Docosahexaenoic acid inhibits cancer cell growth via $p27^{Kip1}$, CDK2, ERK1/ERK2, and retinoblastoma phosphorylation

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Abstract Docosahexaenoic acid (DHA), a PUFA of the n-3 family, inhibited the growth of FM3A mouse mammary cancer cells by arresting their progression from the late-G_1 to the S phase of the cell cycle. DHA upregulated $p27^{Kip1}$ levels by inhibiting phosphorylation of mitogen-activated protein (MAP) kinases, i.e., ERK1/ERK2. Indeed, inhibition of ERK1/ERK2 phosphorylation by DHA, U0126 [chemical MAPK extracellularly signal-regulated kinase kinase (MEK) inhibitor], and MEK^{SA} (cells expressing dominant negative constructs of MEK) resulted in the accumulation of $p27^{Kip1}$. MAP kinase (MAPK) inhibition by DHA did not increase $p27^{Kip1}$ mRNA levels. Rather, this fatty acid stabilized $p27^{Kip1}$ contents and inhibited MAPK-dependent proteasomal degradation of this protein. DHA also diminished cyclin E phosphorylation, cyclin-dependent kinase-2 (CDK2) activity, and phosphorylation of retinoblastoma protein in these cells. Our study shows that DHA arrests cell growth by modulating the phosphorylation of cell cycle-related proteins.—Khan, N. A., K. Nishimura, V. Aires, T. Yamashita, D. Oaxaca-Castillo, K. Kashiwagi, and K. Igarashi. **Docosahexaenoic acid inhibits cancer cell growth via $p27^{Kip1}$, CDK2, ERK1/ERK2, and retinoblastoma phosphorylation.** ***J. Lipid Res.*** **2006.** 47: **2306–2313.**

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During the recent past, there has been an upsurge of information on the role of PUFAs in health and disease (1–4). As far as cancer growth is concerned, the epidemiological studies have demonstrated that n-6 PUFAs exert stimulatory effects (5), whereas the n-3 PUFAs have suppressive activity on cancer cell proliferation (6–8). These observations are supported by other studies, which have demonstrated enhancing effects of high-fat diets (6, 9) and inhibitory effects of dietary n-3 PUFAs (10) on experimental carcinogenesis. In vitro studies have also shown that n-6 PUFAs stimulate the growth of human breast (11, 12) and prostate (13) cancer cell lines, whereas docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) inhibit their growth (14, 15).

The G_1 phase of the cell cycle, regulated by stimulatory and inhibitory growth factors, is the most important step with respect to the control of cell proliferation (16, 17). In normal growth-stimulated cells, the levels of cyclin D and cyclin E are increased, respectively, in the mid-G_1 and late-G_1 phases of the cell cycle (16, 17). Cyclin D and cyclin E associate, respectively, with cyclin-dependent kinase 4/6 (CDK4/6) and CDK2 to form active kinase complexes (16). The activity of CDKs is regulated by their specific dephosphorylation and phosphorylation, and by interactions with distinct CDK inhibitors like Cip and Kip family proteins, which inhibit, respectively, cyclin D/CDK4/6 and cyclin E/CDK2 (18, 19). The activities of cyclin D/CDK4/6 and cyclin E/CDK2 complexes reach their maximum, respectively, in mid-to-late-G_1 and late-G_1-to-S-phase transition (20). Cyclin D/CDK4/6 and cyclin E/CDK2 complexes phosphorylate retinoblastoma protein (pRb) (21, 22). In quiescent cells, pRb is in a hypophosphorylated active form, but after growth stimulation, it starts to become phosphorylated in the mid-G_1 phase, and maximal phosphorylation occurs at the late-G_1-to-S-phase junction (22). The increased phosphorylation of pRb leads to its functional inactivation, resulting in the release of S-phase-specific transcription factors that are bound to

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Abbreviations: AA, arachidonic acid; CDK, cyclin-dependent kinase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ES medium, essential seiyaku medium; FCS, fetal calf serum; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK extracellularly signal-regulated kinase kinase; PMA, phorbol-12-myristate-13-acetate; pRb, retinoblastoma protein.

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and sequestered by unphosphorylated pRb during the G_1 phase of the cell cycle (23).

Nevertheless, there is a lack of information on the mechanism of action of these fatty acids in the modulation of cancer cell proliferation. Therefore, the present study was designed to shed light on the role of DHA, the terminal molecule of n-3 PUFA metabolism, in the modulation of mouse mammary FM3A cancer cell growth.

MATERIALS AND METHODS

Materials

Essential seiyaku (ES) medium was procured from Nissui Pharmaceutical Co. (Tokyo, Japan). Unless otherwise stated, all other chemicals, including DHA (22:6n-3) and arachidonic acid (AA, 20:4n-6) were purchased from Sigma (St. Louis, MO). Anti-cyclin D1, anti-cyclin E, anti-$p27^{Kip1}$, and CDK2 were purchased from Santa Cruz Biotechnology. FUT-175 was kindly supplied by Torii Pharmaceutical Co. (Tokyo, Japan). Anti-phosphorylated anti-total mitogen-activated protein (MAP) kinase (MAPK) (ERK1/ERK2) antibodies were obtained from Cell Signaling. Anti-hypoRb and anti-pRb antibodies were procured from Pharmingen.

