

Lipid Bodies Are Reservoirs of Cyclooxygenase-2 and Sites of Prostaglandin-E₂ Synthesis in Colon Cancer Cells

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Abstract

Lipid bodies (lipid droplets) are emerging as dynamic organelles involved in lipid metabolism and inflammation. Increased lipid body numbers have been described in tumor cells; however, its functional significance in cancer has never been addressed. Here, we showed increased number of lipid bodies in tumor tissues from patients with adenocarcinoma of colon submitted to surgical resection when compared with an adjacent normal tissue. Accordingly, increased numbers of lipid bodies were observed in human colon adenocarcinoma cell lines and in a H-rasV12-transformed intestinal epithelial cell line (IEC-6 H-rasV12) compared with nontransformed IEC-6 cells. The functions of lipid bodies in eicosanoid synthesis in cancer cells were investigated. CACO-2 cells have increased expression of cyclooxygenase-2 (COX-2) when compared with IEC-6 cells. We showed by immunolocalization that, in addition to perinuclear stain, COX-2 and prostaglandin E (PGE) synthase present punctate cytoplasmic localizations that were concordant with adipose differentiation-related protein-labeled lipid bodies. The colocalization of COX-2 at lipid bodies was confirmed by immunoblot of subcellular fractionated cells. Direct localization of PGE₂ at its synthesis locale showed that lipid bodies are sources of eicosanoids in the transformed colon cancer cells. Treatment with either aspirin or the fatty acid synthase inhibitor C75 significantly reduced the number of lipid bodies and PGE₂ production in CACO-2 and in IEC-6 H-rasV12 cells with effects in cell proliferation. Together, our results showed that lipid bodies in colon cancer cells are dynamic and functional active organelles centrally involved in PGE₂ synthesis and may potentially have implications in the pathogenesis of adenocarcinoma of colon. [Cancer Res 2008;68(6):1732–40]

Introduction

Increased lipogenesis has emerged as a common phenotype to numerous human carcinomas and has been associated to poor prognosis in breast, prostate, and colon cancer (1, 2). Altered lipid metabolism in cancer cells involves modulation of multiple lipogenic enzymes (1, 2). In fact, enzymes involved in fatty acid synthesis, as well as metabolic regulators, are potential targets for antineoplastic intervention and chemoprevention (1, 2). In virtually all organisms, including mammalian cells, cytoplasmic lipids are

stored in hydrophobic organelles called lipid bodies or lipid droplets. Cytoplasmic lipid bodies are osmiophilic structures, surrounded by a phospholipid monolayer with a unique fatty acid composition, have a neutral lipid rich core, and contain a variable protein composition (3–5). Although in the past, the presence of lipid bodies in cells were largely associated with lipid storage and transport, it has become apparent that lipid bodies are dynamic and functionally active organelles (4, 5).

Increased numbers of lipid bodies are described pathologic observation in cancer cells both in experimental settings and in clinical conditions (4). However, mechanisms that regulate lipid body formation and their functional significance to the cellular biology of the tumor are not known. Recent studies based on lipid body content have shown that lipid bodies, beside their rich lipid content, contain several functionally diverse types of proteins, including adipose differentiation-related protein (ADRP) and caveolin (6–9), lipid metabolic enzymes and proteins of Rab family (10–12), eicosanoid-forming enzymes (13, 14), and protein kinases, such as phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase, and protein kinase C (PKC; refs. 15–17). Therefore, lipid bodies may function in lipid metabolism, membrane trafficking, and intracellular signaling and might have potential implications to mechanisms of cell proliferation and differentiation.

In inflammatory cells, lipid bodies have important roles in regulating arachidonic acid (AA; 20:4, n-6) metabolism. Indeed, AA, an essential polyunsaturated fatty acid with signaling functions and the precursor of prostaglandins and leukotrienes, is stored in its esterified form at lipid bodies (15, 18). Accumulating evidence indicates that AA metabolism plays an important role in colon carcinogenesis. Unesterified AA itself is a signal for apoptosis of colon cancer cells, and conversely, enzymatic conversion of AA or esterification of AA into lipid pools depletes unesterified AA, thereby removing a proapoptotic signal and promoting carcinogenesis (19, 20). Different products of AA metabolism are also implicated in carcinogenesis. As much as 80% to 90% of colon carcinomas show an enhanced cyclooxygenase-2 (COX-2; prostaglandin H synthase) expression compared with normal intestinal mucosa (21–23). COX-2 is the enzyme that catalyzes the rate-limiting step in eicosanoids synthesis, converting AA into prostaglandins (24). Accordingly, high levels of prostaglandins, particularly prostaglandin E₂ (PGE₂), were observed in colorectal cancers (25, 26). Moreover, compelling data from epidemiologic studies, clinical trials, and animal experiments indicate that aspirin and other nonsteroidal antiinflammatory drugs (NSAID) inhibit colorectal carcinogenesis (reviewed in refs. 27, 28).

Leukocyte lipid bodies associated with inflammatory responses were shown to compartmentalize enzymes involved in AA release (cPLA₂; ref. 15) and metabolic conversion into prostaglandins

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(COX) and leukotrienes (5-lipoxygenase; LTC₄ synthase; refs. 13, 14). Moreover, quantitative correlations between an enhanced generation of eicosanoids and levels of lipid body formation have been established in different cell types, suggesting that lipid bodies might function as specific sites involved in eicosanoids synthesis by cells engaged in inflammation (14, 29, 30). Recently, direct assessment of newly synthesized eicosanoids into its locale of synthesis has placed lipid bodies as specific sites of LTC₄ and PGE₂ synthesis within inflammatory leukocytes (31, 32).

In the present study, the occurrence and functions of lipid bodies in colon cancer were investigated. Here, we showed that human colon adenocarcinoma cell lines and colon cancer biopsies from patients present a drastic increase in lipid body numbers. Lipid bodies in these cells contain COX-2 and are structurally distinct cytoplasmic sites for PGE₂ production in an adenocarcinoma cell line. Furthermore, treatment with either aspirin or fatty acid synthase (FAS) inhibitor inhibited PGE₂ production and cell proliferation that correlated to an inhibition of lipid body formation in cancer cells. Together, our results place lipid bodies as functional organelles involved in prostaglandin synthesis in cancer cells and might have roles to the pathogenesis of colon cancer.

