

# Regulation of interleukin-2 signaling by fatty acids in human lymphocytes

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**Abstract** Docosahexaenoic (DHA; C22:6 n-3), eicosapentaenoic (EPA; C20:5 n-3), palmitic (PA; C16:0), and stearic (SA; C18:0) acids decrease lymphocyte proliferation in concentrations of >50  $\mu$ M, as observed in our previous study. However, oleic acid (OA; C18:1 n-9) and linoleic acid (LA; C18:2 n-6) increase lymphocyte proliferation at 25  $\mu$ M. In this study, the effect of these FAs on the interleukin-2 (IL-2) signaling pathway in human lymphocytes was investigated. Cells were isolated from heparinized venous blood of healthy human donors by density-gradient sedimentation. Cells were stimulated with 5  $\mu$ g/ml concanavalin A and treated with FAs in the absence or presence of IL-2 for 1 hour. CD25- $\alpha$  externalization was analyzed by flow cytometry, and Janus kinase 1 (JAK1), JAK3, signal transducer and activator of transcription (STAT) 5, extracellular signal-regulated kinases (ERKs) 1 and 2, Akt, and protein kinase C (PKC)- $\zeta$  phosphorylation were analyzed by Western blotting. The expression of CD25- $\alpha$  at the cell surface was increased by DHA, SA, and PA but was unaffected by EPA, OA, and LA. PA, SA, DHA, and EPA decreased JAK1, JAK3, STAT5, and Akt phosphorylation induced by IL-2, but OA and LA did not cause any effect. OA and LA increased ERK1/2 phosphorylation, whereas the other FAs caused a marked decrease. PKC- $\zeta$  phosphorylation was decreased by OA and LA and was not altered by the remaining FAs. **In conclusion**, the inhibitory effect of PA, SA, DHA, and EPA on lymphocyte proliferation observed in our previous study was attributable to a decrease in JAK/STAT, ERK, and Akt pathways activated by IL-2. Probably, OA and LA stimulated lymphocyte proliferation by increasing ERK1/2 phosphorylation through PKC- $\zeta$  activation. The inhibition of JAK1, JAK3, STAT5, ERK1/2, and Akt phosphorylation caused by DHA, SA, and PA is associated with an alteration of CD25 expression at the cell surface.—Gorjão, R., S. M. Hirabara, T. M. de Lima, M. F. Cury-Boaventura, and R. Curi. Regulation of interleukin-2 signaling by fatty acids in human lymphocytes. *J. Lipid Res.* 2007. 48: 2009–2019.

**Supplementary key words** oleic acid • linoleic acid • palmitic acid • stearic acid • eicosapentaenoic acid • docosahexaenoic acid • Janus kinase-signal transducer and activator of transcription • mitogen-activated protein kinase • protein kinase B

Lymphocyte proliferation and growth is a tightly regulated process: the T-cell antigen receptor controls the G<sub>0</sub>/G<sub>1</sub> cell cycle transition and stimulates T-cells to produce interleukin-2 (IL-2) and express interleukin-2 receptors (IL-2Rs). The IL-2-IL-2R interaction drives G<sub>1</sub>-S phase progression and T-cell clonal expansion (1). The high-affinity receptor IL-2R consists of three subunits, the  $\alpha$ ,  $\beta$ , and  $\gamma$  chains. The IL-2-induced heterodimerization of  $\beta$  and  $\gamma$  chains results in intermolecular transphosphorylation of their corresponding receptor associated Janus kinase 1 (JAK1) and JAK3 (2, 3). Members of the STAT (signal transducer and activator of transcription) family of transcription factors, downstream effectors of the JAK, are also regulated by IL-2 (4). IL-2-induced tyrosine phosphorylation of STAT5 allows src homology 2 domain-mediated homodimerization or heterodimerization, with a resultant induction of nuclear migration and sequence-specific DNA binding by the STATs (5). The STAT5 serine phosphorylation is rapidly induced by IL-2, and tyrosine phosphorylation is required for the transcriptional activity of this factor. The serine/threonine kinase inhibitor H7 clearly blocks IL-2-mediated signals by a mechanism not yet elucidated (6). IL-2 signal transduction also involves the activation of other distinct signaling pathways: the Shc/Ras/Raf/mitogen-activated protein kinase (MAPK) cascade and the phosphatidylinositol 3-kinase (PI3K) signaling pathway. The activation of these two pathways is necessary for cell proliferation and differentiation.

Fatty acids can control leukocyte functions such as cytokine production (7), macrophage phagocytosis (8), and lymphocyte proliferation (9). Some FAs (e.g., n-3) are gen-

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- $\kappa$ B, nuclear factor  $\kappa$ B; OA, oleic acid; PA, palmitic acid; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol myristate acetate; SA, stearic acid; STAT, signal transducer and activator of transcription.

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erally considered beneficial for human health because of their immunosuppressive effects in some autoimmune and inflammatory diseases (10–12). The n-3 FAs eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) cause a marked decrease in lymphocyte proliferation at 50  $\mu$ M and promote an inhibition of cell cycle progression induced by IL-2 (9). On the other hand, n-6 FAs have proinflammatory properties by increasing intercellular adhesion molecule-1 expression (13), natural killer cell activity (14), and IgE production (15). Linoleic acid (LA; C18:2 n-6) promotes an increase of lymphocyte proliferation at low concentration (25  $\mu$ M) and increases the stimulatory effect of IL-2 on lymphocyte proliferation (9).

The effect of FAs on lymphocyte function has been studied extensively, but the mechanisms involved are still unclear. EPA and DHA have been shown to inhibit the MAPK pathway in Jurkat T-cells during an antigenic challenge. Denys and colleagues (16–18) showed that EPA and DHA decreased the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) 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 (19). These results suggest that these FAs may exert their inhibitory action on ERK1/2 activation by reducing PKC activity. Denys, Hichami, and Khan (19) showed that DHA and EPA inhibit membrane recruitment of PKC- $\alpha$  and PKC- $\epsilon$ . These PKC isoforms are coupled to MAPK activation upstream of ERK1/2, which leads to a decrease in nuclear translocation of nuclear factor  $\kappa$ B (NF- $\kappa$ B), resulting in an inhibition of IL-2 gene expression and cell proliferation. Other studies have demonstrated that these FAs may exert suppressive effects on the phosphorylation of other MAPKs. Zeyda et al. (20) studied the effect of PUFAs on MAPK phosphorylation and the transcriptional activity of AP-1, NF-AT, and NF- $\kappa$ B in Jurkat and primary T-cells after 2 days of treatment. The authors found that PUFAs promote a highly selective inhibition of *c-jun* N-terminal kinase phosphorylation and activation, whereas phosphorylation of other MAPKs, such as p38 MAPK, remained essentially unaltered by PUFA treatment in Jurkat and peripheral blood T-cells. These studies suggest that PUFAs have a selective action on some intracellular proteins involved in T-cell activation.

