Clin Exp Immunol 160: 185-198.

99. Jung JS, Shin KO, Lee YM, Shin JA, Park EM, et al. (2013) Anti-inflammatory mechanism of exogenous C2 ceramide in lipopolysaccharide-stimulated microglia. Biochim Biophys Acta 1831: 1016-1026.

100. Truman JP, Al Gadban MM, Smith KJ, Hammad SM (2011) Acid sphingomyelinase in macrophage biology. Cell Mol Life Sci 68: 3293-3305.

101. Chen CL, Lin CF, Chang WT, Huang WC, Teng CF, et al. (2008) Ceramide induces p38 MAPK and JNK activation through a mechanism involving a thioredoxin-interacting protein-mediated pathway. Blood 111: 4365-4374.

102. Powell DJ, Hajduch E, Kular G, Hundal HS (2003) Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKCzeta-dependent mechanism. Mol Cell Biol 23: 7794-7808.

103. Zhang Y, Li X, Becker KA, Gulbins E (2009) Ceramide-enriched membrane domains--structure and function. Biochim Biophys Acta 1788: 178-183.

104. McMurray DN, Jolly CA, Chapkin RS (2000) Effects of dietary n-3 fatty acids on T cell activation and T cell receptor-mediated signaling in a murine model. J Infect Dis 182: S103-S107.