Materials and Methods

Human subjects. Samples of colon cancer and adjacent grossly normal-appearing tissue were obtained from three patients at the time of surgery from patients undergoing colon surgical resection at Brazilian National Cancer Institute. Control specimens were collected from the accompanying normal mucosa distant at 5 to 10 cm from the carcinoma. Samples for electron microscopy were immediately fixed as described below. This study was carried out with approval of the Brazilian National Cancer Institute's Ethic Committee.

Cell culture. CACO-2, LOVO, HT-29, and HCT-116 cells were grown in DMEM containing 10% fetal bovine serum (FBS). IEC-6 cells were also grown in DMEM containing 10% FBS supplemented with 0.1 unit of insulin. All cells were grown at 37°C in a humidified 5% CO₂ incubator.

Production of recombinant retroviruses and infection of IEC-6 cells. The retroviral expression vector pBabe-H-rasV12 has been previously described and was kindly donated by Dr. Lowe (Cold Spring Harbor Laboratory; ref. [33]). BD EcoPack2 ecotropic packing cell line (BD Biosciences) was transiently transfected with retroviral vector by calcium phosphate precipitation for 24 h. Cell-free virus-containing supernatant was collected 48 h after transfection and concentrated using Amicon Ultracel 50k (Millipore). The supernatant was supplemented with 8 µg/mL polybrene (FLUKA Chemie) and immediately used for spin infection (2 × 45 min at 380 × g) of 5 × 10⁴ IEC-6 cells. Infected cells were incubated at 37°C for further 24 h and selected for puromycin (Sigma-Aldrich) resistance (7.5 µg/mL) for 14 d.

Lipid body staining and enumeration. Cells (5 × 10⁶ per well) were left unstimulated and untreated or treated with aspirin (500 µg/mL) or C75 (50 µg/mL) for 24 h. Lipid bodies were stained as previously described in each triplicate well ([29]). In brief, cells (10⁵ cells/mL) were fixed in 3.7% formaldehyde in calcium/magnesium-free HBSS (pH 7.4), rinsed in 0.1 mol/L cacodylate buffer, stained in 1.5% OsO₄ (30 min), rinsed in distilled water (dH₂O), immersed in 1.0% thiocarbohydrazide (5 min), rinsed in 0.1 mol/L cacodylate buffer, restained in 1.5% OsO₄ (3 min), rinsed, and then dried and mounted. The morphology of fixed cells was observed, and lipid bodies were enumerated by light microscopy with 100× objective lens in 50 consecutively scanned cells.

Electron microscopy. Lipid bodies were analyzed as previously described ([34]). Briefly, samples were fixed for 1 h in 1% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.2). Then, samples were washed with 0.1 mol/L cacodylate buffer (pH 7.2) followed by a second wash with 0.1 mol/L imidazole buffer (pH 7.5). Samples were then postfixed for 30 min with 2%

OsO₄ diluted in 0.1 mol/L imidazole buffer, washed in the 0.1 mol/L imidazole, dehydrated in a graded series of acetone and embedded in PolyBed 812 resin. Ultrathin sections were stained for 2 min with lead citrate and examined by transmission electron microscopy. Lipid bodies were enumerated by electron microscopy in 100 consecutively scanned cells.

Isolation of lipid bodies by subcellular fractionation. Lipid bodies were isolated by subcellular fractionation based on the buoyancy property of these lipid-rich organelles in sucrose gradients as described ([15, 16]). CACO-2 cells (10⁸/mL) in 20 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L EGTA, 100 mmol/L KCl buffer (pH 7.4) containing 10 µg/mL leupeptin, 10 µg/mL benzamidin, 0.7 µg/mL pepstatin, and 0.1 mmol/L phenylmethylsulfonylfluoride were disrupted by nitrogen cavitation at 700ψ for 5 min at 4°C. The cavitate was collected dropwise and mixed with an equal volume of disruption buffer containing 1.08 mol/L sucrose. The homogenates were centrifuged at 1800 rpm for 5 min to remove the nuclei. The supernatants were overlaid sequentially with 2.0 mL each of 0.27 mol/L sucrose buffer, 0.135 mol/L sucrose buffer, and Top solution [25 mmol/L Tris-HCl, 1 mmol/L EDTA, and 1 mmol/L EGTA (pH 7.4)] and centrifuged at 35,000 rpm at 4°C for 1 h. Seven discrete fractions were collected sequentially from top to bottom: a top layer of lipid body (fraction 1), the mid-zone between lipid bodies and cytosol (fractions 2–3), the cytosol (fractions 4–5), and the microsomal and pellet (fractions 6–7). Proteins from subcellular fractions were concentrated by precipitation with 20% trichloroacetic acid overnight at 4°C. The precipitates were then washed twice with cold acetone. The protein contents in the fractions were analyzed by DC Protein Assay (Bio-Rad). The activity of lactate dehydrogenase was measured as cytosolic marker using the CytoTox 96 kit (Promega).

Western blotting. Proteins from cellular fractions or total cell lysates prepared in reducing and denaturing conditions were separated by SDS-PAGE on polyacrylamide gel. After transfer onto nitrocellulose membranes, nonspecific binding sites were blocked with 5% nonfat milk in TBS-Tween [TBST; 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.05% Tween 20]. Membranes were probed with anti-ADRP monoclonal antibody (mAb; AP125, RDI), anti-COX-2 mAb (C-20; Santa Cruz Biotechnology), anti-H-Ras mAb (F132; Santa Cruz Biotechnology), and anti-β-actin mAb (AC-40; Sigma-Aldrich) in TBST with 1% nonfat dry milk. Proteins of interest were then identified by incubating the membrane with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) in TBST with 1% milk. The membrane was developed with enhanced chemiluminescence (Amersham Biosciences) reagent, and the luminescence was captured by exposure to an autoradiography film.

Immunolocalization. CACO-2 cells (2 × 10⁴/mL) were fixed in 3% formaldehyde at room temperature for 10 min and permeabilized with 0.05% saponin/HBSS solution for 10 min. The unspecific sites were blocked with 1% normal human serum for 10 min. The cells were incubated for 1 h simultaneously with goat polyclonal anti-COX-2 (C-20; Santa Cruz Biotechnology) or goat polyclonal anti-PGE synthase (PGES; S-16; Santa Cruz Biotechnology) and mouse monoclonal anti-ADRP (AP125; RDI) diluted in 0.05% saponin/HBSS solution. Then, cells were blocked with 1% donkey serum for 10 min before simultaneous incubation with Cy3-conjugated affiniPure F(ab') fragment donkey anti-mouse and Cy2-conjugated affiniPure F(ab') fragment donkey anti-goat (Jackson ImmunoResearch Laboratories) for 45 min. Then, cells were washed several times with HBSS, mounted in an aqueous mounting medium, and visualized with 100× objective by both phase contrast and fluorescence microscopy.