Studies involving the effects of FAs on signaling pathways activated by T-cell receptor stimulation have been performed (17, 19–21). However, the effect of FAs on cytokine signaling pathways, such as IL-2 signaling, is still unclear. Proteins related to IL-2R-activated pathways may be modulated by FAs, which could explain some of their immunomodulatory effects. We have shown previously that DHA, EPA, palmitic acid (PA), and stearic acid (SA) decreased the stimulatory effect of IL-2 on lymphocyte proliferation, increasing the percentage of cells in G<sub>1</sub> phase and decreasing the proportion of cells in S and G<sub>2</sub>/M phases after 48 h of treatment, whereas LA and oleic acid (OA) at low concentration (25  $\mu$ M) caused an additive effect to IL-2, increasing cell proliferation (9).

In this study, we investigated whether the effects of FAs on human lymphocyte proliferation stimulated by IL-2 reported previously (9) are related to alterations in the IL-2 signaling pathway. The effects of 25  $\mu$ M OA (C18:1 n-9) and LA (C18:2 n-6) and 50  $\mu$ M PA (C16:0), SA (C18:0), DHA (C22:6 n-3), and EPA (C20:5 n-3) on the IL-2 signaling pathway in human lymphocytes after 1 h of treatment were examined. These FA concentrations were chosen based on a previous study in which we observed that OA and LA at 25  $\mu$ M increased lymphocyte proliferation. This finding is in opposition to that caused by the other FAs at 50  $\mu$ M, leading to a decrease of cell proliferation that did not promote cell death (9).

## MATERIALS AND METHODS

### Chemicals

RPMI-1640 medium, HEPES, fetal bovine serum, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). PA, SA, OA, LA, DHA, EPA, concanavalin A (ConA), Histopaque-1077, glutamine, and recombinant IL-2 were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-pJAK1 (Tyr 1022/1023), anti-pJAK3 (Tyr 980), anti-pSTAT5 (Tyr 694), anti-pSTAT5 (Ser 726), anti-pERK1/2 (Tyr 204), anti-pAkt (Ser 473), anti-JAK1, anti-JAK3, anti-STAT5, anti-ERK1/2, anti-Akt, anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pPKC- $\zeta$ , anti-PKC- $\zeta$ , and anti-CD25 were obtained from Cell Signaling Technology (Beverly, MA). FITC-conjugated anti-CD25 antibody was purchased from Pharmingen-BD Biosciences (San Diego, CA). Salts for buffer preparation were from Labsynth (Diadema, Brazil).

### Study design

The study was approved by the Ethical Committee of the Institute of Biomedical Sciences, University of São Paulo. 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.

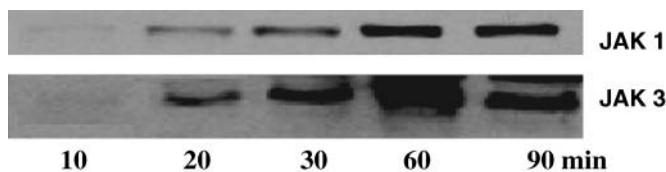
### Isolation of peripheral blood lymphocytes

Peripheral blood lymphocytes were isolated as described previously (9). Blood was diluted in PBS (1:1), and this suspension was layered on 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<sub>4</sub>Cl, 10 mM NaHCO<sub>3</sub>, and 0.1 mM EDTA, pH 7.4, and washed once with PBS.

The peripheral blood mononuclear cells were maintained in RPMI-1640 medium to allow the adherence of monocytes to the plates to obtain a pure lymphocyte suspension (~98%).

### Culture conditions

The 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 (10,000  $\mu$ g/ml), penicillin (10,000 IU/ml), and sodium bicarbonate (24 mM). Cells were grown in 25 ml flasks containing  $1 \times 10^6$  cells/ml. The cells were kept in a humidified atmosphere at 37°C containing 5% CO<sub>2</sub>.



**Fig. 1.** Time course of Janus kinase 1 (JAK1) and JAK3 phosphorylation in human lymphocytes stimulated with interleukin-2 (IL-2). Lymphocytes were stimulated with concanavalin A (ConA) for 24 h and then washed and incubated with 30 ng/ml IL-2 for 10, 20, 30, 60, and 90 min.

### Fatty acid treatment

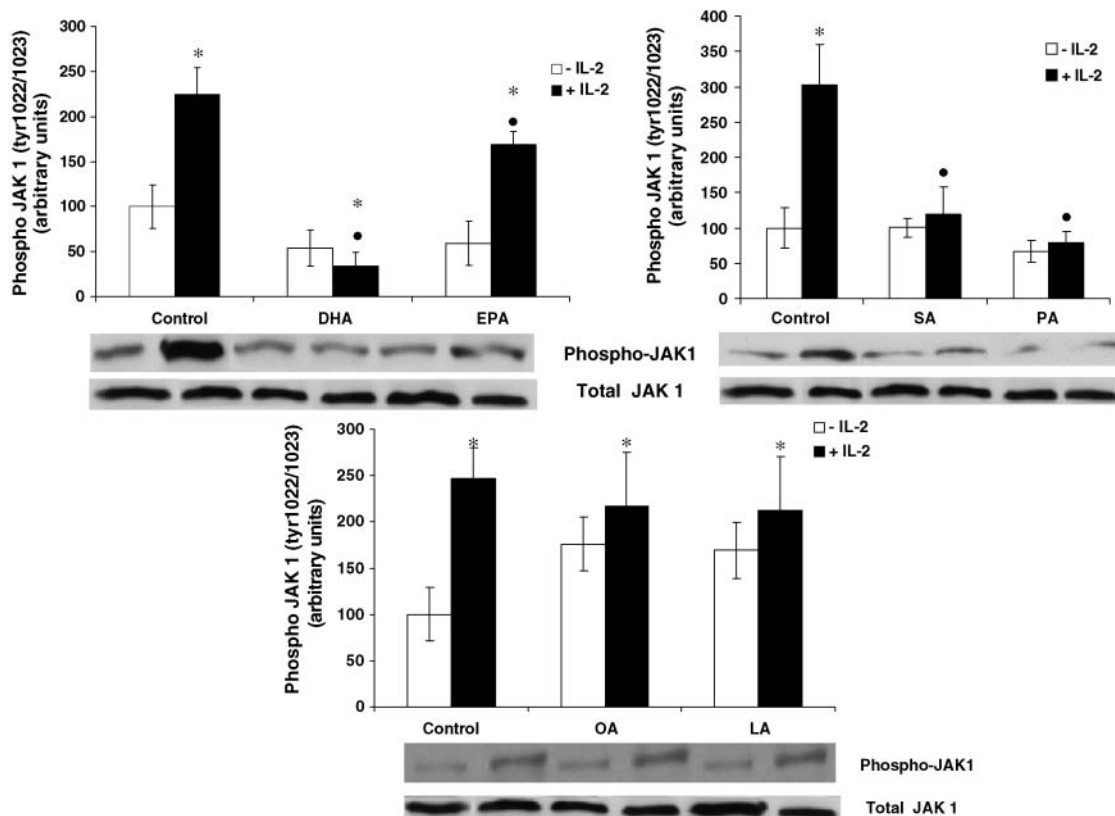
The assays to evaluate the effects of FAs on IL-2-activated intracellular signaling were performed by incubating the cells with 5  $\mu$ g/ml ConA for 24 h to stimulate the expression of the  $\alpha$  subunit of the IL-2R (CD25). Afterward, lymphocytes were washed with PBS and then cultured with different FAs in the presence and absence of IL-2 (30 ng/ml) for 1 h. This concentration of IL-2 was chosen because it promoted the highest response of lymphocyte proliferation (data not shown).