Plasmids

The dominant negative (S222A) hemagglutinin-1-tagged MAPK extracellularly signal-regulated kinase kinase-1 [MEK-1 (MEK^{SA})] and constitutively active (S218D/S222D) MEK-1 (MEK^{DD}) constructs were kindly provided by Alain Eychène (Orsay, France) and Stéphane Manenti (Toulouse, France). They were cloned into the $pcDNA_3$ expression vector between the *Hind*III and *Xba*I restriction sites. After transformation into *Echerichia coli* DH5α efficiency-competent cells (Invitrogen; Paisely, UK), plasmid constructs were purified by using Plasmid Midi and Maxi kits (Qiagen GmbH, Germany) according to the instructions of the manufacturer.

Cell culture

Mouse mammary FM3A cancer cells were cultured routinely (1×10^4 cells/ml) in ES medium supplemented with 50 U/ml streptomycin, 100 U/ml penicillin G, and 2% (v/v) heat-inactivated fetal calf serum (FCS) at 37°C in a humidified chamber containing 95% air and 5% CO_2, as described by Ayusawa, Iwata, and Seno (24). Cell numbers were determined by hemocytometer. DHA, dissolved in ethanol at a concentration of 0.01% (v/v), was kept under nitrogen in order to minimize its oxidation.

Cell proliferation assays

The cells were serum-starved for 6 h, washed, and further cultured in the aforesaid conditions in the ES medium containing 0.2% FCS with or without DHA at the concentrations stated in the figure legends. Trypan blue was employed to check cell viability and to count cell numbers.

Transient cell transfection

Routinely cultured (1×10^4 cells/ml) mouse mammary FM3A cancer cells were washed and then plated at 1×10^4 cells/well in 24-well dishes, and transient transfection was performed using X-treme-GENE Q2 transfection reagent (Roche Applied Science; Paris, France). For cell transfection, 25.7 μg of plasmid DNA was diluted with 750 μl of DNA-dilution reagent, which was further mixed with 750 μl of transfection reagent. The mixed solution was added drop by drop to the cells. Nontransfected cells (control) received all transfection reagents except cDNA. After 36 h, control and transfected cells were used for experiments. The control cells were transfected with $pcDNA_3$ empty vector.

Cell cycle analysis

The cells were serum-starved for 6 h, washed, and cultured for 12 h in ES medium containing 0.2% FCS with or without DHA. After washing, the cells were fixed in cold 90% ethanol for the cell cycle studies by FACScan flow cytometer (Beckton-Dickinson). The measurement of the cell cycle progression was performed essentially according to the protocol of Ffrench et al. (25) by staining the cell nuclei with propidium iodide (PI). Before the addition of PI, the cells were treated with RNase. After eliminating the cell debris, the different stages of the cell cycle were deduced from the histograms, using the software provided by the manufacturer.

Real-time RT-PCR quantification of $p27^{Kip1}$ expression

Total RNA was extracted using Trisol (Invitrogen Life Technologies; Groningen, the Netherlands) according to the manufacturer's instructions. The integrity of the RNA was electrophoretically checked by ethidium bromide staining and by the optical density (OD) absorption ratio $OD_{260nm}/OD_{280nm} > 1.9$. One microgram of total RNA was reverse transcribed with Superscript II RNase H reverse transcriptase using oligo (dT) according to the manufacturer's instructions (Invitrogen Life Technologies). Real-time PCR was carried out on the iCycler iQ real-time detection system (Bio-Rad; Paris, France), and amplification was undertaken using SYBR® Green I. Oligonucleotide primers, used for $p27^{Kip1}$ mRNA analysis, were as follows: upstream primer 5′-CGGCGGCAAGGTTTGGAGAGG-3′; downstream primer, 5′-GGAGGAGGCAGGAGGAGGTGG-3′. The sequences of the PCR primers for β-actin were as follows: upstream primer, 5′-GGCACCACACCTTCTACAATGAGC-3′; downstream primer, 5′-CGACCAGAGGCATACAGGGACAG-3′. The amplification was carried out in a total volume of 25 μl containing 12.5 μl SYBR® Green supermix PCR buffer [50 mM KCl, 20 mM Tris-HCl (pH 8.4), 3 mM $MgCl_2$], 0.2 mM each dNTPs, 0.63 U iTaq DNA polymerase, SYBR® Green, 1.10 nM fluorescein, and 12.5 μl containing 0.3 μM of each primer and diluted cDNA. RT-PCR was performed at an annealing temperature of 62°C, 30 cycles. Results were evaluated by iCycler iQ software including standard curves, amplification efficiency, and cycle threshold.