Immunodetection of PGE₂ at its sites of production. Immunodetection of PGE₂ was performed as previously described ([32]). In brief, cells (2 × 10⁴/mL) were fixed and permeabilized during 1 h at 37°C with water-soluble 1-ethyl-3-(3-dimethylamino-propyl) carbodimide (EDAC; 0.5% in HBSS). Then, cells were washed with HBSS and blocked with 2% donkey serum for 15 min before incubation with anti-PGE₂ mAb (Cayman Chemicals) for 45 min. The cells were washed with HBSS and incubated with Cy3-conjugated affiniPure F(ab') fragment donkey anti-mouse for 45 min. Lipid bodies were fluorescent labeled with Bodipy 493/503 (Molecular Probes), added together with the secondary antibodies. The

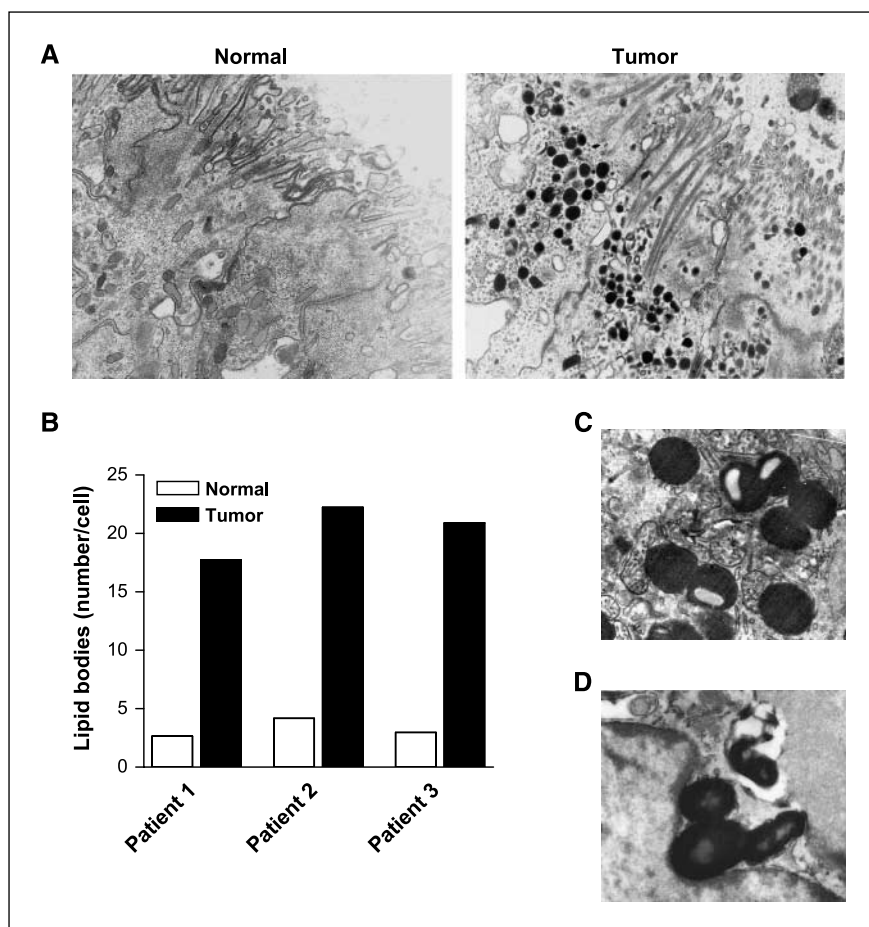


Figure 1. Analysis of lipid bodies of paired samples of human colon cancer and normal tissue. Pairs of samples of colon cancer and adjacent nonneoplastic tissue obtained at the time of surgery from patients undergoing colon surgical resection as described. *A*, analysis of thin sections of the adjacent normal tissue (*Normal*) and colon cancer (*Tumor*) examined by transmission electron microscopy. *B*, quantitative analyses of the lipid body numbers of the adjacent normal tissue and colon cancer. Lipid bodies were enumerated by electron microscopy and presented as mean from 100 consecutively scanned cells in tissues from three independent patients. *C* and *D*, electron microscopy analysis of the lipid bodies of the colon cancer sample. The presented images are representative from three independent patients.

cells were washed with HBSS, then mounted in an aqueous mounting medium, and visualized with 100 \times objective by both phase contrast and fluorescence microscopy. The specificity of the PGE₂ immunolabeling was ascertained by the incubation (10 min before EDAC) with the COX inhibitor indomethacin (10 μ g/mL) to avoid the synthesis of PGE₂ and by the use of an isotype control (MOPC 31c; BD Pharmingen).

Cell proliferation assay. Cells (5×10^4 per well) were left unstimulated and untreated or treated with aspirin (500 μ g/mL) or C75 (50 μ g/mL) for 24, 48, 72, and 96 h. After treatment, cell proliferation was assayed in each triplicate well. Briefly, cells were fixed with ethanol for 10 min, stained with 0.05% violet crystal in 20% ethanol for 10 min, solubilized with methanol, and read by spectrometer at 595 nm.

EIA. Cells (2×10^6 per well) were left unstimulated and untreated or treated with aspirin (500 μ g/mL) or C75 (50 μ g/mL) for 24 h. After the treatment, PGE₂ was assayed in each triplicate well supernatant. Determination of PGE₂ levels by enzyme immunoassay was accomplished using PGE₂ monoclonal enzyme immunoassay kit (Cayman Chemical).

Statistical analysis. Statistical analysis of values from control and treated groups was determined using an unpaired Student's *t* test for single comparison or an ANOVA followed by the Student-Newman-Keuls for multiple comparisons. $P < 0.05$ was considered to be statistically significant.