The FAs were previously diluted in ethanol. The concentration of ethanol was always <0.5%. This concentration was not toxic to the cells (data not shown), as also reported by Siddiqui et al. (22). The concentrations of the FAs used were not toxic

but caused significant effects on the proliferation of human lymphocytes: 50  $\mu$ M for EPA, DHA, PA, and SA and 25  $\mu$ M for OA and LA (9).

### Western blotting

Lymphocytes were incubated for 1 h as described above in the absence or presence of 30 ng/ml IL-2 and 50  $\mu$ M DHA, EPA, SA, and PA or 25  $\mu$ M OA and LA. After the incubation period, IL-2 stimulation was stopped by the addition of ice-cold PBS, and cells were pelleted by short centrifugation. Lymphocytes ( $1 \times 10^7$  cells) were homogenized in 120  $\mu$ l 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. Samples were boiled for 5 min and centrifuged at 13,000 *g* for 40 min at 4°C. Aliquots of the supernatants were used for the measurement of total protein content, as described by Bradford (23). Equal amounts of proteins of each sample (40  $\mu$ g) were diluted in Laemmli buffer containing DL-dithiothreitol (1 M), heated in a boiling-water bath for 3 min, and separated using a 12% SDS-polyacrylamide gel (24). Western blotting was carried out according to the method described by Towbin, Staehelin, and Gordon (25). The proteins on the gel were transferred to a nitrocellulose membrane at 120 V for 1 h. Nonspecific bonds were blocked by incubating the membranes with 5% defatted 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 in basal solution and then incubated in



**Fig. 2.** Effects of 50  $\mu$ M docosahexaenoic (DHA), eicosapentaenoic (EPA), stearic (SA), and palmitic (PA) acids and 25  $\mu$ M oleic (OA) and linoleic (LA) acids on JAK1 phosphorylation induced by IL-2. After densitometry analysis, data were normalized to the respective controls, which received a value of 100% in each experiment. The values are presented as means  $\pm$  SEM of four experiments. \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control (in the absence of FAs and IL-2); \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control treated with IL-2.

basal solution containing 3% defatted milk at room temperature for 3 h with the following antibodies: anti-pJAK1 (1:1,000 dilution, Tyr 1022/1023), anti-JAK1 (1:1,000), anti-pJAK3 (1:1,000, Tyr 980), anti-JAK3 (1:1,000), anti-pSTAT5 (1:500, Tyr 694) or anti-pSTAT5 (1:400, Ser 726), anti-STAT5 (1:1,000), anti-pERK1/2 (1:500, Tyr 204), anti-pAkt (1:500, Ser 473), anti-pPKC- $\zeta$  (1:1,000), and anti-pPKC- $\zeta$  (1:1,000) or anti-CD25 (1:1,000). Membranes were washed again (three times for 10 min each) 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 the washings, membranes were incubated with substrate for peroxidase and chemiluminescence enhancer (ECL Western Blotting System Kit) for 1 min and immediately exposed to X-ray film. Films were then developed in the conventional manner (25, 26). Quantitative analysis of blots was performed using Scion Image software (Scion Corp., Frederick, MD).

### Flow cytometric analysis

After 1 h of treatment with FA and IL-2, lymphocytes ( $1 \times 10^6$  cells) were resuspended in PBS and labeled with FITC-conjugated anti-CD25 antibody (1:50) (Pharmingen-BD Biosciences), and the cell suspension was incubated for 1 h at 4°C in the dark. Negative control cells were incubated with isotype-matched nonreactive IgG<sub>1</sub> antibody. Afterward, these cells were washed with PBS and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Juan, CA). Fluorescence was mea-

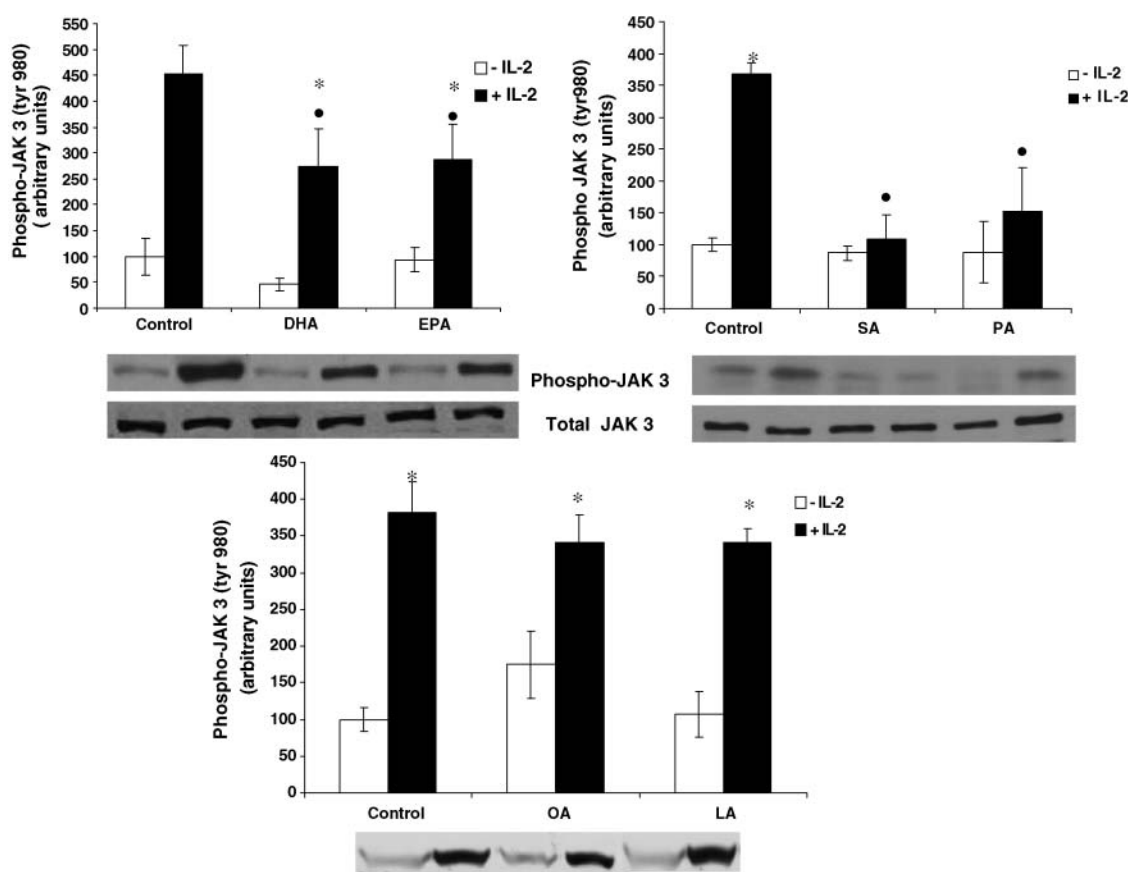
sured using FL1-H channel (green fluorescence = 530/30 nm). Ten thousand events were analyzed per experiment. Cells with FITC fluorescence were evaluated using Cell Quest software (Becton Dickinson).