Western blot of cell cycle proteins and MAPK

The cells were serum-starved for 6 h, washed, and cultured in ES medium containing 0.2% FCS with or without DHA (10 μM). The cells were cultured for times as indicated in the figure legends. After washing, the cells were lysed with 50 μl of buffer containing the following: HEPES, 20 mM, pH 7.3; EDTA, 1 mM; EGTA, 1 mM; NaCl, 0.15 mM; Triton X-100, 1%; glycerol, 10%; phenylmethylsulfonyl fluoride, 1 mM; sodium orthovanadate, 2 mM; antiprotease cocktail, 2 μl in 1 ml of buffer (Sigma). After centrifugation (2,500 $g \times 1$ min), cell lysates were used immediately or stored at −80°C. Protein concentration was determined by the method of Lowry et al. (26). Denatured proteins (20 μg) were separated by SDS-PAGE (10%) and transferred to polyvinylidine difluoride membranes. Membranes were blocked by incubation with cream-free milk overnight at 4°C. Immunodetection was performed by using anti-$p27^{Kip1}$, anti-phosphorylated ERK1/ERK2, anti-total ERK1/ERK2, anti-cyclin D1, anti-cyclin E, and anti-CDK2 (2 μg/ml). After treating the membranes with peroxidase-conjugated secondary antibodies (goat anti-rabbit for anti-$p27^{Kip1}$, goat anti-mouse for ERK1/ERK2, and goat anti-rat

for all other proteins), peroxidase activity was detected with ECL reagents (Amersham, France).

Assays of CDK2 kinase activity

The cell lysates prepared for Western blot assays were precleared by the incubation with protein A-Sepharose-4B followed by centrifugation (10,000 $g \times 15$ min). CDK2 kinase assays were performed by the phosphorylation of H1 histone, as per the protocol of Terano et al. (27). The supernatant was incubated with anti-CDK2 antibody and precipitated with protein A-Sepharose-4B. The H1 histone kinase assays were performed at 30°C for 30 min in the following buffer: 100 μg/ml H1 histone, ATP (50 μM), and [^{32}P]ATP (5 μCi). Phosphorylated H1 histone was analyzed by SDS-PAGE (10%) and autoradiography.

Detection of state of hypo- or hyperphosphorylation of pRb

The hypophosphorylated pRb (hypo-pRb) was determined by the ratio of hypo-pRb to total pRb by flow cytometry as described by Siddiqui et al. (28). In brief, the cells were serum-starved for 6 h, washed, and cultured in ES medium containing 0.2% FCS with or without DHA (10 μM) for different times, as mentioned in the figure legends. The cells were washed with PBS for 15 min and then fixed with 1% (v/v) formaldehyde in PBS for 15 min at 4°C. Cells were then washed with PBS and suspended in 1 ml of ice-cold 80% (v/v) ethanol and incubated for 2 h at −20°C. The ethanol-fixed cells were washed again and suspended in 1 ml of 0.25% (v/v) Triton X-100 in PBS on ice for 5 min. After centrifugation (800 $g \times 5$ min), cells were incubated with two antibodies: 0.5 μg/100 μl of FITC-conjugated anti-hypoRb, which recognized the hypophosphorylated form; and PE-conjugated anti-pRb, which recognized total pRb protein. Quantification of the ratio of hypo-pRb to total pRb was performed using a FACScan flow cytometer (Beckton-Dickinson).

Statistical analysis

Results are shown as means ± SD for quadruplicate assay samples, reproduced independently at least three times. Statistical analysis of data was carried out with Statistica (version 4.1; Statsoft, Paris, France). The significance of the differences between mean values was determined by one-way ANOVA, followed by a least-significant-difference test.

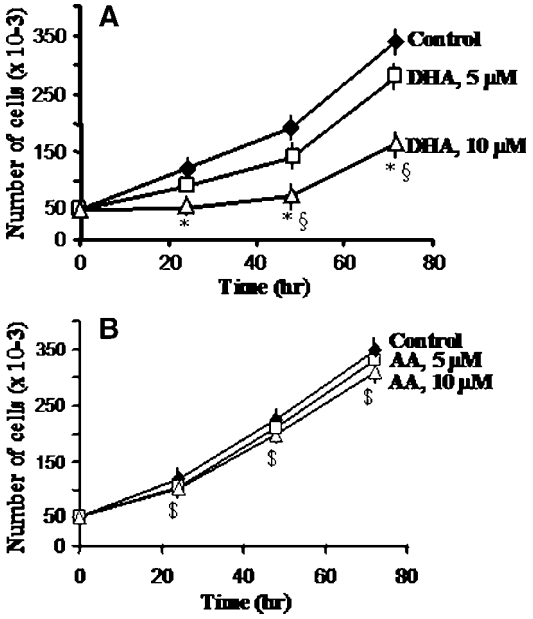

Fig. 1. Effects of docosahexaenoic acid (DHA) and arachidonic acid (AA) on growth of FM3A cells. The cells were serum-starved for 6 h, washed, and cultured for different times in an eicosapentaenoic acid (ES) medium containing 0.2% fetal calf serum (FCS) with or without DHA (A) and AA (B), as described in Materials and Methods. The cell number was counted by employing the trypan blue exclusion test. Results are means ± SD of three experiments, reproduced independently in triplicate. The values are significantly different as compared with control cells ($^*P < 0.001$) or to cells treated with 5 μM DHA ($^{\S}P < 0.001$) at different corresponding time periods. $, insignificant differences between control and AA-treated cells.

