Research Article

Peroxisome Proliferator-Activated Receptor γ–Mediated Up-regulation of Syndecan-1 by n-3 Fatty Acids Promotes Apoptosis of Human Breast Cancer Cells

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Abstract

Diets enriched in n-3 polyunsaturated fatty acids (n-3 PUFA) may protect against breast cancer but biochemical mechanisms are unclear. Our studies showed that the n-3 fatty acid docosahexaenoic acid (DHA) up-regulated syndecan-1 (SDC-1) in human breast cancer cells, and we tested the hypothesis that DHA-mediated up-regulation of SDC-1 induces apoptosis. DHA was delivered to MCF-7 cells by n-3 PUFA–enriched low-density lipoproteins (LDL) or by albumin in the presence or absence of SDC-1 small interfering RNA. The n-3 PUFA induced apoptosis, which was blocked by SDC-1 silencing. We also confirmed that SDC-1 up-regulation and apoptosis promotion by n-3 PUFA was mediated by peroxisome proliferator-activated receptor γ (PPARγ). Using a luciferase gene driven by either a PPAR response element or a DR-1 site present in the SDC-1 promoter, reporter activities were enhanced by n-3 LDL, DHA, and PPARγ agonist, whereas activity of a luciferase gene placed downstream of a mutant DR-1 site was unresponsive. Cotransfection with dominant-negative PPARγ DNA eliminated the increase in luciferase activity. These data provide strong evidence that SDC-1 is a molecular target of n-3 PUFA in human breast cancer cells through activation of PPARγ and that n-3 PUFA–induced apoptosis is mediated by SDC-1. This provides a novel mechanism for the chemopreventive effects of n-3 PUFA in breast cancer. [Cancer Res 2008;68(8):2912–9]

Introduction

Although still controversial, several human epidemiologic studies have shown that a high dietary intake of fish is associated with a lower incidence of cancers, including breast cancer (1–3). The omega-3 polyunsaturated fatty acids (n-3 PUFA) contained in fish oil are proposed to be the effectors of this protection. The two main PUFA constituents are eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which are converted by fish from α-linolenic acid (α-LNA; 18:3n-3) of ingested cold water vegetation. Animal studies clearly support the idea that dietary supplementation with fish oil or its constituent fatty acids not only slows the growth of both xenograft (4, 5) and chemically induced tumors (6, 7) but also increases sensitivity to chemotherapy (8, 9) and inhibits metastases (4, 5). DHA in particular was shown to be a potent enhancer of tumor cell chemosensitivity (10, 11). Biochemical mechanisms for the antitumor properties of n-3 PUFA are still unclear but may include changes in eicosanoid metabolism, reduction in oncogene expression, and modulation of lipid peroxidation (reviewed in refs. 12, 13).

It is known that fatty acids can regulate gene transcription. We have previously reported that the transmembrane heparan sulfate proteoglycan syndecan-1 (SDC-1) is up-regulated by n-3 PUFA (14). SDC-1 has been shown to act as a tumor suppressor molecule by inhibition of cell growth and induction of apoptosis (15, 16). Proteolytic cleavage of the SDC-1 core protein at the cell surface generates a soluble ectodomain that retains antigrowth properties that depend on the presence of both core protein and heparan sulfate glycosaminoglycan chains (15, 16). Growth inhibition has been shown to require close proximity of SDC-1 ectodomain to the cell surface and to involve delay of cell G_1-S progression and down-regulation of cyclin D1 (16). Although there are inconsistent reports (17–19), the expression of SDC-1 is generally down-regulated in malignant tumors (20, 21), and lower levels of expression have been associated with high metastatic potential in malignant mesothelioma (22), invasive esophageal cancer (23), head and neck cancer (24), lung cancer (25), and laryngeal cancer (26). SDC-1 expression was markedly reduced in squamous cell carcinoma and there was an inverse relationship between the level of SDC-1 expression and tumor aggressiveness (27). Furthermore, survival rate was decreased in patients with SDC-1–negative colorectal cancer (28) and was inversely associated with SDC-1 levels in gastric cancer (29). *In vitro* studies have shown that SDC-1 inhibits the invasion of tumor cells into type 1 collagen (30, 31) and that expression of SDC-1 in myeloma cells suppresses matrix metalloproteinase-9 (32). A reduction in SDC-1 promoted normal murine mammary epithelial-mesenchymal transition (31). Therefore, the decrease of SDC-1 expression may be an important step from tumorigenesis to the metastatic phenotype. However, there is little information on factors that regulate SDC-1.

Insight into a potential mechanism underlying the effects of n-3 PUFA on SDC-1 gene expression was suggested by the finding that the proximal promoter of the *SDC-1* gene contains a specific sequence termed a DR-1 element (33). This sequence is recognized by several members of the nuclear hormone receptor superfamily, including peroxisome proliferator-activated receptors (PPAR), which participate in activating the basic transcriptional machinery to regulate target genes. Fatty acids are known to bind and activate PPARs (34–36). There are three PPAR subtypes (α, β/δ, and γ) with different tissue distributions and physiologic involvements. Processes such as lipid and glucose homeostasis, inflammation, cell growth and differentiation, and apoptosis have all been shown as regulatory targets for the PPARs (reviewed in refs. 37, 38). In human breast cancer cell lines, the activation of PPARα (39) as well as

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PPARβ/δ (40) stimulated proliferation, whereas ligands for PPARγ were growth inhibitory (41, 42).

In the human breast cancer cell line MCF-7, n-3 PUFA not only inhibited cell proliferation and increased apoptosis but also promoted SDC-1 expression (14, 43). The SDC-1 response was mimicked by the PPARγ agonist troglitazone and blocked by a PPARγ antagonist. Present studies were undertaken to show a novel pathway connecting n-3 PUFA, PPARγ regulation of SDC-1, and apoptosis. An important consideration in these studies was the delivery of n-3 PUFA to the cells by a method designed to model the *in vivo* process. Tumor cells obtain dietary PUFA by two major pathways: uptake of fatty acid-albumin complexes that form after lipolysis of triglyceride-rich lipoproteins and receptor-mediated endocytosis of low-density lipoproteins (LDL). Therefore, we have used LDL isolated from the plasma of monkeys fed a diet supplemented with fish oil to examine a mechanism for n-3 PUFA–induced apoptosis.

