Eicosapentaenoic Acid Demethylates a Single CpG That Mediates Expression of Tumor Suppressor CCAAT/Enhancer-binding Protein δ in U937 Leukemia Cells*

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Polysaturated fatty acids (PUFAs) inhibit proliferation and induce differentiation in leukemia cells. To investigate the molecular mechanisms whereby fatty acids affect these processes, U937 leukemia cells were conditioned with stearic, oleic, linolenic, α-linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acids. PUFAs affected proliferation; eicosapentaenoic acid (EPA) was the most potent on cell cycle progression. EPA enhanced the expression of the myeloid lineage-specific transcription factors C/EBPβ and C/EBPδ, PU.1, and c-Jun, resulting in increased expression of the monocytic lineage-specific target gene, the macrophage colony-stimulating factor receptor. Indeed, it is known that PU.1 and C/EBPs interact with their consensus sequences on a small DNA fragment of macrophage colony-stimulating factor receptor promoter, which is a determinant for expression. We demonstrated that C/EBPβ and C/EBPδ bind the same response element as a heterodimer. We focused on the enhanced expression of C/EBPδ, which has been reported to be a tumor suppressor gene silenced by promoter hypermethylation in U937 cells. After U937 conditioning with EPA and bisulfite sequencing of the −370/−20 CpG island on the C/EBPδ promoter region, we found a site-specific CpG demethylation that was a determinant for the binding activity of Sp1, an essential factor for C/EBPδ gene basal expression. Our results provide evidence for a new role of PUFAs in the regulation of gene expression. Moreover, we demonstrated for the first time that re-expression of the tumor suppressor C/EBPδ is controlled by the methylation state of a site-specific CpG dinucleotide.

Increasing evidence from animal and in vitro studies indicates that fatty acids, especially the long-chain polysaturated fatty acids (PUFAs), affect carcinogenesis (1). n-3 PUFAs inhibit the growth of tumor cells both in vivo and in vitro (2, 3), decrease metastasis and cachexia (4, 5), and increase the cytotoxic effects of some chemotherapeutic agents (6), although the results are not always consistent (7, 8). In addition, n-3 PUFAs reduce cell proliferation and induce differentiation and apoptosis in hepatocarcinoma and leukemia cells (9–12). Although not completely known, several molecular mechanisms whereby n-3 PUFAs may modify the carcinogenic process have been proposed. These include alteration in cell membrane composition and function, modulation of mitochondrial calcium homeostasis, suppression of the biosynthesis of proinflammatory molecules, influence on signal transduction, alteration of hormone-stimulated cell growth, inhibition of angiogenic mediators, production of free radicals and reactive oxygen species, and influence on transcription factor activity and gene expression (2, 13–17).

In hepatocarcinoma cells, n-3 PUFAs modulate the expression of CCAAT/enhancer-binding protein (C/EBP) transcription factors (18, 19). C/EBPs (α, β, δ, γ, ε, and ζ) represent a family of master regulators that play an essential role in controlling cell proliferation and differentiation processes of several cell types, including myeloid cells (20). Members of the C/EBP family are structurally related, each consisting of an N-terminal transactivating region, a central basic DNA-binding domain, and a C-terminal leucine zipper motif for dimerization (20). During hematopoiesis, C/EBPα, C/EBPβ, and C/EBPδ are predominantly expressed in the granulocyte and monocyte lineages, whereas C/EBPε is found in the middle to later stages of differentiation of granulocytes and T cells (21–25). The most compelling evidence for a crucial role of the C/EBPs in myeloid cell differentiation and maturation has come from studies on knock-out mice. C/EBPα-deficient mice fail to undergo myeloid differentiation beyond the myeloblast stage and, therefore, lack mature neutrophils (26), whereas the phenotype of C/EBPβ-deficient mice indicates a potential role in the activation and/or differentiation of macrophages (27). Moreover, overexpression of C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε induce granulocyte differentiation in myeloblastic cell lines (28), suggesting that in myeloid cells C/EBP family mem-
ers can compensate in vivo for the lack of one of the other C/EBP proteins.