Results

Lipid bodies are increased in human colon cancer. Ultrastructural evaluation of lipid bodies were performed by electron microscopy of paired samples of human colon cancer and normal tissue distant from the tumor. Patients' pairs of samples of cancer and nonneoplastic tissues were processed as

described in material and methods. Analysis of adenocarcinoma of colon from patients showed round, nonmembrane-bound lipid bodies exhibiting variable osmiophilia, ranging from light to strong electron dense organelles that were prominently increased in number and size in tumor tissues when compared with a normal tissue from the same patient submitted to surgical resection (Fig. 1A). Electron microscopy quantitative analyzes showed a dramatic increase in lipid body numbers in all studied patients (Fig. 1B). Moreover, lipid bodies in tumor tissues were imaged as heterogeneous organelles. They frequently displayed lucent areas and granular and/or membranous internal structures or showed irregular surfaces (Fig. 1). Images suggesting budding, coalescence, or fusions of lipid bodies were identified (Fig. 1). Remarkably, lipid bodies from tumor tissues showed clear interaction with nucleus, ER, and mitochondria (Fig. 1C and D).

Adenocarcinoma cell line CACO-2 presents an increased number of lipid bodies, COX-2 overexpression, and high levels of PGE₂ production. To evaluate the possible involvement of lipid bodies in the pathogenesis of colon cancer, the presence of lipid bodies in a human colon adenocarcinoma cell line (CACO-2 cells) was compared with a nontransformed rat intestinal epithelial cell line (IEC-6 cells). Both cell lines were maintained in culture medium by 24 h and stained with osmium tetroxide. As shown in Fig. 2A, CACO-2 cells present enhanced lipid body formation when compared with IEC-6 cells, which have few or absence of these cytoplasmic structures. Electron microscopy analysis showed cytoplasmic organelles that were identified by their shape,

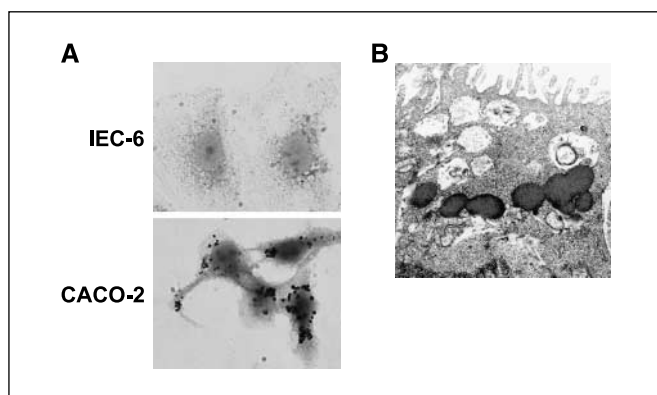


Figure 2. Analysis of lipid bodies in IEC-6 and CACO-2 cell lines. *A*, IEC-6 and CACO-2 cells were grown without stimulation for 24 h and stained for lipid bodies as described. Then, cells were analyzed by phase contrast microscopy with 100 \times objective lens. *B*, analysis of lipid bodies by electron microscopy in unstimulated CACO-2 cells as described in *A*. These results are representative from three independent experiments.

osmiophilia, and lack of a limiting membrane as lipid bodies (Fig. 2*B*). Images suggesting fusion of lipid bodies with each other were frequently observed, as well as, interactions with other organelles including ER, nuclei, and cytoplasmic vacuoles (Fig. 2*B* and data not shown).

Quantitative analysis of lipid bodies shows a 4-fold to 5-fold increase of lipid body numbers in CACO-2 cells when compared with IEC-6 cells (Fig. 3*A*). To evaluate if increased lipid body numbers were a unique feature of CACO-2 cells or if it was a common aspect to other human colon adenocarcinoma cell lines, LOVO, HT-29, and HCT-116 cells were analyzed (Fig. 3*A*). Increased lipid body numbers in all these cancer cell lines were observed when compared with IEC-6 cells (Fig. 3*A*). Several evidences suggest that the inflammatory process and COX-2 have a central role in colorectal carcinogenesis. COX-2 expression was evaluated in CACO-2 and IEC-6 cell lines. Western blot analysis showed increased expression of COX-2 protein in CACO-2 cells when compared with IEC-6 cells (Fig. 3*B*). Furthermore, CACO-2 cells also presented an enhanced production of PGE₂ when compared with IEC-6 cells (Fig. 3*C*).

Lipid bodies are sites for PGES and COX-2 localization and PGE₂ production in adenocarcinoma cell line. Lipid bodies in inflammatory cells have been shown to function as main sites involved in the oxidative metabolism of AA and eicosanoid synthesis (reviewed in ref. 5). To address the relationship between COX-2 and lipid bodies in cancer cells, the cellular compartment for COX-2 localization was evaluated. Initially, CACO-2 cells were subject to subcellular fractionation, and then cellular fractions were immunoblotted with anti-COX-2. ADRP was used as a marker of lipid bodies for colocalization purpose. ADRP is ubiquitously expressed in many cells and tissues as a major component of lipid droplets (6, 7). As shown in Fig. 4*A*, COX-2 protein localizes in fractions where lipid bodies and nuclei are isolated. On the other hand, ADRP, a protein that is associated to lipid bodies in different cell types, only localizes in the lipid body fraction (Fig. 4*A*).

We then used indirect immunofluorescence microscopy to confirm the localization of COX-2 in intact cells with specific conditions for fixation and permeabilization, which prevent dissolution of lipid bodies. In CACO-2 cells, lipid bodies are readily visualized by a strong ring shape staining for ADRP, indicating a staining pattern localized to the peripheral surface of lipid bodies

similar to the staining pattern for ADRP-associated lipid body in adipocytes and other cells (6, 7). CACO-2 cells stained with anti-COX-2 polyclonal antibody, in addition to perinuclear membrane, showed focal punctate cytoplasmic staining (Fig. 4*B*, top). The presence of COX-2 within lipid body was confirmed by ADRP and COX-2 colocalization within CACO-2 cells (Fig. 4*B*, top). Interestingly, COX-2 lipid body staining was not restricted to lipid body surface, suggestive that COX-2 pervades lipid body core (Fig. 4*B* insert). PGE₂ is formed from AA by cyclooxygenase-catalyzed formation of PGH₂ and further transformation by downstream terminal enzyme PGES (35). Next, we evaluated the localization of the inducible isoform of PGES, mPGES, in CACO-2 cells. As shown in Fig. 4*B* (bottom), mPGES shows a similar localization pattern as the observed for COX-2 with colocalization with ADRP-labeled lipid bodies in addition to perinuclear staining.