## RESULTS

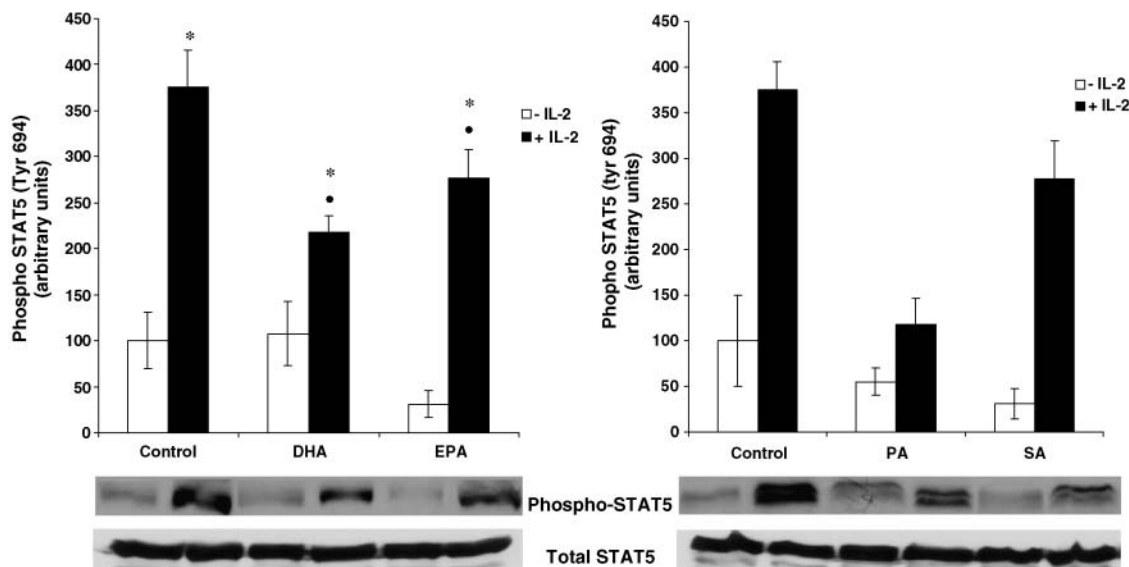
### JAK1, JAK3, and STAT5 phosphorylation

The effects of IL-2 on JAK1 and JAK3 phosphorylation in human lymphocytes were evaluated for up to 90 min of incubation. The highest phosphorylation of JAK1 and JAK3 promoted by IL-2 was observed at 60 min (Fig. 1). DHA, EPA, SA, and PA at 50  $\mu$ M caused marked decreases (85, 24, 58, and 75%, respectively) of JAK1 phosphorylation (Figs. 2, 3) induced by IL-2. On the other hand, OA and LA at 25  $\mu$ M did not cause any effects. The total expression of the three proteins was not altered by the fatty acids.

DHA, EPA, PA, and SA caused decreases (42, 26, 69, and 27%, respectively) of STAT5 tyrosine residue phosphorylation induced by IL-2 (Fig. 4). Phosphorylation of serine residue was also decreased by EPA, DHA, PA, and SA (45, 52, 24, and 20%, respectively) (Fig. 5). Both residues (tyrosine and serine) must be phosphorylated for



**Fig. 3.** Effects of 50  $\mu$ M DHA, EPA, SA, and PA and 25  $\mu$ M OA and LA on IL-2-induced JAK3 phosphorylation. After densitometry analysis, data were normalized to the respective controls, which received a value of 100% in each experiment. The values are presented as means  $\pm$  SEM of four experiments. \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control (in the absence of FAs and IL-2); \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control treated with IL-2.



**Fig. 4.** Effects of 50  $\mu$ M DHA, EPA, SA, and PA and 25  $\mu$ M OA and LA on signal transducer and activator of transcription (STAT) 5 tyrosine 694 phosphorylation induced by IL-2. After densitometry analysis, data were normalized to the respective controls, which received a value of 100% in each experiment. The values are presented as means  $\pm$  SEM of four experiments. \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control (in the absence of FAs and IL-2); •  $P < 0.05$ , for comparison between the treatments with fatty acids versus control treated with IL-2.

the highest transcriptional activity of STAT5 induced by IL-2 (6). OA and LA did not cause significant effects (data not shown).

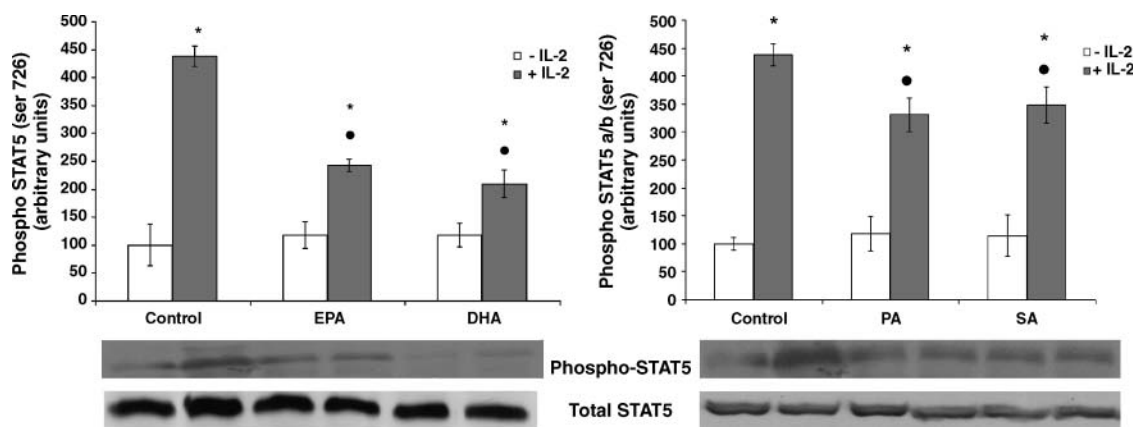
#### ERK1/2 phosphorylation

The phosphorylation of ERK1/2 induced by IL-2 was decreased (by 83% and 27%) by DHA and EPA treatment, respectively (Fig. 6). DHA also caused a decrease of 62% in cells not stimulated with IL-2. PA and SA promoted decreases of 64% and 38% in the phosphorylation of these proteins (Fig. 6). The total content of ERK1/2 was not changed by these FAs. On the other hand, OA and LA caused increases of  $\sim$ 25% in the ERK1/2 phosphoryla-