Besides C/EBPs, other transcription factors and co-activators contribute to myeloid cell fate (29). First of all, PU.1 drives the transcription of monocyte-specific genes, including the macrophage colony-stimulating factor (M-CSF) receptor (30, 31). PU.1 and C/EBPs can bind to and activate the M-CSF receptor promoter, and their combinatorial activities are essential to mediate the M-CSF receptor expression level (32). In addition, the co-activator partner protein c-Jun cooperates with PU.1 (33) and C/EBPs (34) during monocyte differentiation, although it is able itself to induce partial monocyte differentiation in a variety of myeloid cell lines (35, 36). c-Jun does not directly bind to the M-CSF receptor promoter but enhances the ability of PU.1 to transactivate it (37). Synergism among PU.1, C/EBPs, and c-Jun is essential to activate monocyte target genes (34). Among these, M-CSF receptor is critical for monocyte cell survival and proliferation and is activated early during the monocyte differentiation process (38–40).

In the present study, we evaluated the effects of fatty acid conditioning of the U937 promonocytic cell line on proliferation, cell cycle progression, and the differentiation program in relation to chain length and the number of double bonds. We found that eicosapentaenoic acid (EPA) treatment reduced cell cycle progression and induced monocyte-specific M-CSF receptor expression by enhancing C/EBPo, C/EBPr, PU.1, and c-Jun expression. Considering that C/EBPo was reported to be a tumor suppressor gene (41, 42) that is silenced by promoter hypermethylation in U937 cells and re-expressed by proximal promoter demethylation (43), we analyzed the same promoter region (−370 to −20) after EPA conditioning of U937 cells. We found a site-specific Cpg demethylation that was a determinant for the binding activity of Sp1 transcription factor to induce C/EBPo gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials**

Stearic acid (18:0; SA), oleic acid (18:1, n-9; OA), linoleic acid (18:2, n-6; LA), α-linolenic acid (18:3, n-3; LNA), arachidonic acid (20:4, n-6; AA), eicosapentaenoic acid (20:5, n-3; EPA), docosahexaenoic acid (22:6, n-3; DHA), bovine serum albumin fraction V (BSA; fatty acid-free), and 5-aza-2’-deoxycytidine were from Sigma.

**Preparation of Albumin-bound Fatty Acids**

A stock solution of each fatty acid (5 or 10 mM) was prepared by diluting the free fatty acid in ethanol and precipitating it with the addition of NaOH (final concentration, 0.25 M). The precipitate was dried under nitrogen, reconstituted with 0.9% (w/v) NaCl, and stirred at room temperature for 10 min with defatted BSA (final concentration, 10% (w/v) in 0.15 M NaCl). Each solution was adjusted to pH 7.4 with NaOH and stored in aliquots at −20 °C protected from light under nitrogen. The fatty acid/BSA molar ratio was 3:1 or 6:1.

**Cell Culture**

The human promonocytic cell line U937 (CRL-1593.2) obtained from the American Type Culture Collection (Mannas-sas, VA) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin at 37 °C in a humidified incubator aerated with 5% CO2. U937 cells were seeded at a density of 0.3 × 106 cells/ml for all experiments. Cells were incubated with fatty acid/BSA solutions at the indicated concentrations and times.

**Flow Cytometry Analysis**

**Cell Cycle and Apoptosis**—U937 cells were treated with fatty acids for 24 h (50–200 μM final concentration) and analyzed by flow cytometry as indicated below. After washing, the 200 × g cell pellets were resuspended in 1 ml of hypotonic PI solution (50 μg ml−1 in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma). The samples were placed overnight in the dark at 4 °C, and the PI fluorescence of individual nuclei was measured using an EPICS XL-MCL™ flow cytometer (Beckman Coulter, Inc., Miami, FL). Analysis of apoptosis was performed as described by Nicoletti et al. (44), and data were processed by an Intercomp computer and analyzed with SYSTEM II™ software (Beckman Coulter, Inc.). The cell cycle was analyzed by measuring DNA-bound PI fluorescence in the orange-red fluorescence channel (FL2) through a 585/42-nm bandpass filter with linear amplification. Analysis of distribution profiles was performed with ModFit LT software (Verity Software House, Topsham, ME) to determine fractions of the population in each phase of the cell cycle (G0/G1, S, G2/M). At least 15,000 events were collected for each sample. Cells were gated on FL2-area versus FL2-width plots to exclude aggregates and debris from analysis (45).

**Forward and Side Scatter**—Intact U937 cells were recovered after treatment with 100 μM fatty acids, resuspended in 1 ml of PBS, and identified by forward and right angle (side) light scatter using the same flow cytometer equipped with a 15-milliwatt argon ion laser (488 nm). Cell viability was determined by counting triplicate samples for trypan blue dye-excluding cells.