Materials and Methods

Preparation of LDL and fatty acids-bovine serum albumin complexes. LDLs were isolated from plasma of adult African green (vervet) monkeys fed n-3 PUFA (fish oil)-enriched diets for 3 to 5 y. Diets, maintenance, and clinical measurements of the animals are published (43). Animal care and procedures were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine, and animals were maintained by standard animal care procedures based on institutional guidelines. LDL isolation and characterization procedures have been previously described (43). To measure EPA and DHA concentrations in n-3 LDL preparations, 100 μL LDL was added to 20.6 μg C17:0 (as triheptadecanoate glyceride) and then saponified, extracted, and derivatized by standard procedures. LDL fatty acid methyl esters were quantified by gas-liquid chromatography, and EPA and DHA concentrations were calculated relative to C17:0. Based on these measurements, in all experiments when cells were treated with 100 μg/mL n-3 LDL, they were exposed to final concentrations of 52 μmol/L EPA and 21 μmol/L DHA from the LDL. EPA and DHA were purchased as sodium salts (Sigma Chemical Co.). Fatty acid–free bovine serum albumin (BSA; Sigma) was prepared as a 125 μmol/L solution in DMEM/Ham's F12. Fatty acid salts were solubilized to 600 μmol/L stocks in the BSA medium as described (4:1 ratio of fatty acid to BSA) and stored in aliquots at −20°C under argon (43). Tandem mass spectroscopy analysis of the stock preparation of DHA showed <15% peroxidation products. With regard to DHA stability, over a 48-h treatment of cells, the DHA (as percentage of cell phospholipids) increased from 2.0 (time 0) to 3.7 (12 h), 5.7 (24 h), and 3.5 (48 h), indicating reasonable persistence over the time periods studied.

Preparation of SDC-1 ectodomain. The SDC-1 ectodomain expression construct was designed to encode a COOH-terminal polyhistidine fusion protein and was created using a two-step cloning process as follows. The SDC-1 ectodomain cDNA was amplified by PCR using the SDC-1 plasmid (OriGene Technologies, Inc.) as the source of template, 5′-gcagaattcggcagcatgaggcgcgcggcgctct-3′ as the forward primer, and 5′-gcaggatcctttcctgtccaggaggccctgtga-3′ as the reverse primer. The resulting cDNA was cut with *Bam*HI and *Eco*RI restriction endonucleases and ligated into the pTcam4 expression plasmid (44). In the second round of PCR, 5′-gcaaagcttgaattcggcagcatgaggcgcgcg-3′ (forward) and 5′-gcaaagcttgaattcggcagcatgaggcgcgcg-3′ (reverse) primers were used to amplify the SDC-1 ectodomain cDNA from the modified pTcam4 plasmid. The amplified cDNA was treated with *Hin*dIII and *Xho*I and subsequently ligated into the pCEP4 (Invitrogen Corp.) expression plasmid. The resulting plasmid was transfected into 293-EBNA cells and stable expressing cells selected by the addition of 200 μg/mL hygromycin B. The cells were then amplified and grown to confluence in T175 flasks. On confluence, the culture medium was replaced with serum-free DMEM. Medium was then collected every 48 h and fresh medium was added back to the cells. Under these conditions, the transfected cells remain viable for several weeks, allowing for the collection of conditioned medium over an extended period of time. After collecting several liters of conditioned medium, recombinant SDC-1 ectodomain was purified using the following procedure. Conditioned medium was first concentrated using a Millipore Pellicon 2 tangential flow system (Millipore Corp.). Concentrated conditioned medium was then subjected to anion exchange chromatography using an Amersham Biosciences ÄKTAexplorer chromatography system equipped with a 5-mL HiTrap Q column and bound SDC-1 ectodomain eluted with a linear gradient of 0 to 2 mol/L sodium chloride in a buffer consisting of 20 mmol/L Tris and 0.2% CHAPS (pH 8.0).

Cell culture. MCF-7 cell line was obtained from the American Type Culture Collection and maintained in DMEM/F12 supplemented with 5% fetal bovine serum (FBS), 10 mg/mL porcine insulin (Sigma), penicillin/streptomycin, and L-glutamine at 37°C in 5% CO_2. In experiments measuring mRNA, cleaved poly(ADP-ribose) polymerase (PARP) levels, and PPARγ protein synthesis, cells were seeded in six-well plates at a density of 5×10^5 per well in growth medium. After 6 h, the medium was changed to DMEM/F12 with 0.5% FBS for 18 h to up-regulate LDL receptors. PPARγ protein was determined after medium supplementation with 100 μg/mL n-3 LDL, 30 μmol/L DHA, 30 μmol/L EPA, or 5 μmol/L troglitazone (Cayman Chemical) for 12 and 48 h.

Apoptosis assays. MCF-7 cells were seeded in six-well plates at a density of 5×10^5 per well in DMEM/F12 with 0.5% FBS for 18 h and then exposed to n-3 LDL, DHA, EPA, or troglitazone for 48 h or to SDC-1 ectodomain for 72 h. The SDC-1 small interfering RNA (siRNA)-transfected cells were also exposed to n-3 LDL, DHA, or troglitazone for 48 h. Apoptotic activity in adherent cells was determined by cleaved PARP using Western blots. Alternatively, apoptosis was measured with the Caspase-Glo 3/7 Assay in which 30 μL of Caspase-Glo 3/7 reagent were added to each well and incubated for 1 h at room temperature. Luminescence was measured by a Reporter Microplate Luminometer (Turner Biosystems). Treated cells were also stained with trypan blue (Invitrogen), and percentage of dead cells was counted by hemocytometer.

Real-time PCR. RNA isolation and cDNA synthesis and real-time reverse transcription-PCR (RT-PCR) were as previously described (14). SDC-1 primers were 5′-ggagcaggacttcacctttg (forward) and 5′-ctcccagcacctctttcct (reverse). Peptidyl prolyl isomerase B (PPIB) housekeeping gene primers were 5′-gcccaaagtcaccgtcaa (forward) and 5′-tccgaagagaccaaagatcac (reverse). Care was taken to design primers so as to minimize amplification from any contaminating genomic DNA. The forward SDC-1 primer spans an exon/intron junction. The PPIB primers are on different exons separated by an ∼800 bp intron. Under the conditions of real-time PCR, we detected a single PPIB product of 82 bp by gel electrophoresis, and melt curve analyses performed at the end of the real-time PCR reproducibly show a single peak. SDC-1 data were normalized to the housekeeping control PPIB and are presented relative to control.