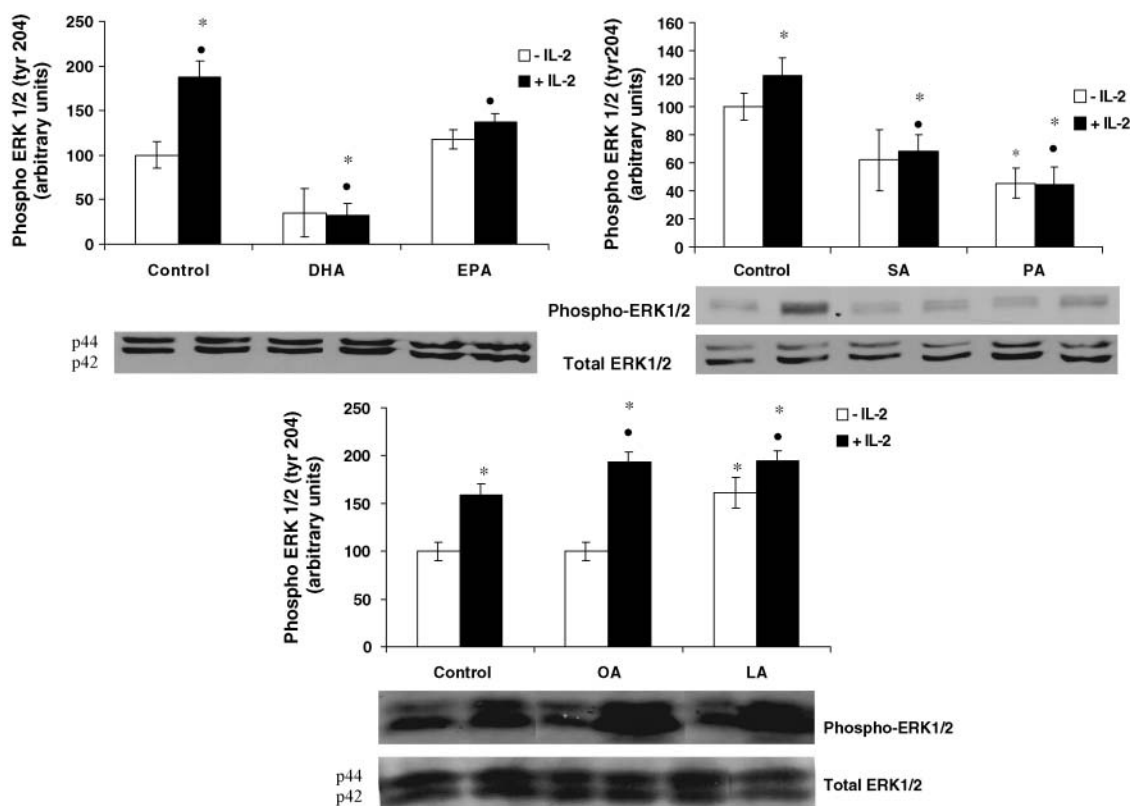
tion induced by IL-2 (Fig. 6). LA also increased (by 62%) ERK1/2 phosphorylation in unstimulated cells.

#### Akt phosphorylation

DHA and EPA exerted inhibitory effects on Akt phosphorylation induced by IL-2 (decreases of 86% and 31%, respectively) (Fig. 7). PA and SA also decreased Akt phosphorylation in cells stimulated (decreases of 78% and 72%, respectively) or not stimulated (decreases of 62% and 75%, respectively) with IL-2. OA and LA did not alter Akt phosphorylation after 1 h of treatment (Fig. 7). This observation suggests that these FAs probably exert their stimulatory effect through the Ras/ERK1/2 pathway.



**Fig. 5.** Effects of 50  $\mu$ M DHA, EPA, SA, and PA and 25  $\mu$ M OA and LA on STAT5 serine 726 phosphorylation induced by IL-2. After densitometry analysis, data were normalized to the respective controls, which received a value of 100% in each experiment. The values are presented as means  $\pm$  SEM of four experiments. \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control (in the absence of FAs and IL-2); •  $P < 0.05$ , for comparison between the treatments with fatty acids versus control treated with IL-2.



**Fig. 6.** Effects of 50  $\mu$ M DHA, EPA, SA, and PA and 25  $\mu$ M OA and LA on extracellular signal-regulated kinase (ERK) 1/2 phosphorylation induced by IL-2. After densitometry analysis, data were normalized to the respective controls, which received a value of 100% in each experiment. The values are presented as means  $\pm$  SEM of three determinations from four experiments. \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control (in the absence of FAs and IL-2); •  $P < 0.05$ , for comparison between the treatments with fatty acids versus control treated with IL-2.

### PKC- $\zeta$ phosphorylation

OA and LA promoted stimulatory effects on PKC- $\zeta$  phosphorylation in the absence (increase of 50%) and presence (increase of 100%) of IL-2. The total expression of this protein was not altered by these FAs. PA, SA, DHA, and EPA did not alter PKC- $\zeta$  phosphorylation (Fig. 8).