**[3H]Thymidine Incorporation Assay**

Triplicate samples of 1 × 10⁵ U937 cells suspended in 200 μl of RPMI 1640 medium were cultured in the presence or absence of 100 μM fatty acids for 24 h. [3H]thymidine (specific activity, 6.7 Ci/mmol) (Amersham Biosciences) was added to the cultures at 2.5 μCi/well. After a 4-h incubation, cells were harvested with a multiple suction-filtration apparatus (Mash II) on a fiberglass filter (BioWhittaker) and counted in a β counter apparatus (Packard Instrument Co.).

**Real Time PCR**

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s guidelines. cDNA was prepared by using the QuantiTect reverse transcription kit (Qiagen). Quantitative gene expression analysis was performed using an Mx3000P™ Real-Time PCR System with Brilliant® SYBR® Green qPCR Master Mix (Stratagene) and ROX as the reference dye. Quantitative PCRs were performed under conditions standardized for each primer. To minimize variability, every time point was investigated with four replicates, and the amplification of three independent treatments was performed. The following primers were used for real time qPCR analysis:
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C/EBPα (5′-AGGAGCAGAACGAGATC-3′), C/EBPαrev (5′-GTCGACAGTGCAAGTCTTC-3′), C/EBPβ (5′-ACGCAAGACAGAAGATC-3′), C/EBPβrev (5′-TGCTCCACTTCTTGGTC-3′), C/EBPβ (5′-GATGAGCAGACAGAAGATC-3′), C/EBPβrev (5′-GTCAGGACAGAAGATC-3′), C/EBPβ (5′-CTTATGGCTTCGCTGTA-3′), and M-CSF receptor (5′-GTGTCTGCTGTCTCTTG-3′) were used. The expression level of C/EBPα was determined based on the threshold cycle (Ct) for each PCR product. Data were quantitatively analyzed according to the formula $2^{-\Delta\Delta Ct} = 2^{-\Delta Ct(Ms) - \Delta Ct(Mo)}$ (47).

DNA Isolation and Quantitative DNA Methylation Analysis of C/EBPα CpG Island

Genomic DNA from unsupplemented U937 cells or U937 grown for 24 h with 100 μM OA or 100 μM EPA was extracted using a FlexiGene DNA kit in accordance with the protocol instructions (Qiagen). DNA methylation levels were quantified using the Methyl-Profiler qPCR Primer Assay for human C/EBPα (MePH28341-1A) (SABiosciences-Qiagen) in accordance with the protocol provided by the manufacturer. Primers are designed by an optimized computer algorithm to ensure that the amplicon contains cutting sites for both methyl-sensitive and methyl-dependent enzymes and are specifically designed for analyzing the DNA methylation status of CpG islands using restriction enzyme digestion (DNA Methylaton Enzyme kit MeA-03, SABiosciences-Qiagen) followed by SYBR Green-based real time PCR detection. Briefly, each genomic DNA was subjected to four separate treatments according to the instructions provided by the manufacturer. (i) For the mock digest (Mo), no enzymes were added in the reaction. The product of the mock digest represented the total amount of input DNA for real time PCR detection. (ii) For the methylation-sensitive digest (Ms), cleavage was carried out with a methylation-sensitive enzyme, which digested unmethylated DNA. The remaining hypermethylated DNA was detected by real time PCR. (iii) For the methylation-dependent digest (Md), cleavage was carried out with a methylation-dependent enzyme, which digested methylated DNA. The remaining unmethylated DNA was detected by real time PCR. (iv) For the double digest (MsMd), both enzymes were added, and all DNA molecules (both methylated and unmethylated) were digested. This reaction measures the background and the fraction of input DNA refractory to enzyme digestion. The four mixtures were incubated at 37 °C overnight. The enzymes were inactivated at 65 °C for 20 min. The resulting DNA was stored at −20 °C or utilized for real time PCR as described above. The specific RT-PCR program was as follows: 95 °C for 10 min and 40 cycles of 97 °C for 15 s and 72 °C for 60 s as indicated in the instruction manual. The relative amount of each DNA fraction (methylated and unmethylated) was calculated using a standard ΔCt method, normalizing the amount of DNA in each digestion against the total amount of input DNA in the mock digest. The amount of hypermethylated target DNA copies ($C_{HUM}$) is defined as $(2^{-\Delta Ct(MsMd) - \Delta Ct(Mo)} - C_{R})/(1 - C_{R})$ where $C_{R}$ represents the amount of target DNA copies that are resistant to enzyme digestion and is defined as $2^{-\Delta Ct(MsMd)} - C_{R}$.