Western blot analysis. Cells were washed twice with ice-cold PBS and lysed for 10 min on ice; debris was then removed by centrifugation and equivalent amounts of protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membranes were blocked with TBST [10 mmol/L Tris-base, 100 mmol/L NaCl, 0.1% Tween 20 (pH 7.5)] containing 5% nonfat dry milk for 2 h at room temperature and then washed thrice with TBST for 5 min, exposed to anti-PARP (Cell Signaling Technology) and anti-PPARγ (Abcam) antibodies in TBST containing 3% BSA at 4°C overnight followed by three washes with TBST, and then incubated with horseradish peroxidase–conjugated secondary antibody for 1 h, washed with TBST, and developed using the SuperSignal West Pico kit (Pierce). The band densities on photographic films were analyzed using Quantity One software. For PARP cleavage measurement, band densities were normalized to β-actin and presented as the fractional change from control values.

SDC-1 siRNA transfection. Three SDC-1 siRNAs, 12432, 12527, and 142557, were purchased from Ambion. For transfection, 50 nmol/L siRNA was added to 2.0×10^5 cells in six-well plates using Lipofectamine 2000 (Invitrogen) and DMEM/F12 lacking serum and antibiotics. Control cells were transfected with a negative control siRNA with no known mRNA target designed by Ambion. At 6 h after transfection, each well was supplemented

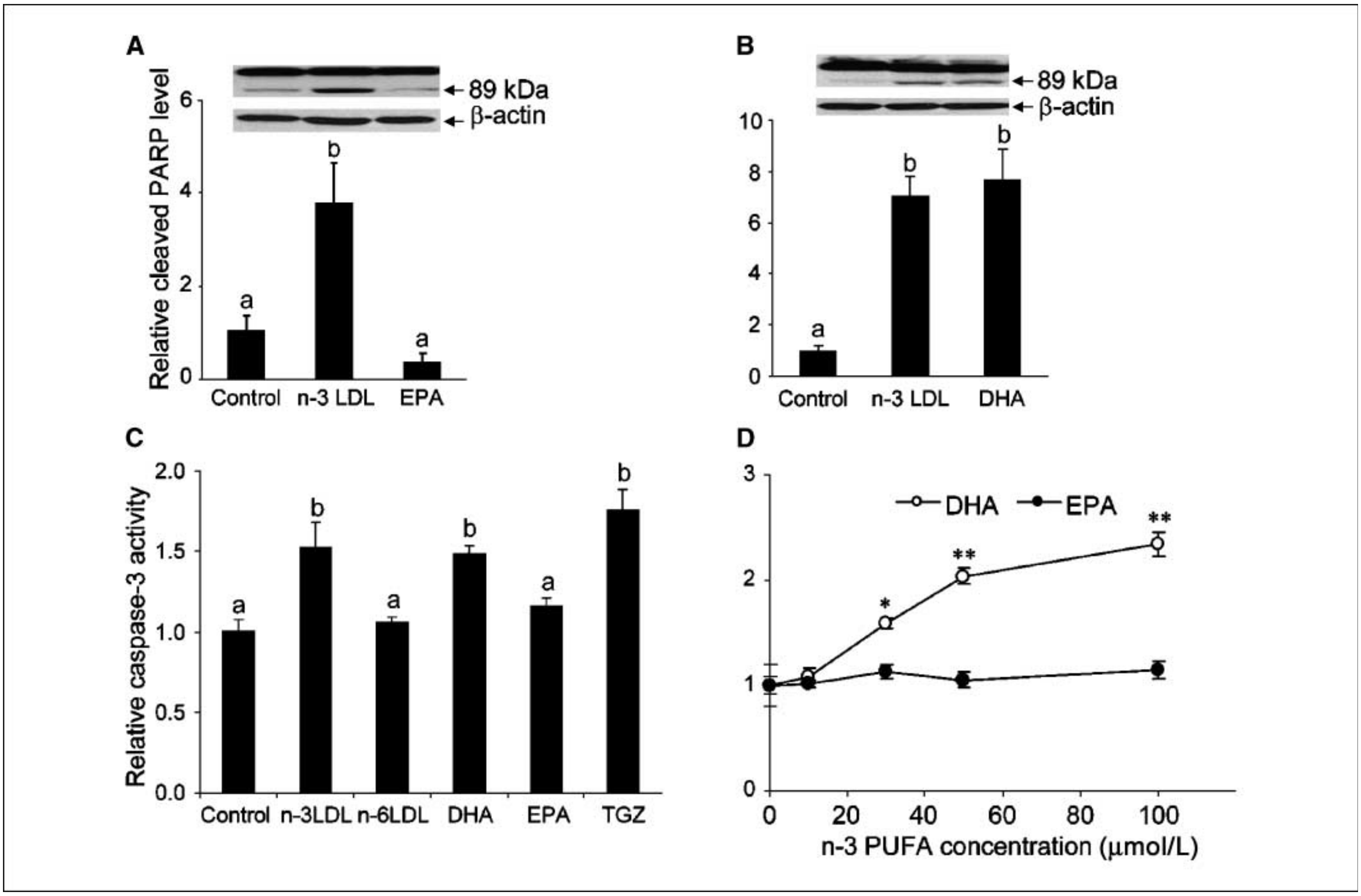

Figure 1. Effects of n-3 PUFA on apoptosis of MCF-7 cells. Cells were preincubated in medium containing 0.5% FBS for 18 h and then medium was supplemented with n-3 LDL (100 μg/mL) and DHA or EPA (30 μmol/L fatty acid, 7.5 μmol/L BSA) for 48 h. *A* and *B,* measurement of apoptosis by Western blot identifying the 89-kDa PARP cleavage product and graphical presentation of intensity of 89-kDa band relative to β-actin. *C* and *D,* measurement of apoptosis by Caspase-Glo 3/7 Assay in MCF-7 cells treated for 48 h with n-3 LDL, n-6 LDL, DHA-BSA, or EPA-BSA. *TGZ,* troglitazone. In *D*, the 4:1 molar ratio of fatty acid to BSA was maintained for each dose. *Points,* mean of experiments performed in triplicate; *bars,* SE. Values with different letters are significantly different ($P < 0.05$). *, $P < 0.05$; **, $P < 0.001$.

with 2 mL of complete growth medium. Forty-eight hours after transfection, the medium was replaced with 2 mL of DMEM/F12 containing 0.5% FBS and cells were treated with n-3 LDL (100 μg/mL), DHA (30 μmol/L), and troglitazone (5 μmol/L) for an additional 48 h. Cells were prepared for Western blot analysis as described above. Data are shown using SDC-1 siRNA 142557. Similar results were obtained with siRNA 12432, whereas siRNA 12527 failed to silence SDC-1 mRNA and did not inhibit n-3 PUFA–induced apoptosis.