RESULTS

DHA inhibits growth of FM3A mouse mammary cancer cells

To assess the effects of DHA on cell growth, we employed physiological concentrations of this agent. We observed that DHA at 5 μM and 10 μM exerted dose-dependent inhibitory effects on the proliferation of FM3A cells (**Fig. 1A**). It is noteworthy that AA, an n-6 PUFA, failed to significantly inhibit cell growth at both 5 μM and 10 μM (Fig. 1B). For further experiments, we employed DHA only at 10 μM, because this is the concentration that exerts cytostatic effects. However, at a concentration of 20 μM, this fatty acid induced cell necrosis (results not shown).

DHA inhibits cell cycle progression

Because the effects of DHA were cytostatic, we were tempted to assess whether this fatty acid exerts its effects on cell cycle progression. We, therefore, conducted cell cycle studies. **Figure 2** shows that the number of cells in the S phase of the cell cycle was increased in serum-stimulated cells. On the other hand, DHA treatment of these cells decreased the number of cells in the S phase and increased those in the G0/G$_1$ phase of the cell cycle.

DHA upregulates p27^{Kip1} levels by inhibiting the phosphorylation of MAP kinases

To study the mechanisms responsible for cell cycle arrest, the upstream events known to play a role in the phosphorylation of pRb were investigated. In fact, p27^{Kip1} proteolysis leads to the phosphorylation of pRb and the release of E2F-1 transcription factor. **Figure 3A** shows that quiescent cells abundantly express p27^{Kip1} protein, although these cells do not express a detectable quantity of phosphorylated ERK1/ERK2. Stimulation of cell proliferation by serum is associated with p27^{Kip1} downregulation and MAPK phosphorylation. Addition of DHA to serum-stimulated cells diminished MAPK phosphorylation and upregulated p27^{Kip1} levels in FM3A cells (Fig. 3A).

Figure 3B shows that U0126, a MAPK inhibitor, also inhibited serum-stimulated downregulation of p27^{Kip1} by inhibiting MAPK phosphorylation. Phorbol-12-myristate-13-acetate (PMA) is known to activate MAPK via activation

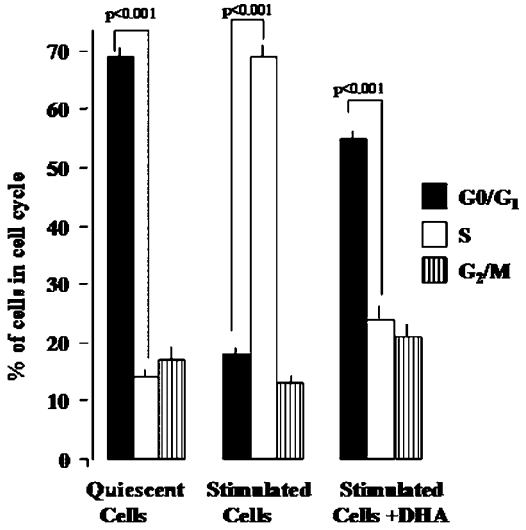

Fig. 2. Effects of DHA on cell cycle progression of FM3A cells. The cells, with or without DHA (10 μM), were cultured for 12 h, as shown in Fig. 1. The analysis of cell cycle progression was assessed by a FACScan flow cytometer, as described in Materials and Methods. Results are means ± SD of three experiments, reproduced independently in triplicate.

of protein kinase C (29). Interestingly, DHA further inhibited the PMA-induced downregulation of $p27^{Kip1}$ by inhibiting PMA-stimulated ERK1/ERK2 phosphorylation in these cells (Fig. 3B). DHA did not induce an additive accumulation of $p27^{Kip1}$ levels in the presence of U0126. In order to strengthen our notion that MAPK activation is directly implicated in the regulation of $p27^{Kip1}$ levels, we transiently transfected FM3A cells with constitutive MEK mutants (MEK^{DD}) and dominant negative MEK mutants (MEK^{SA}). Figure 3B shows that MAPK phosphorylation in MEK^{DD} cells diminished $p27^{Kip1}$ levels, whereas cells expressing MEK^{SA} diminished ERK1/ER2 phosphorylation and exhibited increased $p27^{Kip1}$ levels. Furthermore, addition of DHA to MEK^{DD} cells failed to curtail MAPK phosphorylation and to downregulate $p27^{Kip1}$ levels. In these experiments, DHA was used at 10 μM, a concentration that is only sufficient to diminish endogenous MAPK phosphorylation.