Bisulfite Modification of Genomic DNA and Sequencing

Genomic DNA from unsupplemented U937 cells or U937 grown for 24 h with 100 μM OA or 100 μM EPA was extracted as described above. Bisulfite modification of genomic DNA was done as described (48, 49). Briefly, 1 μg of genomic DNA (50-μl
volume) was denatured by using mild heat at alkaline pH by adding 3.5 μl of 3 M NaOH and incubating for 10 min at 37 °C. Immediately, 10 μl of 10 mM hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (Sigma) were added and incubated for 12–16 h at 50 °C. Bisulfite-treated DNA was purified and eluted in 50 μl of H2O. The conversion of uracil was completed by alkaline desulfonation by adding 5 μl of 3 M NaOH and incubating at 37 °C. The treated DNA was purified using a QIAquick gel extraction kit and eluted in 30 μl of H2O. Sequencing primers for bisulfite-modified DNA were designed by using the on-line program MethPrimer (available at the University of California San Diego web site). Bisulfite-modified DNA was amplified using sequencing primers for the C/EBPβ promoter. The primers for the first PCR were as follows: forward primer, 5′-GATTTTATTTTAATTTYGAGGAGY-3′ (Y = C/T); reverse primer, 5′-AAACTATACCTCRCRTCTAAACCCCAACC-3′ (R = C/T). Following the initial amplification, an aliquot of the initial PCR products was used as template DNA in a nested PCR. The primers for the nested PCR were as follows: forward, 5′-GATTTTATTTTAATTTYGAGGAGY-3′ (Y = C/T); reverse primer, 5′-CTTTTCTACCCCTRACTAARTA-3′ (R = C/T). Nested PCR conditions were as follows: 95 °C for 5 min and 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s followed by 10 min at 72 °C. The amplified products were confirmed by electrophoresis on an agarose gel. The nested 350-bp PCR products (−370/−20) were subcloned into pCR2.1 TA cloning vector (Invitrogen). Single clones were selected and cultured, and plasmid DNA was isolated using a GeneElute plasmid miniprep kit (Sigma) and sequenced using T7 primer at the Genechron-Ylichron S.r.l. Laboratory (Rome, Italy). Eight to 10 clones were sequenced for each sample.

Electrophoretic Mobility Shift Assays and Antibody Interference Assays

Nuclear extracts from U937 cells were prepared, and electrophoretic mobility shift/antibody interference assays were performed as described previously (50). Complementary pairs of site-specific methylated and unmethylated oligonucleotides were from Integrated DNA Technologies-TEMA Ricerca (Bologna, Italy). Oligonucleotides were annealed by boiling for 5 min at 95 °C followed by cooling and end-labeled with [γ-32P]dATP (Amersham Biosciences) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Labeled fragments were separated from residual [γ-32P]dATP using Quick Spin columns (Roche Applied Science) according to the manufacturer’s instructions. The upper strand C/EBPβ promoter oligonucleotide sequence used was 5′-CGGCGGGCCGTCACGTCAGCCGGGGCTAG-3′ (underlined C corresponds to the C demethylated after EPA treatment). Binding reactions were carried out by mixing 20 μg of nuclear extract with 4 μl of 5X binding buffer (40 mM HEPES, pH 8.0, 200 mM KCl, 0.4 mM EDTA, 20 mM MgCl2, 40% glycerol, 20 mM diethiothreitol, 0.05% bromophenol blue) in a total volume of 18 μl and incubating at 4 °C for 1 h. Approximately 90,000 cpm (2 μl of ~0.5 ng/μl [γ-32P]dATP-labeled probe were added per reaction and incubated for 15 min at room temperature. The reaction mixtures were loaded onto a 6% polyacrylamide gel (0.75 mm thick) and separated for 3 h at 200 V in 0.5X Tris borate-EDTA. The gel was dried for 30 min under vacuum and exposed to autoradiograph film using an Instant Imager autoradiography system (PerkinElmer Life Sciences). For antibody interference assays, 3.0 μl of anti-Sp1 antibody (Santa Cruz Biotechnology Inc. sc-59) was incubated with the nuclear extract and binding buffer for 1 h at 4 °C in a total volume of 18 μl. After addition of the probe, subsequent steps were identical to those for the band shift assays.