Wild-type and mutant SDC-1 DR-1-luciferase constructs. The one-copy reporter construct DR-1 from the SDC-1 promoter [pGL3-(DR-1)WT; ref. 33] was generated by annealing the oligonucleotides 5′-cggttcttcccc-tttgctctctcggccgtttccgctacacccgagct-3′ and 5′-cgggtgtagcggaaacggccgaga-gagcaaaggggaagaaccggtac-3′ before ligation into *Kpn*I- and *Sac*I-digested pGL3-promoter vector (Promega). The one-copy mutant DR-1 reporter construct [pGL3-(DR-1)] was generated using the same method and the oligonucleotides 5′-cggttctAGTActGCcCTcactcggccgtttccgctacacccgagct-3′ and 5′-cgggtgtagcggaaacggccgagTgagGGCagTACTagaaccggtac-3′. Mutations are capitalized.

Transfection and luciferase assay. MCF-7 cells were cotransfected with the PPAR response element (PPRE)-TK-luciferase reporter plasmid and a control plasmid containing the *lacZ* gene or with pcDNA3-dominant-negative (d/n) PPARγ cDNA (L468/E471, kindly provided by Dr. V Chattergee, University of Cambridge, Cambridge, United Kingdom) and pGL3-luc-DR-1 (wild-type or mutant) using FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. Transfected cells were incubated with 0 μg/mL n-3 LDL, 100 μg/mL n-3 LDL, 30 μmol/L DHA, and 5 or 10 μmol/L of troglitazone for 8 or 24 h. Luciferase activities were measured using a luciferase assay kit (Promega) in a TD-20e luminometer (Turner Biosystems). β-Galactosidase activity was measured using β-Galactosidase Enzyme Assay kit (Promega) in SpectroMAX. Luciferase activities were normalized to β-galactosidase activity and are presented as the percentage of luciferase activity measured in the presence of stimulus relative to the activity of control cells with no stimulation.

Data analysis. Data were analyzed by ANOVA and Student's *t* test. The assays were carried out in triplicate and the averages are shown together with SE.

Results

DHA induces apoptosis in MCF-7 cells. We have previously shown that n-3 PUFA–enriched LDL, but not n-6 PUFA–enriched LDL, can induce apoptosis in MCF-7 cells (43). The n-3 LDL was shown to contain two predominant species of n-3 PUFA, EPA and DHA, and both were effectively delivered to the cells by the LDL. To clarify the role of the individual n-3 PUFA in apoptosis induction, MCF-7 cells were incubated with n-3 LDL, EPA-BSA, or DHA-BSA for 48 h and apoptosis was measured by PARP cleavage. Consistent with previous observations, n-3 LDL caused a 4- to 7-fold increase in PARP cleavage product compared with control cells (Fig. 1*A* and *B*). EPA-BSA was not effective in induction of apoptosis (Fig. 1*A*), whereas a similar concentration of DHA-BSA resulted in an increase in PARP cleavage similar to that of n-3 LDL (Fig. 1*B*). Apoptosis was confirmed in cells treated with n-3 LDL and DHA by Caspase-Glo 3/7 Assay (Fig. 1*C* and *D*). As shown in Fig. 1*D*, apoptosis induction by DHA was dose dependent, whereas no apoptotic effect was observed with EPA at similar doses for 48 h or in cells treated for up to 72 h (data not shown).

SDC-1 induces apoptosis in MCF-7 cells. Consistent with the effect on apoptosis shown in Fig. 1, previous studies have shown that both n-3 LDL and DHA-BSA significantly up-regulated the expression of SDC-1 in MCF-7 cells (14). To test the hypothesis that n-3 PUFA–induced apoptosis in MCF-7 cells is mediated by SDC-1, studies were first conducted to show that SDC-1 is a proapoptotic stimulant in this model system. Cells were cultured for 72 h in the presence of human recombinant SDC-1 ectodomain, which resulted, as shown in Fig. 2*A*, in a dose-dependent increase in apoptosis of the cells as measured by PARP cleavage. To confirm that SDC-1 is a mediator in n-3 LDL–induced and DHA-induced apoptosis of MCF-7 cells, a SDC-1 siRNA was transfected into the cells to silence SDC-1 expression. This reagent effectively inhibited SDC-1 mRNA for up to 96 h after transfection (Fig. 2*C*). As shown in Fig. 2*B*, the SDC-1 siRNA blocked the ability of n-3 LDL and DHA to enhance PARP cleavage, which was markedly stimulated in cells transfected with a control siRNA. It was also apparent that the level of intact PARP was different in cells treated with SDC-1 and control siRNA but further studies are required to determine the significance of this. In any case, the absolute as well as relative levels of PARP cleavage stimulated by n-3 PUFA were blocked by SDC-1 silencing and the ratio of cleaved to intact PARP was increased by n-3 LDL and DHA only in the presence of the nontargeting siRNA (0.26 ± 0.06 and 0.24 ± 0.05 for control cells; 0.52 ± 0.07 and 0.27 ± 0.05, $P = 0.03$, for n-3 LDL–treated cells; 0.44 ± 0.06 and 0.13 ± 0.07, $P = 0.02$, for DHA-treated cells in the presence of nontargeting and SDC-1 siRNA, respectively). Furthermore, a trypan blue exclusion assay confirmed that the SDC-1 siRNA inhibited n-3 PUFA–induced cell death (Fig. 2*D*).