Because CACO-2 cells have increased COX-2-enriched and PGES-enriched lipid bodies and enhanced PGE₂ formation, we hypothesized that lipid bodies were domains for compartmentalized PGE₂ synthesis. To investigate intracellular sites of newly formed PGE₂ in colon cancer cells, a recently developed strategy for direct *in situ* immunolocalization of PGE₂ synthesis were used (32). EDAC was used to cross-link eicosanoid carboxyl groups to amines in adjacent proteins, and immobilized PGE₂ was immunofluorescently detected. CACO-2 cells showed intense and punctate immunofluorescent staining for PGE₂ (Fig. 4*C*, top). As shown in Fig. 4*C* (top right), PGE₂ intracellular site of production match

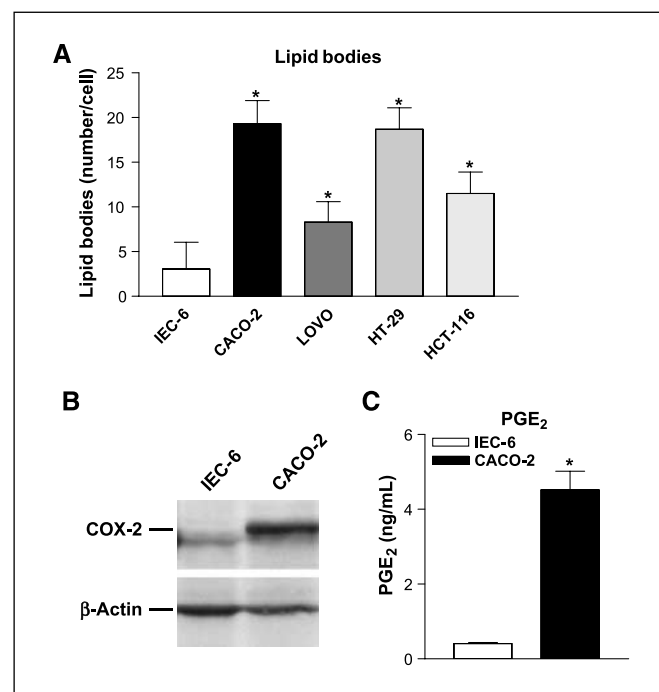


Figure 3. Quantitative analysis of lipid bodies, COX-2, and PGE₂ in colon cancer cell lines. *A*, quantitative analyses of the lipid body numbers in unstimulated IEC-6 and CACO-2, LOVO, HT-29, and HCT-116 cells. The lipid bodies were enumerated in 50 consecutively scanned cells. Columns, mean; bars, SD. *, significantly different values relative to IEC-6 cells ($P < 0.05$). *B*, analysis of COX-2 (top) and β -actin (bottom) expression in total cell lysates of unstimulated IEC-6 and CACO-2 cells by Western blot. β -Actin levels were used for control of protein loading. *C*, analysis of PGE₂ production in unstimulated IEC-6 and CACO-2 cells. In each supernatant, PGE₂ was assayed by EIA. Columns, mean; bars, SD. *, significantly different values relative to IEC-6 cells ($P < 0.05$). These results are representative from three independent experiments.