Statistical Analyses

All average results are presented as mean ± S.D. One-way or two-way (where appropriate) analyses of variance with Bonferroni’s post-test were used. Values were considered significant at p < 0.05.

RESULTS

Effects of PLFAs on Cell Viability, Proliferation, and Cellular Morphology of U937 Cells—Variable effects of PLFAs on proliferation, differentiation, and apoptosis in leukemia cells have been reported in relation to cell line and fatty acid concentration (9, 11, 12). To study the effects of fatty acids on the percent distribution in the various phases of the cell cycle, U937 cells were analyzed by flow cytometry after incubation with fatty acids of increasing carbon chain length and double bond number. Twenty-four hour treatment with LNA, AA, EPA, and DHA increased significantly the percentage in G0-G1 phase in a concentration-dependent manner compared with untreated cells. EPA exhibited the greatest effect when compared with LNA, AA, and DHA (Fig. 1A). Contrarily, SA, OA, and LA had no effect at any of the concentrations studied (Fig. 1A). For untreated U937 cells, 43.4% were in G0-G1 phase, 44.5% were in S phase, and 11.9% were in G2-M phase (Fig. 1B). After 24-h supplementation with LNA, AA, EPA, and DHA (100 μM final concentration), the percentage of cells in G0-G1 phase increased (maximal effect was 64% with EPA) paralleled by a decrease in the percentage of cells in S and G2-M phases (33 and 2.5%, respectively, with EPA). Only minor changes were observed in SA-, OA-, and LA-treated cells in the same experimental conditions (Fig. 1B). No fatty acid treatment, except SA (51), induced U937 apoptosis up to a 200 μM concentration for 24, 48, and 72 h (not shown). When cells were exposed to 100 μM LNA, AA, EPA, and DHA for 24 h, a significant reduction in the cell number was observed as compared with untreated cells (p < 0.001), whereas SA, OA, and LA had no significant effect on cell proliferation (Fig. 1C). The increased number of cells in G0-G1 phase and the decrease of total cell number induced by LNA, AA, EPA, and DHA treatments were in agreement with the reduced DNA synthesis. Indeed, LNA, AA, EPA, and DHA inhibited significantly [3H]thymidine incorporation (p < 0.001). As expected, no effect on [3H]thymidine incorporation was found in SA, OA, and LA treatments (Fig. 1D). EPA treatment produced the greatest effect on reduction of cell cycle progression.

Flow cytometry analyses, measuring forward and side scatter, were used to obtain information about the morphology of U937 leukemia cells after fatty acid treatments. Analysis of the cells showed a significant increase both in the forward and side scatter.
EPA Induces Tumor Suppressor C/EBPα Expression

EPA Increases C/EBP, PU.1, and c-Jun Expression—We examined whether the effects on cell viability, proliferation, and cellular morphology induced by PUFA conditioning could be related to enhanced expression of lineage-specific transcription factors involved in the cell cycle progression and differentiation process of myeloid cell lines. Fatty acid treatments (100 μM) had no effect on the content of any of the C/EBPα isomers (Fig. 2A). LNA, AA, EPA, and DHA increased the protein content of C/EBPβ, C/EBPδ, PU.1, and c-Jun, whereas SA, OA, and LA had a lesser or no effect and were unable to simultaneously induce all four transcription factors (Fig. 2A). The enhanced protein expression levels of C/EBPβ, C/EBPδ, c-Jun, and PU.1 let us to suppose that U937 cells undergo the early phase of the myeloid differentiation process. Indeed, C/EBPβ, C/EBPδ, c-Jun, and PU.1 are involved in the transcriptional control of granulocyte and monocyte development.

To determine whether PUFA treatment affected C/EBP, PU.1, and c-Jun protein levels due to transcriptional events, RT-PCR was performed. Western blot analysis of U937 cells after 2- and 5-day treatment with 100 μM OA or EPA. Asimultaneous and significant increase of C/EBPβ, C/EBPδ, PU.1, and c-jun mRNA levels was observed after 3 h of EPA treatment that further increased up to 24 h (Fig. 2B). On the contrary, OA did not induce any significant change. In agreement with the unchanged protein content, OA and EPA conditioning had no effect on C/EBPα mRNA levels.