PPARγ is a key factor in DHA or n-3 LDL up-regulation of SDC-1 expression. In previous studies, we have shown that the PPARγ agonist troglitazone was an effective stimulator of SDC-1 expression, whereas the PPARδ agonist GW610742 had no effect. Moreover, the PPARγ antagonist GW259662 blocked the effect of n-3 LDL on SDC-1 expression (14). To directly implicate PPARγ in n-3 LDL and DHA regulation of SDC-1, MCF-7 cells were transfected with a d/n PPARγ cDNA or its vector pcDNA3. Figure 3*A* shows a marked overexpression of PPARγ protein in the transfected cells. We then assessed the activity of the d/n PPARγ construct by determining SDC-1 mRNA level after stimulation with n-3 LDL and DHA for 8 h (Fig. 3*B*). In cells transfected with the empty vector, both n-3 LDL and DHA up-regulated SDC-1 expression. No such

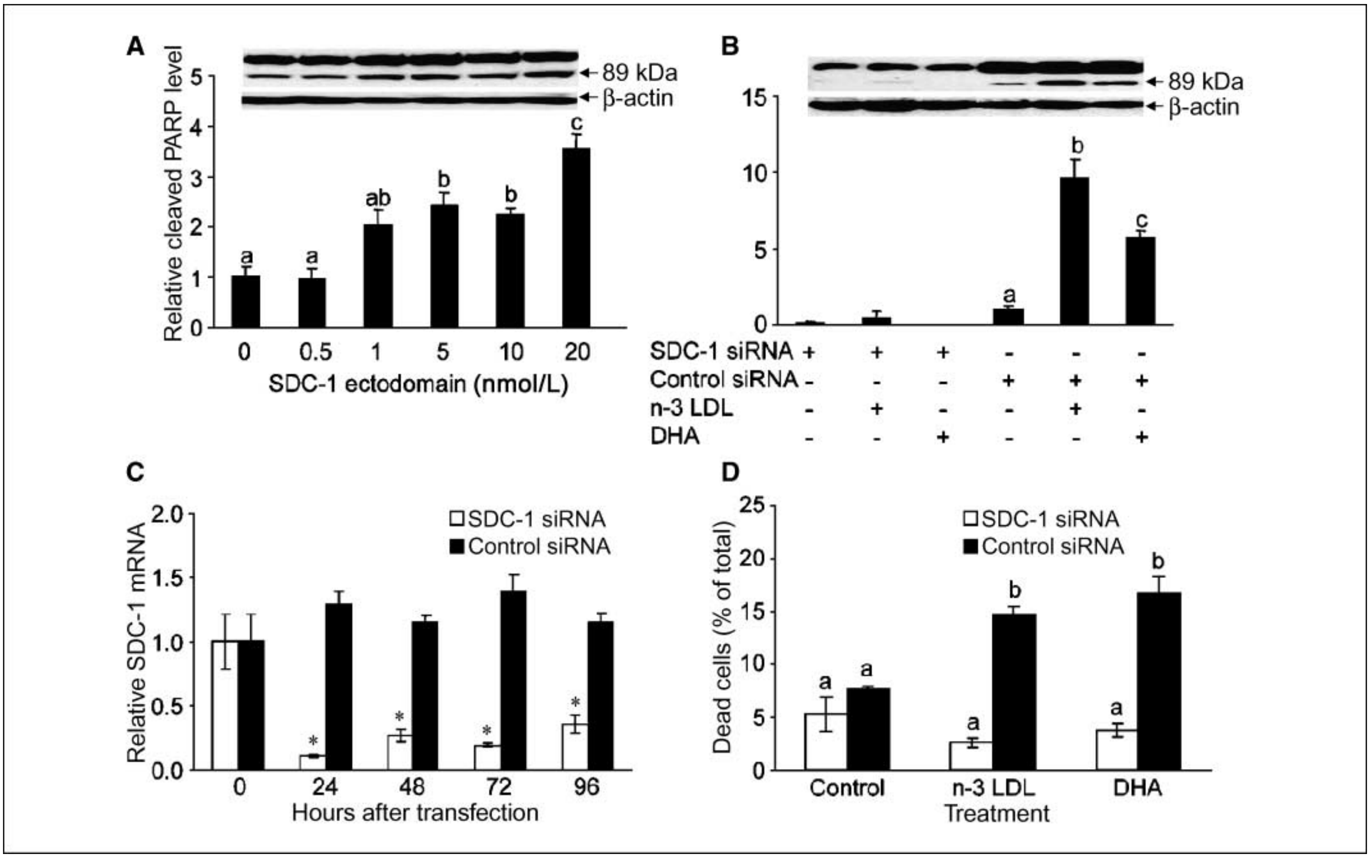

Figure 2. SDC-1 induces apoptosis of MCF-7 cells. *A,* cells were incubated in medium containing indicated concentrations of SDC-1 ectodomain for 72 h. *B,* cells were transfected with SDC-1 siRNA or negative control siRNA for 48 h and then incubated with n-3 LDL (100 μg/mL) and DHA (30 μmol/L fatty acid, 7.5 μmol/L BSA) for 48 h. Apoptosis was measured by PARP Western blot analysis. Data are presented relative to control. *Columns,* mean of three independent experiments performed in triplicate; *bars,* SE. *C,* MCF-7 cells were transfected with SDC-1 siRNA or negative control siRNA for the indicated times and SDC-1 mRNA was measured by real-time RT-PCR. *D,* cells transfected with SDC-1 siRNA or negative control siRNA were assayed for percentage cell death by trypan blue exclusion assay. Values with different letters are significantly different ($P < 0.05$). *, $P < 0.05$.