DHA does not increase $p27^{Kip1}$ mRNA levels

Because DHA was found to upregulate $p27^{Kip1}$ levels in FM3A cells, we were tempted to assess whether this fatty acid increases its mRNA levels. Hence, we employed quantitative real-time RT-PCR. Serum stimulation of cell proliferation increased $p27^{Kip1}$ mRNA levels for 9 h, after which levels remained unchanged until 12 h (results not shown). Serum-stimulated cells containing DHA and U0126 did not possess lower quantities of $p27^{Kip1}$ mRNA than the serum-stimulated-only cells (Fig. 3C). These observations indicate that inhibition of MAPK phosphorylation does not increase $p27^{Kip1}$ mRNA quantities; rather, this phenomenon may trigger an accumulation of $p27^{Kip1}$ protein content, as shown in Fig. 3B.

DHA stabilizes and inhibits the degradation of $p27^{Kip1}$ protein

There are several mechanisms of $p27^{Kip1}$ degradation; however, the best characterized is the ubiquitin-dependent degradation by proteasome (18, 19). Hence, we used a chemical inhibitor of the proteasome, MG132, to inhibit $p27^{Kip1}$ degradation. **Figure 4A** shows that treatment of FM3A cells with MG132 induced $p27^{Kip1}$ accumulation. When the cells were treated with MG132 and MEK inhibitor, U0126, or DHA, no further increase in $p27^{Kip1}$ was observed.

To assess directly the effects of DHA on $p27^{Kip1}$ degradation, we first investigated the stability of $p27^{Kip1}$ in the presence of the protein synthesis inhibitor cycloheximide. We determined the half-life of the $p27^{Kip1}$ protein. Figure 4B shows that $p27^{Kip1}$ is completely degraded in 4 h in the exponentially growing cells. Furthermore, we performed another experiment in which the control cells, after 1 h of seeding, were added with cycloheximide alone or with U0126 or DHA. After 2 h of further culture, cell lysates were prepared in order to determine the effects of these agents on $p27^{Kip1}$ degradation. The Western blot detection of $p27^{Kip1}$ levels showed that DHA and MEK inhibition by U0126 impaired the degradation of $p27^{Kip1}$ protein levels in FM3A cells (Fig. 4C).

DHA inhibits expression of CDK2 and cyclin E

The ubiquitin-dependent proteasome degradation of $p27^{Kip1}$ occurs after phosphorylation by CDK2 on Thr-187. Cyclin E is an activator of CDK, and its accumulation is known to activate CDK2 in the late-G_1 phase of the cell cycle. We, therefore, detected phosphorylated CDK2 and assayed its kinase activity. CDK2 protein was increased by serum stimulation in FM3A cells, and DHA diminished the phosphorylated form (33 kDa) of CDK2 (**Fig. 5A**). Furthermore, DHA diminished the serum-induced CDK2 kinase activity in FM3A cells (Fig. 5B). DHA failed to influence the expression of cyclin D1 in serum-stimulated cells (Fig. 5A). We failed to detect other isoforms of cyclin D in these cells (results not shown). The phosphorylation of cyclin E was enhanced during stimulation of FM3A cells, whereas DHA curtailed the degree of the same (Fig. 5A).

DHA induces hypophosphorylation of pRb

Because phosphorylation of pRb is necessary for the progression through the cell cycle, we assessed the time-course of phosphorylation of pRb. In serum-starved cells, pRb appears in the hypophosphorylated form; however, after 6 h of stimulation, another band of lower mobility appears, which corresponds to the hyperphosphorylated form and persists for 24 h (results not shown). **Figure 6** shows that DHA inhibits hyperphosphorylation of pRb in FM3A cells.

DISCUSSION

The present study was conducted to elucidate the effects of DHA on FM3A mouse mammary cancer cell prolifer-