EPA Enhances M-CSF Receptor Expression—The increased C/EBPβ, C/EBPδ, PU.1, and c-jun mRNA levels in response to EPA suggested a potential transcriptional effect on their target genes. M-CSF receptor is expressed very early during the monocyte differentiation process; PU.1, C/EBPs, and co-activator partner protein c-Jun are the transcriptional activators of M-CSF receptor (30, 32). The M-CSF receptor mRNA levels were evaluated in U937 cells after 1-, 3-, and 24-h treatment with 100 μM OA or EPA. A simultaneous and significant increase of C/EBPβ, C/EBPδ, PU.1, and c-jun mRNA levels was observed after 3 h of EPA treatment that further increased up to 24 h (Fig. 2B). On the contrary, OA did not induce any significant change. In agreement with the unchanged protein content, OA and EPA conditioning had no effect on C/EBPα mRNA levels (Fig. 2B).

EPA Induces C/EBPβ Expression Acting as Site-specific Demethylating Agent—C/EBPβ is silenced by hypermethylation in U937 cells, and the demethylating agent 5-aza-2′-deoxycytidine is able to induce its expression (43). We verified the effect of 5-aza-2′-deoxycytidine on C/EBPβ protein levels in our experimental conditions and compared it with that of EPA. Western blot analysis of U937 cells after 2- and 5-day treatment with 1 μM 5-aza-2′-deoxycytidine as well as after 24-h 100 μM EPA treatment showed increased protein levels (Fig. 4A). We hypothesized that EPA could exert its effects on protein expression by acting as a demethylating agent.

The analysis of C/EBPα gene (−3000/+1269) using the EMBOSS (European Molecular Biology Open Software Suite) or MethPrimer on-line software programs retrieved six putative islands. To verify whether EPA treatment demethylates C/EBPδ CpG islands, the percent content of CpG DNA methylation was quantified using the Methyl-Profiler qPCR Primer Assay. Quantitative RT-PCR indicated that the amount of C/EBPα hypermethylated DNA copies decreased significantly (p < 0.001) after EPA conditioning compared with OA-treated or untreated cells (Fig. 4B). The 5′-upstream promoter region containing a CpG island with high CG content and a demethylated region exhibited a CpG island methylation status consistent with our qPCR data for C/EBPα expression (43). (Fig. 4C) was analyzed.
We performed bisulfite sequencing of this region and found a high degree of methylation in agreement with the low C/EBP protein level. EPA treatment induced a site-specific CpG demethylation in all the sequenced clones (Fig. 4D). The same was not found for OA treatment, which induced random and low level CpG demethylation. Indeed, the specific CpG dinucleotide demethylated after EPA treatment is located in a critical region of C/EBP proximal promoter between the TATA box and an Sp1 binding site (Fig. 4C) and may be functional for C/EBP expression.

To evaluate the influence of this specific CpG methylation on the activity of Sp1 binding to the C/EBP gene promoter, the Sp1 site was investigated by comparative electrophoretic mobility shift assay. C/EBP gene promoter-specific oligos containing an unmethylated or methylated specific CpG (Fig. 5) were incubated with U937 nuclear extracts, and the binding activity was compared. Fig. 5 shows that the signal of the shifted band is stronger with the unmethylated compared with the methylated probe. Incubation of the electrophoretic mobility shift assay reactions with an anti-Sp1 antibody supershifted the unmethylated probe consistent with Sp1-specific binding to the probe. Overall, these experiments indicate that demethylation of a specific CpG can increase Sp1 binding to C/EBP promoter.

DISCUSSION

In this study, we evaluated the effects of PUFAs, relative to saturated and monounsaturated fatty acids, on U937 promyelo-
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Cytic leukemia cells. PUFAs inhibited DNA synthesis and cell cycle progression and promoted changes in cell morphology by increasing size and granularity of U937 cells. At the same time, M-CSF receptor expression increased. M-CSF receptor, specifically induced in the early phase of monocyte differentiation commitment, is expressed on the monocyte-macrophage cell lineage, and as such, it is a useful marker to discriminate between monocyte and granulocyte progenitor cells and their differentiated progeny (39). Expression of the M-CSF receptor gene is under stringent control of both extracellular and intracellular stimuli and appears to occur primarily at the transcriptional level (40). M-CSF receptor mRNA levels are under the control of monocyte lineage-specific transcription factors such as PU.1 and C/EBPs; the activity and specificity of the M-CSF receptor promoter are mediated by a small DNA fragment containing binding sites for PU.1 and C/EBPs (30, 32). CEBP\(\alpha\), C/EBP\(\beta\), and C/EBP\(\delta\) can all bind to and activate the M-CSF receptor promoter, a function that has been ascribed to their highly similar basic leucine zipper domains (52). Moreover, studies on the differences in the binding affinities of recombinant C/EBPs to the M-CSF receptor promoter demonstrated that C/EBP\(\beta\) binding is relatively weaker than CEBP\(\alpha\) and C/EBP\(\delta\) binding (32).