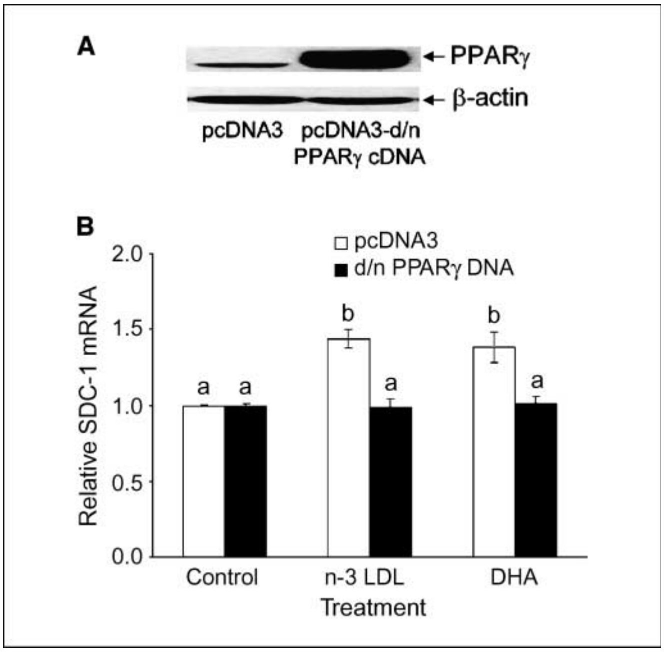

Figure 3. d/n PPARγ cDNA blocks the enhancement effect of n-3 LDL and DHA on SDC-1 expression. MCF-7 cells were transfected with pcDNA3-d/n PPARγ cDNA or empty d/n PPARγ cDNA vector (*pcDNA3*). *A,* PPARγ protein was measured at 12 h by Western blot analysis. *B,* transfected cells were treated with n-3 LDL (100 μg/mL) or DHA-BSA (30 μmol/L fatty acid, 7.5 μmol/L BSA) for 8 h. Total RNA was isolated and SDC-1 mRNA was determined by real-time RT-PCR. *Columns,* mean of triplicate experiments; *bars,* SE. Values with different letters are significantly different ($P < 0.05$).

response occurred in cells transfected with the vector encoding d/n PPARγ cDNA. These data indicate that n-3 LDL and DHA up-regulated SDC-1 expression through PPARγ.

PPARγ activation promotes apoptosis in MCF-7 cells. The observations thus far have indicated that n-3 LDL and DHA induce apoptosis of MCF-7 cells through up-regulation of SDC-1 in a PPARγ-activated transcriptional pathway. The next question addressed the ability of PPARγ to induce apoptosis in these cells. To examine this, cells were treated with the PPARγ agonist troglitazone and cleaved PARP was measured by Western blot analysis. As shown in Fig. 4*A*, a dose-dependent increase in cleavage product was observed in response to troglitazone treatment. Thus, the PPARγ ligand has a similar effect to n-3 PUFA in promoting apoptosis of the breast cancer cells. To explore whether the activated PPARγ induced apoptosis of MCF-7 cells through SDC-1, the cells were transfected with SDC-1 or control siRNA and then were cultured in the presence of 5 μmol/L troglitazone for 48 h (Fig. 4*B* and *C*). The apoptotic effect of troglitazone was inhibited when SDC-1 expression was silenced. These data indicate a critical involvement of SDC-1 in the promotion of apoptosis by n-3 PUFA, troglitazone, and the common target of these agents, PPARγ.

PPARγ is activated by n-3 PUFA and regulates SDC-1 transcription via the DR-1 site. To further clarify the mechanism whereby the PPARγ pathway mediates n-3 PUFA up-regulation of SDC-1, effects on PPARγ expression were examined. Treatment with n-3 LDL and DHA had no effect on PPARγ mRNA level in the cells, and furthermore, Western blot analysis showed that the level of PPARγ protein expression did not differ between control-treated and n-3 PUFA–treated cells or in cells treated with SDC-1 siRNA (data not shown). Thus, it seemed that activation rather than overexpression of PPARγ by n-3 PUFA resulted in up-regulation of SDC-1. To determine if n-3 LDL and DHA could activate a PPAR transcriptional activity, MCF-7 cells were cotransfected with a PPRE-TK-luciferase reporter plasmid and a control plasmid containing the *lacZ* gene. Luciferase activity was determined after treatment with n-3 LDL or n-6 LDL (100 μg/mL), DHA or EPA

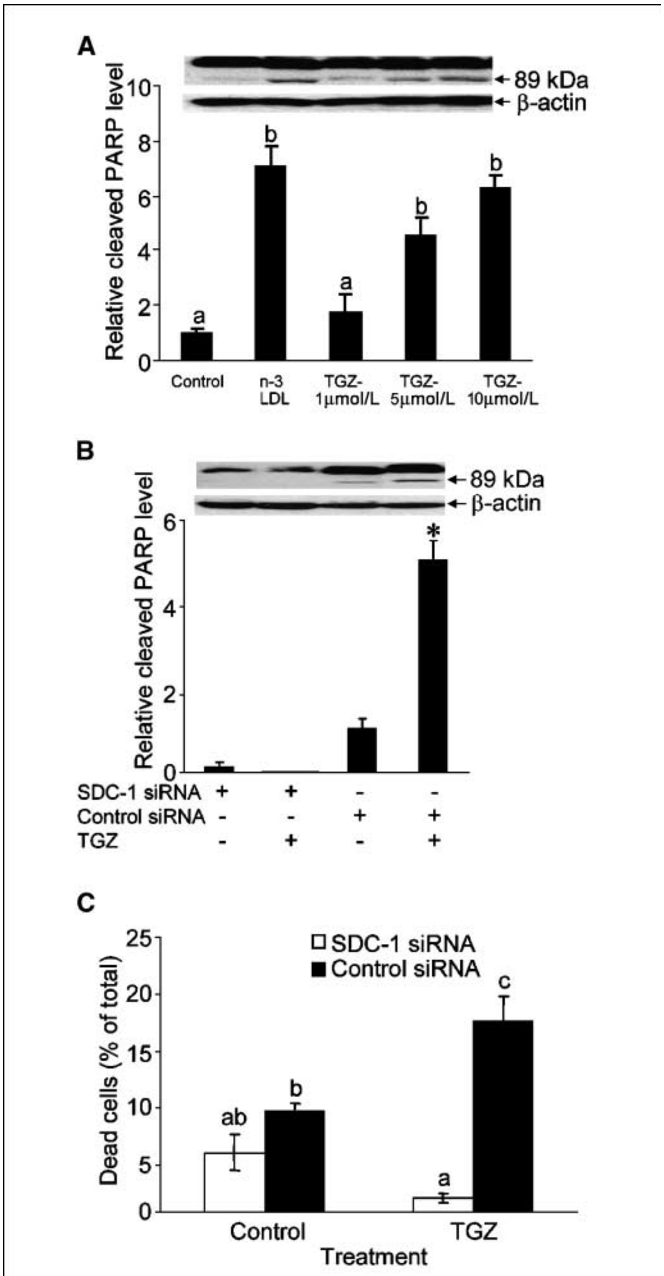

Figure 4. PPARγ-induced apoptosis of MCF-7 cells is eliminated by SDC-1 siRNA. *A,* cells were treated with 100 μg/mL n-3 LDL and 1, 5, or 10 μmol/L of PPARγ agonist troglitazone for 48 h, and apoptosis was measured by PARP Western blot analysis. *B* and *C,* cells transfected with SDC-1 siRNA or negative control siRNA were incubated with 5 μmol/L troglitazone for 48 h, and cleaved PARP levels were compared by Western blot analysis (*B*) or percentage cell death was measured by trypan blue exclusion assay (*C*). *Columns,* mean of triplicate experiments; *bars,* SE. Values with different letters are significantly different ($P < 0.05$). *, $P < 0.05$.