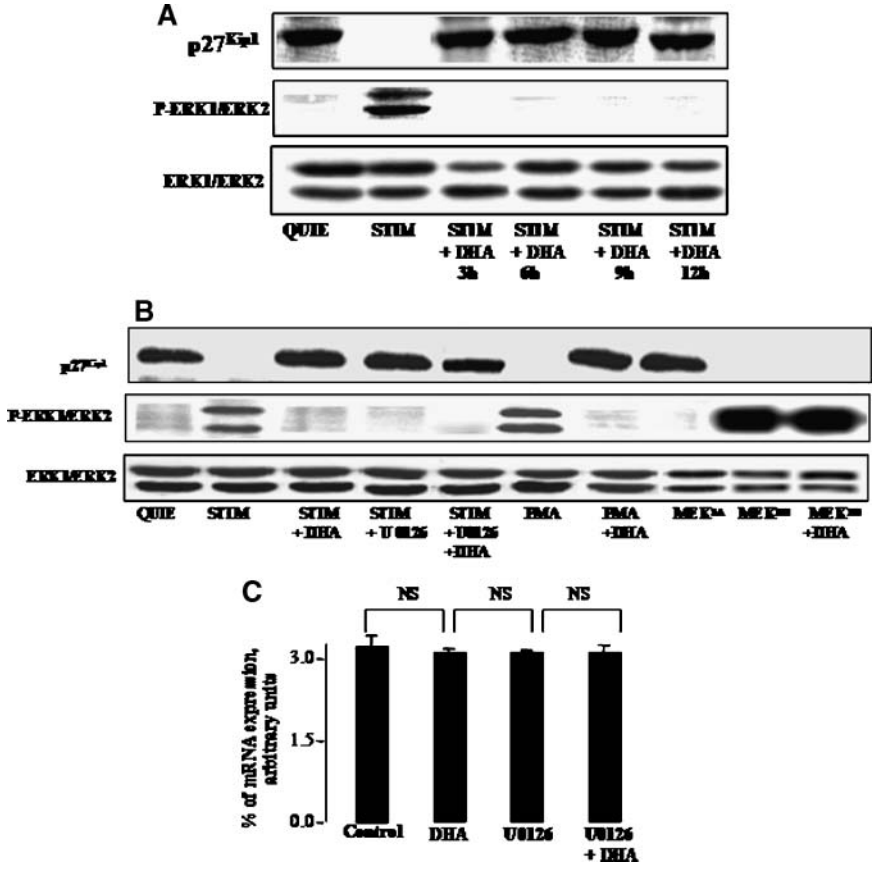

Fig. 3. A: Effects of DHA on mitogen-activated protein (MAP) kinase (MAPK) phosphorylation and $p27^{Kip1}$ expression in FM3A cells. The cells were serum-starved for 6 h (quiescent cells, QUIE), washed, and cultured in the ES medium containing 0.2% FCS with DHA (10 μM) for different times (3 h, 6 h, 9 h, and 12 h). B: Effects of inhibition of MAPK phosphorylation on $p27^{Kip1}$ expression in FM3A cells. The cells were cultured as shown in Fig. 1 for 12 h in the presence or absence of DHA (10 μM), U0126 (10 μM), and PMA (500 nM). MAPK extracellularly signal-regulated kinase kinase^{SA} (MEK^{SA}) and MEK^{DD} represent cells expressing, respectively, dominant negative and constitutively active MEK constructs. C: Effects of DHA and U0126 on expression of KIP1 mRNA in FM3A cells. The cells were cultured with or without DHA (10 μM) and U0126 (10 μM) for 12 h, as shown in Fig. 1. KIP1 mRNA was quantitatively assessed by real-time RT-PCR as described in Materials and Methods. The histograms indicate the expression of mRNA relative to the level of mRNA expressed by cells cultured without serum and the test molecules, considered arbitrarily as 1. STIM, cells stimulated with 0.2% FCS; NS, nonsignificant. Error bars represent standard deviation of three individual experiments.

ation. FM3A cells represent a good model for studying cell growth, because they do not die due to apoptosis (30). In the present study, we employed DHA, because this is the terminal molecule of the n-3 PUFA family. Externally added fatty acids are readily complexed with free albumin. Hence, fatty acids, after their internalization, are metabolized, and their cellular effects are different from those in free fatty acid forms. Because we wanted to assess the effects of free DHA, we minimized the probabilities of its complexation with a high quantity of albumin by employing fetal calf serum at low concentrations. This type of strategy has also been adopted by other investigators (15). We observed that DHA inhibited FM3A cell growth at a relatively physiological concentration, i.e., 10 μM. On the other hand, AA, a PUFA of the n-6 family, did not exert growth-inhibitory effects at this concentration in FM3A cells. Our observations on the inhibition of cell proliferation are in agreement with the results of several other authors (6–8, 31) who have shown that this fatty acid exerts anti-proliferative effects on cancer cells.

To determine whether DHA-induced inhibition of cell growth is due to its specific action on the cell cycle, we assessed cell cycle progression. We observed that there were fewer cells in the S phase of the cell cycle in DHA-treated cells than in the serum-stimulated cells. Similarly, the number of cells in the $G0/G_1$ phase of the cell cycle was higher in DHA-treated cells than in the serum-stimulated cells. Our observations on the specific effects of DHA on the inhibition of cell cycle progression corroborate the findings of Albino et al. (15), who have demonstrated that DHA inhibits the growth of human melanoma cells by inhibiting the entry of these cells into the S phase of the cell cycle. Terano et al. (27) have also observed that both DHA and EPA inhibit the proliferation of vascular