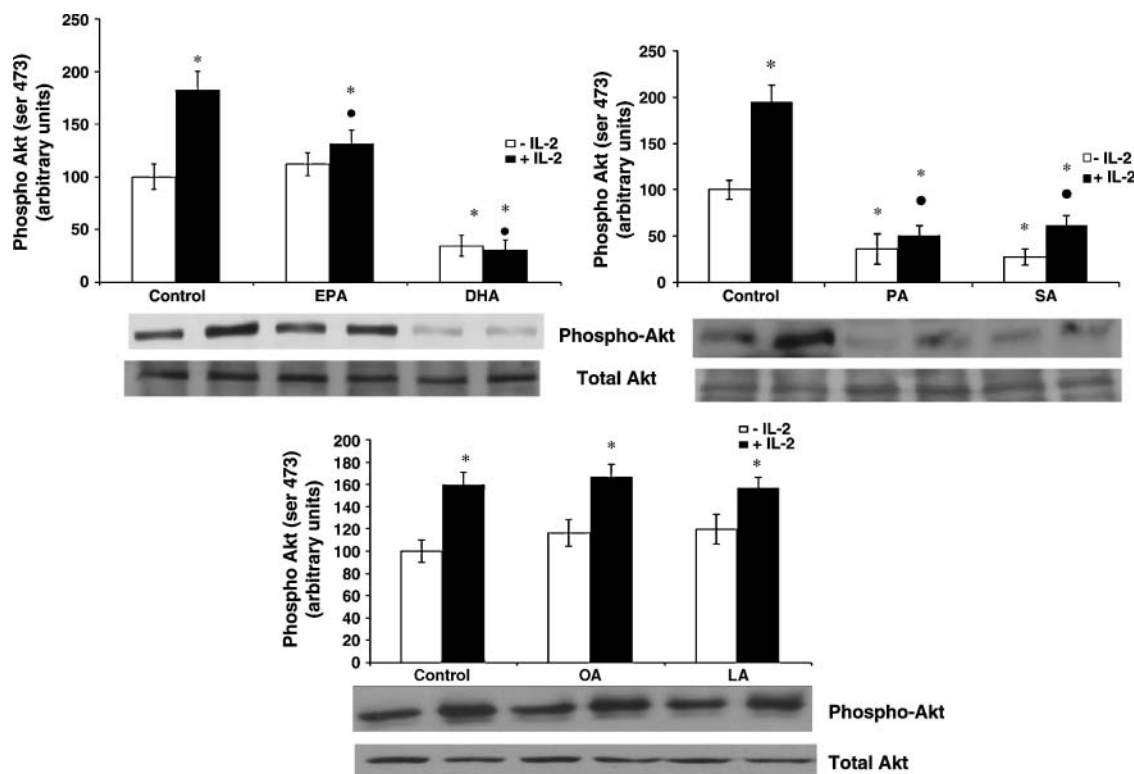
### Expression of IL-2R $\alpha$ -chain

IL-2R  $\alpha$ -chain (CD25) assembles with  $\beta$  and  $\gamma$  subunits to form the high-affinity IL-2R (27). Induction of CD25 expression is required for IL-2-induced signaling events. The histograms in Fig. 9 show the intensity of the sample fluorescence, which corresponds to FITC emission. The inhibition of CD25 expression at the cell surface decreases the fluorescence signal. Only 7.5% of cells were CD25-positive before being stimulated with ConA. ConA-induced surface expression of CD25 was not altered by treatment with IL-2. A significant percentage of cells expressing CD25 were reduced by DHA, PA, and SA treatment compared with the control (Fig. 9). The percentage of cells that were CD25-positive decreased from  $36.5 \pm 5.4\%$  in cells treated with IL-2 to  $12.6 \pm 0.9\%$ ,  $12.4 \pm 1.6\%$ , and  $11.8 \pm 1.1\%$  (expressed as means  $\pm$  SEM) in cells treated with DHA, SA, and PA, respectively. EPA, OA, and LA had no significant effects on CD25 expression after 1 h of treatment (Fig. 9). The total expression of CD25 an-

alyzed by Western blotting was not altered by FA treatment (data not shown).

## DISCUSSION

It is widely known that treatment with tyrosine kinase inhibitors reduces lymphocyte proliferation (28). This finding indicates that JAK1 and JAK3 tyrosine phosphorylation induced by IL-2 plays an essential role in lymphocyte proliferation (28). The present study showed that some FAs inhibit the signaling pathway activated by this cytokine in human lymphocytes after a short period of treatment (1 h). A decrease of JAK1 and JAK3 phosphorylation was observed when human lymphocytes were treated with DHA, EPA, PA, and SA in the presence of IL-2. The reduction of JAK1 and JAK3 phosphorylation may be an important mechanism for the inhibition of lymphocyte proliferation induced by these FAs, as reported in our previous study (9). The total content of these proteins was not altered by the FA treatment, indicating that these FAs inhibit lymphocyte proliferation by altering the protein phosphorylation state. IL-2 is particularly important for the development, expansion, and survival of T-cells, and inhibition of the signaling pathway of this cytokine results in severe immunodeficiency.



**Fig. 7.** Effects of 50  $\mu$ M DHA, EPA, SA, and PA and 25  $\mu$ M OA and LA on Akt phosphorylation induced by IL-2. After densitometry analysis, data were normalized to the respective controls, which received a value of 100% in each experiment. The values are presented as means  $\pm$  SEM of four experiments. \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control (in the absence of FAs and IL-2); •  $P < 0.05$ , for comparison between the treatments with fatty acids versus control treated with IL-2.

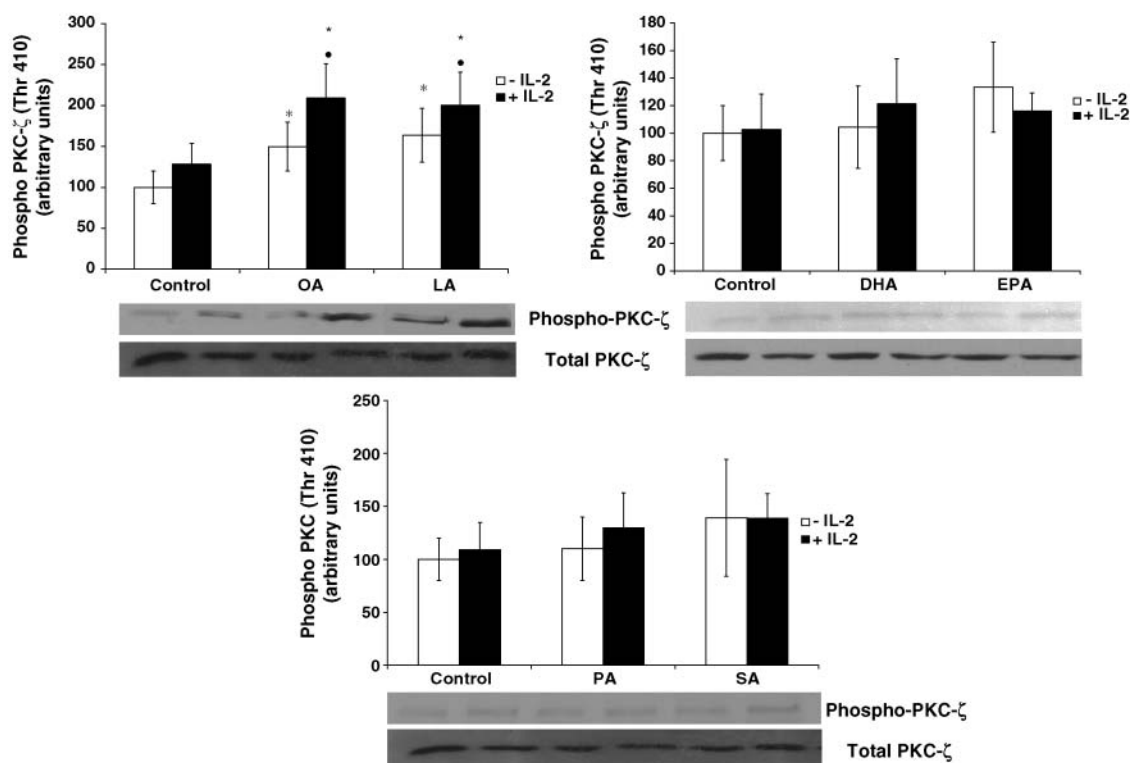
ciency (29). Thus, inhibition of both IL-2 production (30) and IL-2 action in lymphocytes markedly contributes to the potent suppressive effects of DHA and EPA on the function of these cells.