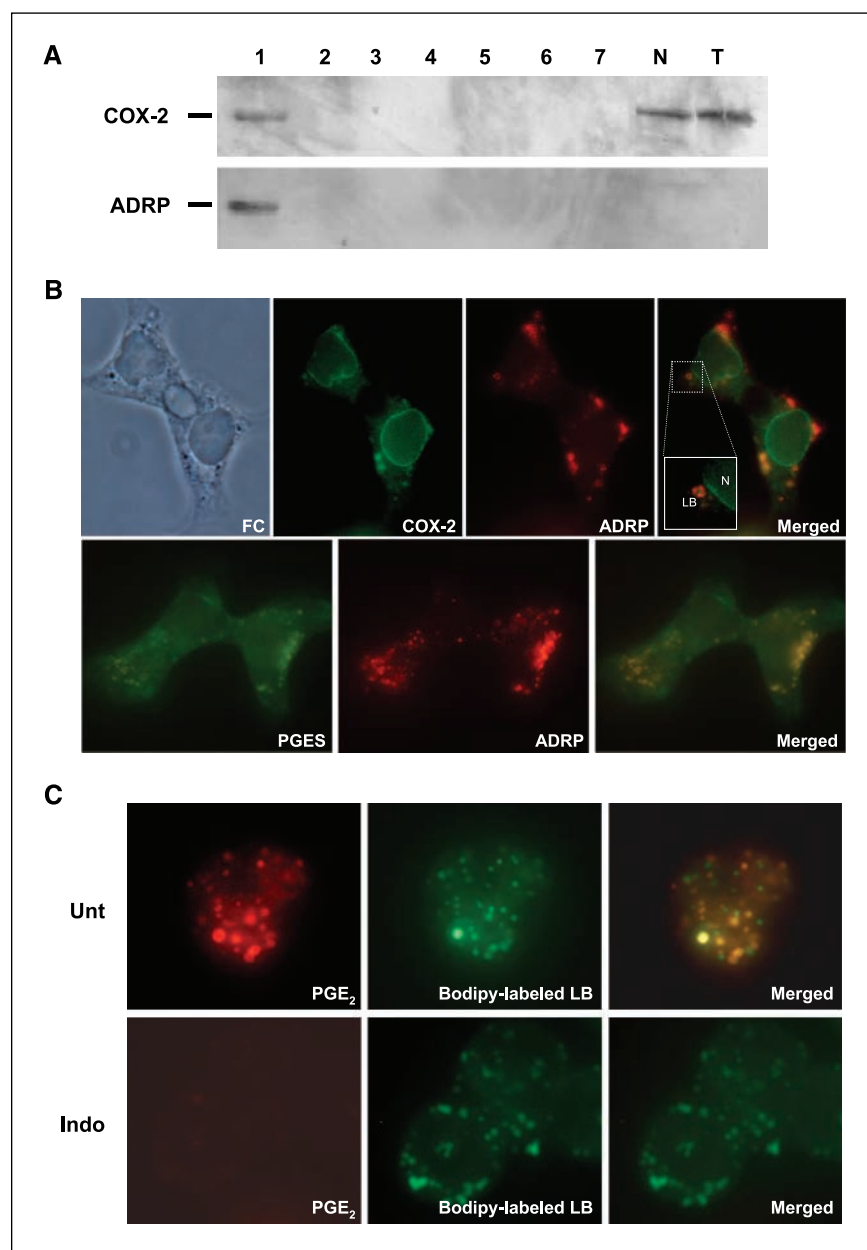


Figure 4. Analysis of COX-2, PGES, and PGE₂ localization in CACO-2 cells. **A**, unstimulated cells were subjected to subcellular fractionation, and fractions were recovered separately. Fractions 1 and 2, lipid bodies; fractions 3 and 4, cytosol; fractions 5, 6, and 7, microsome and membrane; N, nuclear; T, total lysate. Then, fractions were immunoblotted with anti-COX-2 (top) or anti-ADRP (bottom) antibodies. **B**, unstimulated cells were stained with anti-COX-2, anti-PGES, or anti-ADRP antibodies. Top, phase contrast (FC), anti-COX-2 (COX-2), anti-ADRP (ADRP), and merge of COX-2/ADRP (Merged); bottom, anti-PGES (PGES), anti-ADRP (ADRP), and merge of PGES/ADRP (Merged). **C**, unstimulated cells were left untreated (Unt) or treated with indomethacin (Indo) and then stained with anti-PGE₂ antibody and fluorescent Bodipy 493/503. The sequence shows anti-PGE₂ (PGE₂), fluorescent Bodipy 493/503 (Bodipy), and merge of PGE₂/Bodipy (Merged). All results are representative from two independent experiments.

bodipy-stained lipid bodies, placing lipid bodies as sites of PGE₂ synthesis within colon cancer cells. Of note, perinuclear PGE₂-positive staining could also be observed. Specificity of the immunofluorescence for PGE₂ was ascertained by the lack of PGE₂ immunolabeling within indomethacin pretreated CACO-2 cells (Fig. 4C, bottom) and also supported by the absence of immunostaining when an isotype control antibody replaced the anti-PGE₂ mAb (data not shown). These results showed that lipid bodies are intracellular sites for COX-2 and mPGES protein localization and locales of newly formed PGE₂ production in colon cancer cells.

Aspirin and FAS inhibitor C75 inhibited lipid body formation, PGE₂ production, and proliferation in adenocarcinoma cell line. NSAIDs, which inhibit COX, are used as chemopreventive agents for carcinogenesis of colon and cause apoptosis of cancer cells. The mechanism for this response is not

fully understood, but it might result from an absence of a prostaglandin product, an accumulation of AA substrate, or shunting of substrate into a different pathway (28, 36). In addition, NSAIDs, including aspirin and NS398, have been previously shown to inhibit lipid body formation induced by cys-unsaturated fatty acids through COX-independent mechanisms (29, 37). The capacity of aspirin to inhibit lipid body formation in CACO-2 cells was investigated. CACO-2 cells were left untreated or treated with aspirin (500 µg/mL) for 24 hours, and lipid bodies and PGE₂ were evaluated as described above. As shown in Fig. 5A and B, aspirin inhibited lipid body formation and PGE₂ generation in CACO-2 cells. To gain further insights on the functions of lipid bodies in eicosanoid metabolism and roles in cell growth, we analyzed the effect of a noncyclooxygenase inhibitor of lipid body biogenesis.

To this end, CACO-2 cells were treated with the FAS inhibitor C75. FAS is the key biosynthetic enzyme in the fatty acid synthesis.

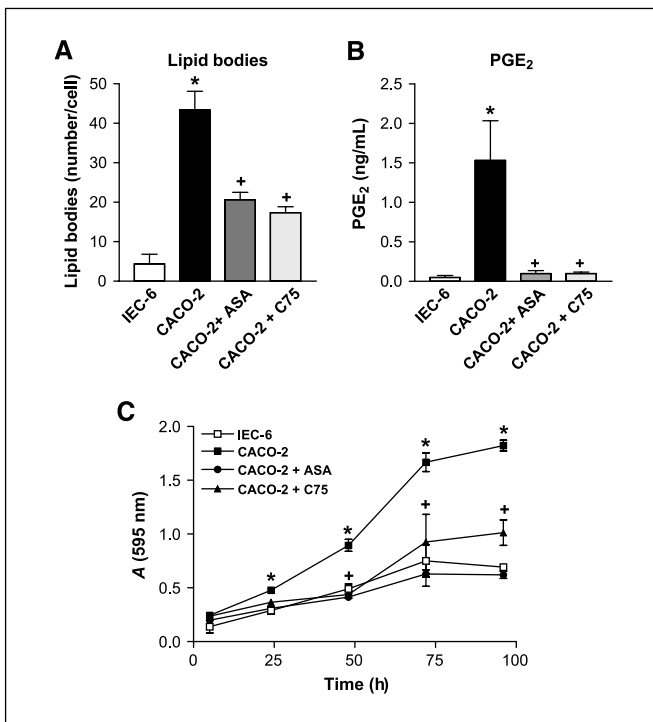


Figure 5. Analysis of lipid bodies, PGE₂ production, and cell growth in CACO-2 cells treated with aspirin and C75. *A*, cells were left unstimulated, and CACO-2 cells were left untreated or treated with aspirin (500 μ g/mL) or C75 (50 μ g/mL) for 24 h. Then, the lipid bodies were enumerated as described in Fig. 3*A*. *B*, cells were left unstimulated and CACO-2 cells were left untreated or treated with aspirin (500 μ g/mL) or C75 (50 μ g/mL) for 24 h. Then, PGE₂ was assayed by EIA as described in Fig. 3*C*. *C*, cells were left unstimulated and CACO-2 cells were left untreated or treated with aspirin (500 μ g/mL) or C75 (50 μ g/mL) for 24, 48, 72, and 96 h. Then, cells were stained with violet crystal and plates were analyzed by spectrometer at 595 nm as described. Points, mean; bars, SD. *, significantly different values relative to IEC-6 cells ($P < 0.05$); +, significantly different values relative to untreated CACO-2 cells ($P < 0.05$). All results are representative from two independent experiments.