(30 μmol/L), or troglitazone (5 μmol/L) as a positive control. DHA, n-3 LDL, and troglitazone significantly increased luciferase activity, indicating a PPAR response that was blocked by cotransfection with a d/n PPARγ cDNA (Fig. 5*A*). To confirm the role of PPARγ in transcriptional regulation of SDC-1, the luciferase reporter gene was placed under the control of either the wild-type [pGL3-(DR-1)WT] or mutant [pGL3-(DR-1)Mut] DR-1 element from the SDC-1 promoter. MCF-7 cells were cotransfected with wild-type or mutant SDC-1 DR-1-luciferase constructs and a d/n PPARγ cDNA. The result showed that pGL3-(DR-1)WT-luciferase reporter was activated by n-3 LDL, DHA, and troglitazone. Cotransfection of the same reporter with d/n PPARγ DNA blocked luciferase activity (Fig. 5*B*). In addition, the transfected reporter gene containing mutant SDC-1 DR-1 site completely abolished the effect of n-3 LDL, DHA, and troglitazone. No significant effect on luciferase activity was observed when the empty pGL3-promoter vector was transfected into MCF-7 cells and treated with n-3 LDL, DHA, or troglitazone (Fig. 5*B*). These data establish SDC-1 as a molecular target for n-3 PUFA and PPARγ.

Discussion

These studies have identified a novel pathway for the chemopreventive properties of n-3 PUFA in human breast cancer cells. The most striking finding is that SDC-1, a key molecular target of n-3 LDL and DHA, is a critical player in the ability of n-3 PUFA to induce apoptosis in these cells. When SDC-1 expression was silenced by siRNA, the ability of n-3 PUFA to induce apoptosis was blocked. In addition, present studies have extended previous observations (14) by more clearly defining the role of PPARγ in the n-3 PUFA up-regulation of SDC-1 expression. The studies reported here delineate the pathway linking n-3 PUFA to cellular apoptosis as follows: the n-3 PUFA DHA activates the nuclear transcription factor PPARγ, which results in transcriptional up-regulation of the SDC-1 target gene, and the SDC-1 protein induces cell apoptosis.

Throughout these studies, we modeled two physiologic processes of fatty acid delivery to the cells: by LDL and by albumin. Our previous studies have shown that LDL, isolated from the plasma of vervet monkeys fed a diet enriched in fish oil, contains two major n-3 PUFA species: EPA and DHA (43). Incubation of MCF-7 cells with this LDL showed that both EPA and DHA were efficiently delivered to the cells and detectable in cell phospholipids by 24 h (43) and as early as 8 h.[4] Proliferation of MCF-7 cells was inhibited by n-3 LDL, which also induced apoptosis in the cells (43). Neither of these effects was achieved by n-6 PUFA–enriched LDL. What was puzzling was that EPA-BSA was ineffective in apoptosis induction, a result confirmed by present studies. Evidence now indicates that, by contrast, DHA-BSA is very effective in induction of apoptosis and that DHA is likely to be the apoptotic stimulus in the n-3 LDL.

DHA is synthesized from EPA by elongation and desaturation. However, the conversion is not a straightforward one (see ref. 45 for review). Specifically, EPA (20:5, n-3) is converted to docosapentaenoic acid (22:5, n-3) by elongase (Elovl)-5, and by Elovl-2 to 24:5, n-3. The next step requires desaturation of the 24:5 at the Δ6 position to produce 24:6, n-3. This product is translocated from the endoplasmic reticulum to the peroxisome where the β-oxidation pathway exerts an acyl chain shortening of C2 to produce DHA (22:6, n-3). Although our previous studies have shown that there is some conversion of EPA to DHA in the MCF-7 cells, the inability of EPA to induce apoptosis suggests either that the pool of DHA derived from EPA may be less available to produce an apoptotic response in the cell or that a critical threshold level of DHA needed for activity cannot be achieved by this pathway. Our recent unpublished studies have shown that, over a 48-h treatment with EPA or DHA, the percentage DHA in MCF-7 cell lipids changes from 1.8 at time 0 to 2.1 and 3.7, respectively, at 48 h. It may be important to note that, in humans, studies have consistently shown that increased consumption of either EPA or its precursor α-LNA (18:3, n-3) does not result in a detectable increase in plasma DHA, thus highlighting the inefficiency of conversion in this pathway (46–48). Further studies are ongoing to clarify the metabolism of n-3 PUFA in relation to apoptosis in these cells. One likely possibility is that DHA is metabolized to a more active component that becomes a key player in the pathway that we have identified.

We specifically showed effects of n-3 LDL and DHA on the level and function of SDC-1. DHA and n-3 LDL significantly up-regulated