Inhibition of IL-2-induced STAT5 phosphorylation by DHA, EPA, PA, and SA occurred at the level of tyrosine and serine residues (Figs. 3, 4). JAK3 activates JAK1, which is then able to phosphorylate the target tyrosine residues in STAT5 molecules docked with the  $\beta$  subunit of the IL-2R. Therefore, STAT5 activation occurs when JAK1 and JAK3 are phosphorylated. Once JAK phosphorylation was decreased by PA, SA, EPA, and DHA treatment, STAT5 phosphorylation was also inhibited (Fig. 4, 5). JAK-STAT signaling by IL-2 is critically important for immune and inflammatory responses and for T-cell expansion and differentiation. Inhibition of the signaling of this cytokine, as occurs in genetic deficiency of the  $\gamma$  subunit of JAK3, results in severe immunodeficiency. The STAT5 molecule does not appear to be the only protein involved in this process. In fact, deletion and mutational experiments of STAT5 DNA binding activity or the prevention of STAT5 activation did not block IL-2R mitogenic signaling (29, 31). Although STAT5 does not seem to be essential for mitogenesis, it is involved in the induction of genes required for cell proliferation and survival (32, 33).

JAK1 and JAK3 activation promotes the phosphorylation of tyrosine residues in the cytoplasmic tail of  $\beta$  and  $\gamma$  subunits of IL-2R. Ras is activated through the binding of

the adaptor Shc to the tyrosine-phosphorylated receptor, which recruits the Grb2-Sos complex and activates the Raf-MEK-ERK pathway. EPA and DHA promoted a decrease of ERK1/2 phosphorylation stimulated by IL-2. This inhibitory effect is attributable to a reduction of JAK1 and JAK3 phosphorylation promoted by these FAs that possibly led to a decrease in the activity of ERK1/2 (or p42/p44 MAPK). Activated ERK1/2 phosphorylates various substrates in the cell compartments. ERK activation plays an essential role in cell growth and provides an integrated response: increases nucleotide synthesis, activates the transcription of many genes acting via transcription factors (e.g., Elk-1, Fos, AP-1, NF-AT, and *c-myc*) and chromatin phosphorylation, stimulates protein synthesis, and finally facilitates the formation of an active cyclin D-CDK4 complex, which is a rate-limiting step for cell growth (34). Khan et al. (35) also showed that DHA arrested the progression from the late G<sub>1</sub> to the S phase of the FM3A mouse mammary cancer cell cycle by decreasing ERK1/2 phosphorylation.

Denys and colleagues (16, 17) showed that EPA and DHA inhibited ERK1/2 phosphorylation, but this occurred by decreasing PKC- $\beta$  activation in PMA-stimulated Jurkat cells. In fact, PKC is the main pathway activated by PMA stimulation in these cells. In our study, these two FAs decreased JAK1 and JAK3 phosphorylation, a signaling pathway stimulated by IL-2 that also results in ERK1/2 activation. The saturated FA, PA, and SA decreased ERK



**Fig. 8.** Effects of 50  $\mu$ M DHA, EPA, SA, and PA and 25  $\mu$ M OA and LA on protein kinase C (PKC)- $\zeta$  phosphorylation induced by IL-2. After densitometry analysis, data were normalized to the respective controls, which received a value of 100% in each experiment. The values are presented as means  $\pm$  SEM of three experiments. \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control (in the absence of FAs and IL-2); \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control treated with IL-2.

phosphorylation stimulated by IL-2. Similarly, Hirabara et al. (2003) showed that PA decreased ERK1/2 phosphorylation induced by insulin in 1 h incubated rat soleus muscle.

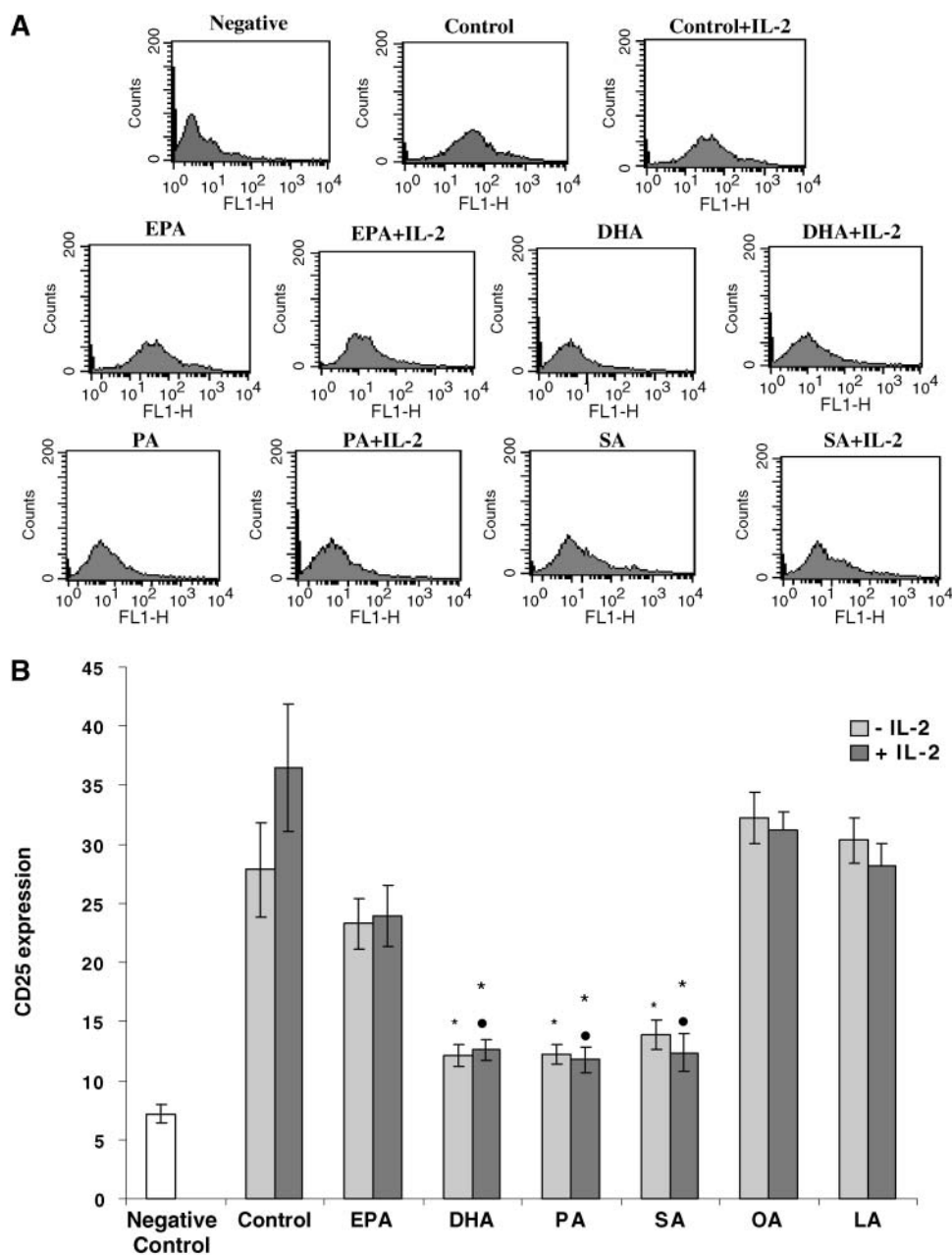
OA and LA enhanced the effect of IL-2 on ERK1/2 phosphorylation, suggesting that this pathway may be involved in the stimulation of lymphocyte proliferation promoted by these FAs, as demonstrated previously (9). Cury-Boaventura et al. (36) observed a decrease in the proliferation of lymphocytes from volunteers who received a lipid emulsion rich in LA. This effect was possibly attributable to the toxicity of the LA, whose plasma level was markedly increased. Thanasak et al. (37) also showed that the treatment of bovine lymphocytes with low concentrations of LA (<25  $\mu$ M) increased lymphocyte proliferation, whereas higher concentrations of this fatty acid decreased it. The results presented here suggest that LA at low concentration acts as a proinflammatory agent by stimulating the MAPK cascade, leading to lymphocyte proliferation. Activation of PKC 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 observed by others (38). OA more potently and completely activates the  $\text{Ca}^{2+}$ -independent and atypical PKC isoforms (39, 40) in platelets. PKC- $\zeta$  has been shown to activate ERK, being associated with mitogenesis in various cell lines (41). Other *cis*-fatty acids, such as LA, also fully activate PKC in the same manner (39). In