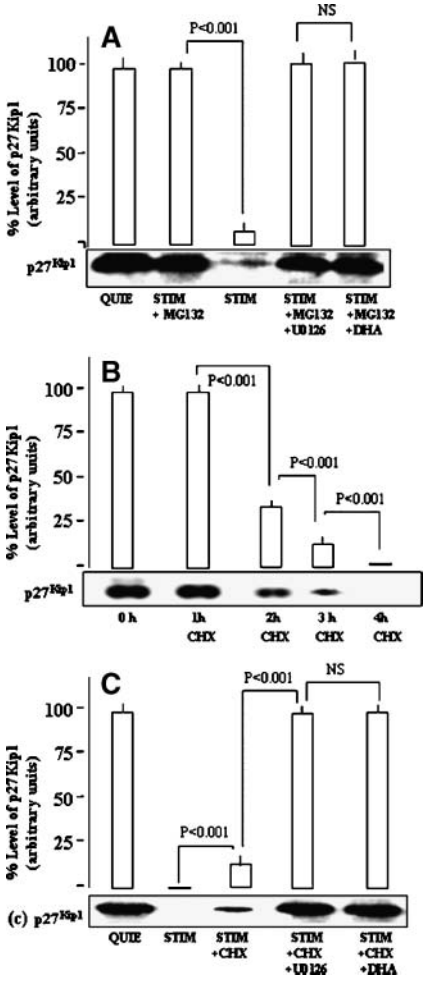

Fig. 4. Effects of DHA and other MEK inhibitors on $p27^{Kip1}$ degradation. A: Cells were cultured as shown in Fig. 1 for 12 h in the presence or absence of DHA (10 μM), MG132 (10 μM), and U0126 (10 μM). STIM, cells stimulated with 0.2% FCS. B: Cells were serum-starved for 6 h (quiescent cells) and stimulated to grow with 0.2% FCS in the presence of cycloheximide (CHX at 75 μM) for different times (0 to 4 h). Cell lysates were prepared, and immunoblotting was performed as described in Materials and Methods. C: Cells were serum-starved for 6 h (quiescent cells, QUIE) and reseeded in the presence of 0.2% FCS (STIM). After 1 h, cycloheximide (CHX at 75 μM) was added to the medium with or without U0126 or DHA. Cell lysates were prepared, after 2 h of culture, and immunoblotting was performed as described in Materials and Methods. NS = nonsignificant.

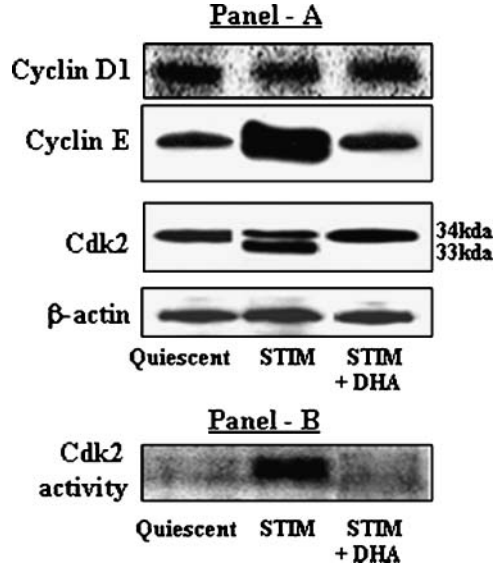

Fig. 5. Effects of DHA on cell cycle-regulated proteins in FM3A cells. The cells, with or without DHA (10 μM), were cultured for 12 h, as shown in Fig. 1. Cell lysates were prepared as described in Materials and Methods. Similar amounts of protein for each sample were loaded onto SDS-PAGE, and cyclin-dependent kinase 2 (CDK2), cyclin E, and cyclin D1 were detected by immunoblotting, as described in Materials and Methods (Panel A). Panel B shows the CDK2 kinase activity. STIM, cells stimulated with 0.2% FCS.

smooth muscle cells by inhibiting the G_1/S progression of the cell cycle.

It has been shown that the proteolysis of $p27^{Kip1}$ begins in the late-G_1 phase of the cell cycle (32). We observed that $p27^{Kip1}$ was downregulated in serum-stimulated cells, whereas DHA treatment blocked this phenomenon. Delmas et al. (33) have shown that ERK1/ERK2 phosphorylation induces the degradation of $p27^{Kip1}$ levels without influencing protein synthesis in choroidal melanoma cells. Moreover, we have demonstrated that DHA inhibits phosphorylation of ERK1/ERK2 (29, 34) and proliferation of T-lymphocytes (35) and NIH/3T3 cells (36). Schley et al. (37) and Xue et al. (31) have also shown that DHA and other n-3 fatty acids modulate cell growth by interfering with ERK1/ERK2 and Akt phosphorylation. To determine whether DHA-induced inhibition of MAPK phosphorylation is responsible for the upregulation of $p27^{Kip1}$, we employed a chemical inhibitor of MEK (U0126) and a MEK dominant negative construct (MEK^{SA}). We activated MAPK phosphorylation by PMA and a constitu-

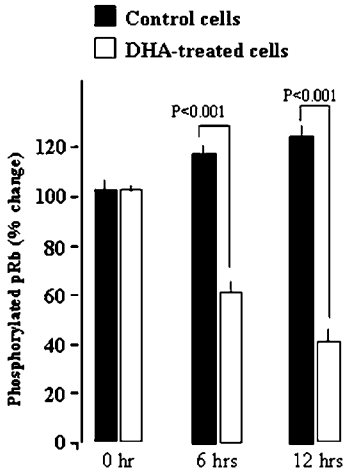

Fig. 6. Effects of DHA on phosphorylation of retinoblastoma protein (pRb) in FM3A cells. The cells, with or without DHA (10 μM) or not (control), were cultured for 0 h, 6 h, and 12 h as shown in Fig. 1. Quantification of the ratio of hypophosphorylated pRb to total pRb was performed by FACScan as described in Materials and Methods. Results are means ± SD of three experiments.