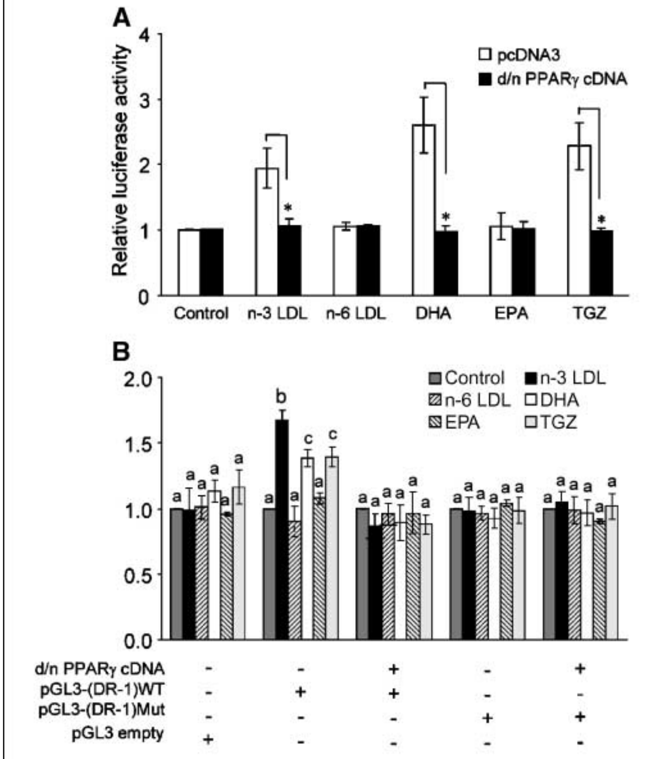

Figure 5. Activation of the human SDC-1 promoter by PPARγ activated by n-3 LDL or DHA. *A,* luciferase activity in MCF-7 cells cotransfected with luciferase driven by a PPAR response element (PPRE×3-TK-luciferase reporter) and a control plasmid containing the *lacZ* gene. Cells were transfected with pcDNA3-d/n PPARγ cDNA or empty d/n PPARγ cDNA vector (*pcDNA3*) and treated with n-3 LDL or n-6 LDL (100 μg/mL), DHA-BSA or EPA-BSA (30 μmol/L fatty acid, 7.5 μmol/L BSA), or troglitazone (10 μmol/L) and luciferase activity was measured at 24 h. *B,* luciferase activity in MCF-7 cells cotransfected with a reporter construct containing the DR-1 from the SDC-1 promoter linked to a minimal promoter-luciferase reporter gene pGL3-(DR-1)WT or the same vector containing the mutant DR-1 [pGL3-(DR-1)Mut] with or without the d/n PPARγ cDNA. Following transfection, cells were treated with n-3 LDL or n-6 LDL (100 μg/mL), DHA-BSA or EPA-BSA (30 μmol/L, 7.5 μmol/L BSA), or troglitazone (10 μmol/L) for 8 h. Luciferase activity values are normalized to β-galactosidase activity and presented as the percentage of luciferase activity measured in the presence of stimulus relative to the activity of control cells with no stimulation. Basal activity was reduced ∼2-fold by the d/n PPARγ cDNA but not changed by mutant DR-1. Values with different letters are significantly different ($P < 0.05$). *, $P < 0.001$.

[4] H. Sun and I.J. Edwards, unpublished data.

expression of SDC-1 in MCF-7 cells, whereas EPA was ineffective. To connect the up-regulation of SDC-1 with apoptosis induction, we showed that exogenous human recombinant SDC-1 ectodomain stimulated apoptosis in a dose-dependent manner. This is distinct from studies of Mali et al. (15), which showed cell growth inhibition but did not show apoptosis with a similar reagent. To show that *SDC-1* was the target gene of n-3 LDL or DHA that was responsible for the proapoptotic effect, SDC-1 siRNA was transfected into the cells. SDC-1 gene silencing blocked the ability of n-3 LDL and DHA to promote apoptosis, thus confirming a role for SDC-1 in n-3 PUFA–induced apoptosis in the breast cancer cells.

The present study verified *SDC-1* as a novel PPARγ target gene. We had previously shown that PPARγ agonist troglitazone, DHA, and n-3 LDL were effective stimulators of SDC-1 mRNA expression, whereas PPARδ agonist GW610742 had no effect. A PPARγ antagonist, GW259662, blocked the effect of n-3 LDL on SDC-1 expression (14). To confirm and further clarify the involvement of PPARγ in n-3 LDL and DHA regulation of SDC-1, we first used a d/n PPARγ cDNA, which effectively eliminated the up-regulation of SDC-1 mRNA by n-3 LDL and DHA. Second, studies were undertaken to show that whereas the n-3 PUFA had no detectable effects on PPARγ message and protein levels, a significant increase in PPARγ activity was measured by reporter assays. To obtain direct evidence for a coupling mechanism between n-3 PUFA and PPARγ in SDC-1 gene up-regulation, we constructed a luciferase reporter gene under the control of either the wild-type [pGL3-(DR-1)WT] or mutant [pGL3-(DR-1)Mut] DR-1 element from the SDC-1 promoter and investigated whether the activity of PPARγ was enhanced by n-3 PUFA to regulate expression of SDC-1. DHA, n-3 LDL, and troglitazone significantly increased activity of luciferase in cells transfected with wild-type SDC-1 DR-1-luciferase genes. Cotransfection of the same reporter with d/n PPARγ cDNA inhibited activity and the transfected reporter construct containing a mutant SDC-1 DR-1 site was not responsive to n-3 LDL, DHA, and troglitazone. DHA, n-3 LDL, and troglitazone had no effect on luciferase activity in MCF-7 cells transfected with empty pGL3-promoter vector. Anisfeld et al. (33), using a similar strategy, identified *SDC-1* as a target gene for the farnesoid X receptor (FXR) in hepatocytes. Although FXR is not expressed in breast epithelial cells, our data are consistent with those studies because the DR-1 element in the proximal promoter of SDC-1 is recognized by several nuclear hormone receptors, including the PPARs. PPARγ is directly activated by fatty acids to regulate gene expression (49, 50); however, our study is the first to show the functional coupling of an n-3 PUFA and PPARγ in up-regulation of SDC-1 and thence SDC-1–dependent apoptosis.

PPARγ ligands have been shown to induce apoptosis in a variety of tumor cells, including human breast cancer cells (41). Thus, the dose-dependent increase in apoptosis of MCF-7 cells caused by troglitazone was not unexpected. The important new finding from our studies was that transfection of the cells with SDC-1 siRNA blocked apoptosis induction by both troglitazone and DHA or n-3 LDL. This indicates that the target gene *SDC-1* is required for the proapoptotic activity of PPARγ and that n-3 LDL and DHA promote apoptosis through a SDC-1–dependent pathway.

In summary, the data presented in this work highlight a novel pathway in which n-3 LDL or DHA promotes apoptosis of human breast cancer cells. Further studies are needed to delineate the pathway by which SDC-1 promotes apoptosis of the MCF-7 cells and to identify other participants in this process.

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