contrast, PKC- $\alpha$ , a  $\text{Ca}^{2+}$ -dependent isoform, is less potently stimulated by OA (40) and has been associated with the differentiation rather than the proliferation of vascular smooth muscle cells (42). In the present study, OA and LA increased PKC- $\zeta$  phosphorylation after 1 h of treatment. Once OA and LA did not alter JAK/STAT phosphorylation, activation of the novel and/or atypical PKC isoforms may explain the stimulatory effect of OA on ERK1/2 phosphorylation induced by IL-2.

Akt phosphorylation was decreased by DHA, EPA, PA, and SA. The effect of DHA was more pronounced than that of the other FAs, whereas OA and LA did not affect this protein phosphorylation (Fig. 7). The activation of the PI3K pathway can potentiate proliferative signaling associated with STAT5 (43). PI3K may activate transcription factors that mediate events downstream of the  $G_1$  cell cycle checkpoint. Several Akt substrates are transcription factors or are involved directly in the regulation of gene transcription (44). Probably, this inhibitory effect on Akt phosphorylation and the JAK-STAT and ERK1/2 pathways induced by DHA, EPA, PA, and SA are responsible for the decreased lymphocyte proliferation observed in our previous study (9).

The  $\alpha$  subunit of IL-2R (CD25- $\alpha$ ) is undetectable in resting T-cells. The expression of this protein is triggered by antigens (45), a stimulus that can be mimicked by lectins such as ConA (46). If a decrease in CD25- $\alpha$  externalization occurs, IL-2R-stimulated pathways are suppressed and






**Fig. 9.** Effects of 50  $\mu$ M DHA, EPA, SA, and PA and 25  $\mu$ M OA and LA on CD25- $\alpha$  lymphocyte surface expression. Lymphocytes were stimulated with ConA for 24 h, washed, and incubated with 30 ng/ml IL-2 for 1 h. Cells were pelleted and labeled with FITC-conjugated anti-CD25 antibody and analyzed by flow cytometry. Negative control cells were incubated with a marked nonreactive control antibody. Histograms from 10,000 events are shown. Fluorescence was measured in channel FL1-H (green fluorescence = 530/30 nm). A: Representative histograms obtained by flow cytometry. B: Percentage of cells expressing CD25- $\alpha$ . The values are presented as means  $\pm$  SEM of three determinations from four experiments. \*  $P < 0.05$ , for comparison between the treatments with fatty acids versus control (in the absence of FAs and IL-2); •  $P < 0.05$ , for comparison between the treatments with fatty acids versus control treated with IL-2.

lymphocyte proliferation is inhibited. DHA, PA, and SA decreased CD25- $\alpha$  content at the plasma membrane surface in lymphocytes after 1 h of treatment (Fig. 9), with no alteration in the total expression of CD25 (data not shown). The CD25 surface membrane expression is necessary to form the high-affinity IL-2R that actually activates the intracellular signal pathways (JAK-STAT, Ras-ERK1/2,

and PI3K-Akt) leading to lymphocyte proliferation. Probably, the inhibition of JAK1 and JAK3, ERK1/2, and Akt phosphorylation promoted by DHA, PA, and SA is attributable to the decrease in CD25- $\alpha$  expression and content in the plasma membrane. Other studies showed that PUFAs can alter receptor localization on the cell surface after 24 h of treatment (21, 47). 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 noteworthy that OA and LA stimulated ERK1/2 phosphorylation but did not change CD25 expression. Measurements using red-fluorescent Alexa Fluor 594 conjugate of cholera toxin subunit B showed that, except for DHA, none of the FAs tested caused marked changes in lipid raft distribution under the conditions of this study (data not shown). Therefore, the FAs tested hardly affected intracellular signaling through changes in lipid rafts.

Although DHA and EPA have promoted an inhibitory effect on lymphocyte proliferation (9), DHA caused a more pronounced decrease of ERK1/2 and JAK1 phosphorylation in relation to EPA (Figs. 2, 6). Other studies have shown that different n-3 FAs can exert divergent effects on immune response. DHA-rich fish oil caused an increase in neutrophil and monocyte phagocytosis (48), whereas others demonstrated that EPA-rich fish oil did not alter this cell function (49). Verlengia et al. (50) showed that the effect of EPA on gene expression in a human B-lymphocyte cell line (Raji) was more pronounced (25.9% of genes investigated were altered) compared with that of DHA (8.4% of genes investigated were altered). We observed here that an alteration in lipid raft organization was induced by DHA, but it did not occur when lymphocytes were treated with EPA. The differences observed in the effects of these two fatty acids may occur because the spatial conformation of DHA is different from that of EPA as a result of its carbon length and degree of unsaturation. Therefore, we cannot generalize the effects of FAs from the same family on lymphocytes.

In conclusion, the findings reported here are important for understanding the mechanisms by which different FAs alter lymphocyte proliferation. The inhibitory effect of PA, SA, and DHA on lymphocyte proliferation was associated with a reduction in the IL-2-induced activation of the JAK/STAT, ERK, and Akt pathways and a decrease in CD25- $\alpha$  expression. EPA decreased the phosphorylation of the IL-2R signaling proteins by a mechanism that did not involve CD25. Probably, OA and LA stimulated lymphocyte proliferation by increasing ERK1/2 phosphorylation through PKC- $\zeta$  activation. 

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