Increased expression of FAS has emerged as a common phenotype in most human cancer, including colon cancer (38, 39) and its increased expression and activity associated to poor prognosis in cancer (reviewed in ref. 2). Moreover, it has been well established that FAS pharmacologic inhibition or knockdown by RNA interference leads to selective cytotoxic to human cancer cells (40–42). The mechanisms involved in FAS-induced killing of cancer cells remain an area of intense investigation. The effect of C75 in lipid body formation and PGE₂ production was analyzed. As shown in Fig. 5*A*, C75 significantly inhibited lipid body biogenesis in CACO-2 cells. Accordingly, C75 was shown to significantly inhibit lipid body formation in preadipocytes (43). Strikingly, although C75 is not an enzymatic inhibitor of COX-2 it significantly inhibited PGE₂ production by CACO-2 cells (Fig. 5*B*), thus suggesting that COX-2 compartmentalization within lipid bodies play roles in the enhanced PGE₂ synthetic capacity by colon cancer cells. It should be noted that cell viability 24 hours after treatment with either C75 or aspirin was greater than 95% as assessed by trypan blue exclusion (not shown).

We next examined the effect of aspirin or C75-induced inhibition of lipid body formation in CACO-2 cellular proliferation. CACO-2 cells were left untreated or treated with aspirin (500 μ g/mL) or C75 (50 μ g/mL) for 24, 48, and 72 hours. As shown in Fig. 5*C*, CACO-2 cells presented an enhanced proliferation when compared with

IEC-6 cells. However, the treatment with either aspirin or C75 significantly decreased the CACO-2 cells proliferation at levels of IEC-6 cells (Fig. 5*C*).

Aspirin and FAS inhibitor C75 inhibited lipid body formation, PGE₂ production, and proliferation in H-Ras transformed IEC-6. To gain further insight into the association of cell transformation and lipid body biogenesis, we transformed IEC-6 cells with a retrovirus construct of H-rasV12. Consistent with published data (44), oncogenic Ras-mediated transformation of intestinal epithelial cells (IEC-6 H-rasV12) shows COX-2 overexpression (Fig. 6*A*) and is associated with an increased cell proliferation phenotype (Fig. 6*B*). As shown in Fig. 6, H-Ras transformed IEC-6 exhibits highly increased lipid body biogenesis (Fig. 6*C*) and enhanced PGE₂ production (Fig. 6*D*) when compared with IEC-6 cells. In addition, treatment with phorbol 12-myristate 13-acetate (PMA; 10 nmol/L for 1 h) increased lipid body formation in IEC-6 cells (4.8 ± 2.5 in untreated to 23.6 ± 3.5 lipid bodies per cell in IEC-6 cells treated with PMA; $P < 0.05$).

As shown in Fig. 5*C* and *D*, pretreatment with either aspirin (500 μ g/mL) or C75 (50 μ g/mL) significantly inhibited lipid body biogenesis and PGE₂ in IEC-6 H-rasV12 cells. Furthermore, the treatment with either aspirin or C75 in parallel to the inhibition of lipid body formation significantly decreased the IEC-6 H-rasV12 cell proliferation to the levels of IEC-6 cells (Fig. 6*B*). Of note, pretreatment with aspirin or C75 failed to inhibit basal lipid body numbers in wild-type IEC-6 (Fig. 6*C*) and inhibited IEC-6 cell growth in <30% within 72 hours (not shown).

Discussion

Lipid bodies are increasingly recognized as dynamic and multifunctional organelles in different cell systems and organisms. The occurrence and functions of lipid bodies in cancer cells are largely unknown. Here, we showed that lipid bodies are increased in neoplastic cells. Furthermore, lipid bodies in colon cancer cells are functionally active organelles that compartmentalize eicosanoid-forming enzymes and are sites for newly formed lipid mediator (PGE₂) production. Together, these findings yielded insights into the functions of lipid bodies in AA metabolism in cancer cells and place lipid bodies as potential targets for therapeutic interventions.

Inflammation is considered a well-established cancer risk factor, and a number of inflammatory conditions predispose to colon cancer, including ulcerative colitis and Crohn's disease (45, 46). Increased biogenesis of lipid bodies has been extensively characterized in cells associated with several human inflammatory diseases (reviewed in ref. 5), including infiltrating leukocytes and intestinal epithelium of patients with Crohn's disease (47). We showed drastic increased numbers of lipid bodies in the tumor tissues from patients with adenocarcinoma of colon submitted to surgical resection when compared with paired normal tissues. Lipid bodies within adenocarcinoma cells appeared as round amorphous osmiophilic inclusions showing variable electron densities and frequently showed an electron-dense rim, devoid of a bilayer membrane, typical features of lipid bodies in other cells. Although our study is limited by its small sample size, it gives support to future studies with large numbers of patients where the relationship between lipid bodies and the different aspects of colon cancer, including the stage, invasiveness, and the outcome could be addressed.

The surface of lipid bodies in cancer cell was shown to be enriched with the protein ADRP, which is in agreement with