We found that EPA treatment induced C/EBP\(\beta\) and C/EBP\(\delta\) expression and promoted C/EBP\(\beta\)/C/EBP\(\delta\) heterodimer binding to the same response element on the M-CSF receptor promoter, resulting in enhanced M-CSF receptor expression. In addition, EPA treatment induced an increase on c-\textit{jun} mRNA and protein levels. This last result is in agreement with previous data indicating that c-\textit{jun} and M-CSF receptor mRNA increase.

**FIGURE 3. Effect of fatty acids on M-CSF receptor expression.** A, U937 cells were treated with 100 \(\mu\text{M}\) fatty acids for 1, 3, and 24 h. mRNA content was evaluated using quantitative RT-PCR as described under “Experimental Procedures.” White bars, unsupplemented U937; gray bars, OA; black bars, EPA. Data are presented as relative expression by calculating \(2^{-\Delta\Delta\text{Ct}}\) normalized to untreated U937 cells. The means \(\pm\) S.D. (error bars) of three separate experiments are shown (*, \(p < 0.001\) versus U937 untreated cells). B, M-CSF receptor protein levels after 24 h of 100 \(\mu\text{M}\) fatty acid conditioning. Total cell lysates (50 \(\mu\text{g}\) of protein) were subject to Western blotting as described under “Experimental Procedures.” One representative of four experiments is shown. C, cells were treated with 100 \(\mu\text{M}\) fatty acids for 24 h. Chromatin immunoprecipitations were performed in untreated (white bars), OA-treated (gray bars), and EPA-treated (black bars) U937 cells. Antibodies against C/EBP\(\alpha\), C/EBP\(\beta\), C/EBP\(\delta\), and PU.1 were used. Real time PCR was performed using M-CSF receptor promoter-specific primers. The results shown are the mean \(\pm\) S.D. (error bars) of three independent experiments.
During monocyte differentiation of U937 cells (53). Interestingly, the M-CSF receptor promoter has no consensus binding site for c-Jun transcription factor (37). As a consequence, c-Jun does not directly bind to M-CSF receptor promoter, but it associates with PU.1 and C/EBPβ/H9252 via its basic domain (34, 37). Moreover, co-activator c-Jun recruitment by C/EBPβ/H9252 to DNA facilitates RNA polymerase II recruitment (34). c-Jun, PU.1, and C/EBPβ/H9252 have been shown to physically interact with each other and enhance the transcription of monocyte-specific genes via binding to their respective sites on DNA (33, 54). c-Jun expression has been shown to be differentially up-regulated during monocyte, but not granulocyte, differentiation of myeloid cell lines, implying a specific role for c-Jun expression in monocyte development (55). Our results confirm that there is not a single master myeloid transcription factor that alone governs myeloid lineage commitment, but multiple transcription factors work cooperatively and coordinately to regulate both temporal and lineage-specific genes. In this light, the simultaneous effects induced by EPA treatment on myeloid lineage-specific PU.1, C/EBPβ/H9252, C/EBPα/H9254, and c-Jun gene expression suggest a complex regulatory network.

FIGURE 4. Effect of EPA on C/EBPβ protein expression and methylation status in U937 cells.

A, Western blot analysis of C/EBPβ in U937 untreated cells (lane 1), cells after 2- (lane 2) and 5-day (lane 3) treatment with 1 μM 5-aza 2'-deoxycytidine, and cells after 24-h 100 μM EPA treatment (lane 4). Cell lysates (50 μg of protein) were loaded. One representative of three experiments is shown. Images of independent blots were acquired using the VersaDoc Imaging System, and signals were quantified using Quantity One software. The -fold change of C/EBPβ protein was compared with control U937 cells and was calculated after correction for β-tubulin loading differences. Data are the mean ± S.D. of three separate experiments.