tively active MEK construct (MEK^{DD}). We observed that PMA and MEK^{DD} induced phosphorylation of ERK1/ERK2 and, concomitantly, downregulated $p27^{Kip1}$ levels. U0126 and MEK^{SA} inhibited MAPK phosphorylation and upregulated $p27^{Kip1}$ levels in these cells. Similarly, DHA inhibited serum- and PMA-induced MAPK phosphorylation and downregulation of $p27^{Kip1}$ levels in FM3A cells. Furthermore, DHA failed to induce an additive accumulation of $p27^{Kip1}$ in the presence of the MEK inhibitor U0126. To determine whether DHA and the MEK inhibitor increase $p27^{Kip1}$ protein by exerting their actions at the mRNA level, we quantitatively determined the KIP1 mRNA expression in these cells. To our surprise, we observed that DHA and U0126 (and also MEK^{SA}, results not shown) did not induce the expression of $p27^{Kip1}$ mRNA. Moreover, DHA did not induce an additive response in the presence of the MEK inhibitor U0126 on expression of $p27^{Kip1}$ mRNA. These observations suggest that the high expression of $p27^{Kip1}$ by DHA and these agents involves posttranscriptional mechanisms, although both of the mechanisms, i.e., transcriptional and posttranscriptional, have been reported to exist in other cells, depending on cell type (33, 38). However, posttranscriptional control, viz. proteolytic degradation, of $p27^{Kip1}$ is a common phenomenon (except in some particular cell systems) that takes place through a ubiquitin-dependent proteasome pathway (38). To determine whether DHA blocks the proteasome degradation of the $p27^{Kip1}$ protein, we employed MG132, the proteasome inhibitor. Indeed, MG132 inhibited the degradation of $p27^{Kip1}$ in FM3A cells. The DHA and MEK inhibitor U0126 did not evoke an additive accumulation of $p27^{Kip1}$ levels in the presence of MG132. These observations suggest that DHA-induced upregulation of $p27^{Kip1}$ is the consequence of the blockade of $p27^{Kip1}$ degradation by this fatty acid and that the proteasome pathway may be implicated in this phenomenon. Although there are also other pathways of $p27^{Kip1}$ degradation, such as calpain-mediated proteolysis (39), we can rule out, at least, the implication of a caspase-dependent pathway, inasmuch as we did not observe, during immunoblotting, the appearance of 22 kDa and 14 kDa bands (40), the caspase-dependent cleavage products of $p27^{Kip1}$ (results not shown). Furthermore, by employing cycloheximide, we evaluated the half-life of $p27^{Kip1}$ degradation and observed that after 4 h, $p27^{Kip1}$ was totally degraded. Hence, the DHA and MEK inhibitor U0126 stabilized $p27^{Kip1}$ protein and blocked its further degradation in the presence of cycloheximide. Because the ERK1/ERK2 cascade is required for $p27^{Kip1}$ downregulation and late-G_1 phase exit (41), our study does show that DHA inhibits MAPK-dependent degradation of $p27^{Kip1}$ in accordance with a recent study (37) showing that MAPK inhibition was found to inhibit the proliferation of six pancreatic cell lines by upregulating $p27^{Kip1}$ levels.

$P27^{Kip1}$ is a specific inhibitor of CDK2/cyclin E. CDK2, via its association with cyclin E, is involved in the regulation of the G_1-to-S transition (18, 19). In our study, DHA did not inhibit cyclin D1 expression, suggesting that the cyclin D/CDK4/6 complex, activated in the early G_1phase but not in late G_1 phase, is not influenced by DHA (16). On the other hand, DHA was found to inhibit cyclin E phosphorylation and CDK2 levels. Moreover, DHA inhibited H1 kinase activity of CDK2. In fact, the CDK2/cyclin E complex is known to phosphorylate pRb, because this complex acts as an Rb-kinase (16, 20). It is well known that pRb is hypophosphorylated in the $G0/G_1$ phase and hyperphosphorylated in the G_1-to-S-phase transition. Phosphorylation of pRb results in the dissociation of pRb and the release of a transcription factor (E2F-1) that induces the S-phase entry (16, 20). In the present study, we observed that DHA inhibited pRb hyperphosphorylation. The inhibition of pRb hyperphosphorylation may be due to DHA-induced reduced activity of CDK2 kinase activity. These observations are in accord with the findings of Albino et al. (15), who have reported that inhibition of cyclin expression, CDK2 kinase activity, and pRb phosphorylation are responsible for the inhibition of the transition of WI-38 cells from the G_1 to the S phase of the cell cycle.

In conclusion, our findings provide new insights into the mechanism of action of DHA in the regulation of cell proliferation. Our observations demonstrate that DHA, a PUFA of the n-3 family, induced cell cycle arrest in the late-G_1 to S phase of the cell cycle by inhibiting the MAPK activation and proteasome degradation of $p27^{Kip1}$ in FM3A cells. DHA also inhibits CDK2 kinase activity and hyperphosphorylation of pRb in these cells.

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