B, cells were treated with 100 μM fatty acids for 24 h, and the methylated DNA levels of C/EBPβ CpG islands were quantified using the Methyl-Profiler qPCR Primer Assay as described under “Experimental Procedures.” The means ± S.D. (error bars) of three separate experiments are shown (*, p < 0.001 versus OA-treated or U937 untreated cells). C, C/EBPβ gene proximal promoter DNA sequence contains a CpG island. The underlined sequences indicate the forward and reverse nested primers utilized for cloning and sequencing after bisulfite reaction. The cloned fragment (350 bp) contains 32 CpGs (in bold). The arrow indicates the CpG demethylated nucleotide after EPA treatment. D, sequencing of the individual clones generated by PCR after bisulfite reaction. Black and white circles represent methylated and unmethylated CpGs, respectively.
Unmethylated Oligo

5’-cg ggg cgg ggc gtc cac gtc agc cgg ggc tag- 3’
3’-gc ccc ggc cgg cac gag cag tgg ggc cgc atc- 5’

Methylated Oligo

5’-cg ggg cgg ggc gtc cac gtc agc cgg ggc tag- 3’
3’-gc ccc ggc cgg cac gag cag tgg ggc cgc atc- 5’

CH3

U M +Sp1 Ab

FIGURE 5. Influence of demethylated CpG on Sp1 binding to C/EBPβ gene promoter. For the electrophoretic mobility shift assay, end-labeled double-stranded unmethylated and methylated oligonucleotides were incubated with U937 nuclear extracts. Sequences in bold italics represent the Sp1 consensus sequence. U, unmethylated oligonucleotides; M, methylated oligonucleotides; +Sp1 Ab, unmethylated oligonucleotides plus Sp1 antibody.

In the present study, both 5-aza-2’-deoxycytidine and EPA conditioning induced C/EBPβ protein expression (Ref. 43 and Fig. 4A). C/EBPβ-enhanced expression is ascribed to demethylation of a site-specific CpG downstream of the Sp1 site in the C/EBPβ proximal promoter (Fig. 4C) required for C/EBPβ basal transcriptional activation (58). Indeed, methylation of this specific CpG impaired Sp1 binding to its consensus sequence (Fig. 5). Our results are consistent with the site-specific CpG demethylation of C/EBPβ proximal promoter in primary breast cancer (59). Moreover, evidence demonstrating gene promoter site-specific methylation as a mechanism of tumor suppressor gene silencing in various types of cancer cells has been reported (60, 61). A single CpG demethylation appears to be the molecular event associated with a putative antitumor gene expression in prostate carcinogenesis (62). On this basis, we can assume that the CpG demethylation events are not all equal within a CpG island. Although it is generally accepted that high concentrations of demethylating agents induce the maximal gene reactivation (63), paradoxically, global demethylation may result in increased tumorigenicity (64). Moreover, a strong demethylation action prevents ascertaining whether any of the CpG dinucleotides exhibits differential susceptibility toward the methylation-demethylation process and hinders locating the critical regulatory sequences within CpG islands. We identified a CpG dinucleotide in the C/EBPβ proximal promoter as a key element that proved useful in identifying hypersensitive regions essential for gene regulation. This site may serve as a dynamic switch to activate the C/EBPβ gene through a possible chromatin conformation change.

Surprisingly, even 3-h EPA treatment was able to significantly induce C/EBPβ, C/EBPδ, PU.1, c-jun, and M-CSF receptor mRNA level increases (Figs. 2B and 3A). These results led us to conclude that the site-specific C/EBPβ promoter demethylation occurred within 3 h, suggesting the involvement of some active demethylation mechanism(s) occurring in the absence of DNA replication (the cell cycle is ~24 h in these cells). The existence of demethylating enzymes has been previously postulated when rapid demethylation of genes occurs (65, 66). The effects induced by EPA treatment on PU.1, C/EBPβ, C/EBPδ, c-Jun, and M-CSF receptor protein levels are similar to the effects of LNA, AA, and DHA treatments. On this basis, a potentially similar mechanism for all PUFA conditioning cannot be excluded. Although the molecular mechanisms underlying the CpG site-specific demethylation process and the potential common PUFA mechanisms need to be investigated, our findings provide for the first time evidence of a tight correlation among C/EBPβ site-specific CpG demethylation, M-CSF receptor expression, and the beginning of the differentiation program induced by EPA in U937 leukemia cells.

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