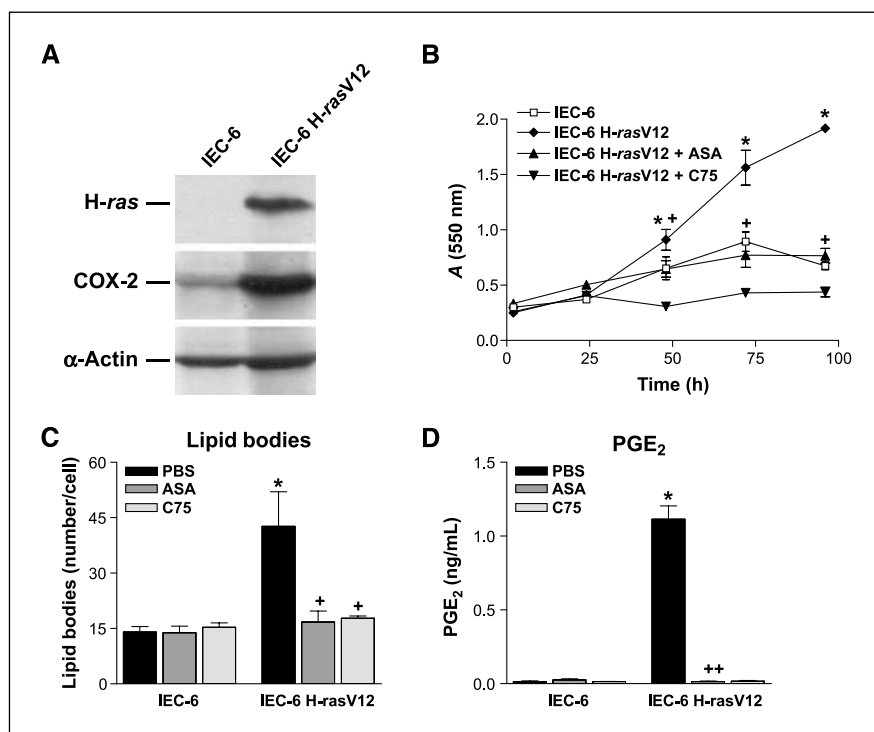


Figure 6. Analysis of lipid bodies, PGE₂ production, and cell growth in H-ras transformed IEC-6 cells treated with aspirin and C75. *A*, analysis of H-Ras (top), COX-2 (middle), and β -actin (bottom) expression in total cell lysates of unstimulated IEC-6 and IEC-6 H-rasV12 cells by Western blot. β -Actin levels were used for control of protein loading. *B*, cells were left unstimulated and untreated or treated with aspirin (500 μ g/mL) or C75 (50 μ g/mL) for 24, 48, 72, and 96 h. Then, cells were stained with violet crystal and plates were analyzed by spectrometer at 595 nm as described. *C*, cells were left unstimulated and untreated or treated with aspirin (500 μ g/mL) or C75 (50 μ g/mL) for 24 h. Then, the lipid bodies were enumerated as described in Fig. 3A. *D*, cells were left unstimulated and untreated or treated with aspirin (500 μ g/mL) or C75 (50 μ g/mL) for 24 h. Then, PGE₂ was assayed by EIA as described in Fig. 3C. Columns, mean; bars, SD. *, significantly different values relative to IEC-6 cells ($P < 0.05$); +, significantly different values relative to untreated IEC-6 H-rasV12 cells ($P < 0.05$). All results are representative from two independent experiments.

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previous demonstrations that ADRP is the main surface protein of lipid bodies in different mammalian cells, including epithelial cells. Accumulating data points to an important role of ADRP in fatty acid uptake, cholesterol, and fatty acid binding and neutral lipid storage (48) and although the biological function of ADRP is not completely known, this protein is a specific probe for lipid bodies enabling its subcellular localization within intact cells and subcellular gradients (6, 7). To assess discrete sites involved in eicosanoid synthesis, eicosanoid-forming enzymes may be immunolocalized at specific intracellular sites. Based on immunolocalization studies of eicosanoid-forming enzymes in many cells, major sites of synthesis of cyclooxygenase pathway-derived eicosanoids are believed to be the ER and perinuclear membranes (24). In addition, extranuclear lipid bodies have been described as sites to where different enzymes involved in prostaglandin synthesis, including cPLA2 and COX-2, translocate upon leukocyte stimulation in inflammatory conditions (13–15, 30). Different evidences support the hypothesis that lipid bodies in neoplastic cells function as distinct intracellular domain for regulated eicosanoid production. First, lipid bodies were sites of COX-2 and PGES compartmentalization. Second, in this study we observed a significant correlation between lipid body numbers and PGE₂ generation in cancer colonic epithelial cells and in H-rasV12 transformed epithelial cells. Moreover, PGE₂ production was shown to occur at lipid bodies. Direct assessment of intracellular sites for prostaglandin generation has been elusive, as prostanoids and other eicosanoids are newly formed, nonstorable, and rapidly released upon cell stimulation. Recently, a new strategy to cross-link newly formed eicosanoids at its sites of synthesis has been described, enabling the immunofluorescent localization of newly formed eicosanoids at their intracellular formation locale and demonstrating that intracellular compartments involved in both leukotriene and prostaglandin synthesis differ according with the cell type studied and the stimulatory conditions used (31, 32). By

investigating intracellular domains involved in prostaglandin synthesis in colon carcinoma cells, we showed that lipid bodies are intracellular domains for PGE₂ synthesis, placing lipid bodies as important sites in the cellular processing and metabolism of AA, in addition to nuclear membrane in colon cancer cells.

Increased lipid body biogenesis may contribute in different ways to cancer progression. As mentioned above, lipid body arachidonylphospholipids might provide a source of substrate AA leading to increased prostaglandin synthesis without requiring the perturbation of the integrity of membranes and directly coupled to cocompartmentalized PGES and COX-2. Lipid bodies could also function as a draining compartment to rapidly take up and reacylate free AA within lipid body lipids, with potentially detrimental outcomes for the host cell as AA has important functions in inducing apoptosis of tumor cells (19, 20).

Together, our results showed that adenocarcinoma of colon beside overexpression of COX-2, have increased numbers of lipid bodies equipped with active PGE₂-synthesizing machinery. These results prompt us to address if lipid body inhibition would have an effect on neoplastic cell proliferation. Different classes of drugs have been described with the capacity to inhibit lipid body formation, although no specific lipid body inhibitor has been identified thus far (reviewed in ref. 5). Thus, to address the hypothesis of lipid body involvement in colon cancer proliferation, parallel assessment of lipid bodies, PGE₂ production, and CACO-2 cell proliferation was obtained with two different classes of drugs that inhibit lipid body formation.

Epidemiologic studies showed that individuals who have taken NSAIDs have a markedly reduced risk of developing colon cancer. In mice, COX-2 inhibitors or traditional NSAIDs lower the incidence of polyps and cancer. It was suggested that inhibition of tumors by NSAIDs involves the common property of COX suppression and the resultant reduction in levels of prostaglandins in tissues (reviewed in refs. 28, 36). Our data show that treatment

with aspirin significantly reduced the number of lipid bodies and PGE₂ levels in CACO-2 and IEC-6 H-*ras*V12 cells (Figs. 5 and 6). The mechanisms involved in aspirin inhibition of lipid body biogenesis are not completely understood. Indeed, previous findings showed that lipid body biogenesis can be induced in COX-deficient cells and aspirin are able to inhibit lipid body formation even in COX-deficient cells, thus suggesting that aspirin inhibitory effect on lipid body biogenesis involves COX-independent mechanisms (29, 37). Clinical and experimental evidence strongly suggest that NSAIDs are anticarcinogenic, antiproliferative, and antineoplastic. In fact, the oncogenic potentials of the prostaglandins were related to its properties to promote cell proliferation and inhibit apoptosis in intestinal epithelial cells (49–51). We observed that CACO-2 cells present a progressive growth when compared with nontransformed epithelial intestinal cells. Inhibition of lipid body formation by either aspirin or FAS inhibitor, correlated to inhibition of PGE₂ generation and cell proliferation (Figs. 5 and 6). Thus, our data provides one more possible mechanism by which NSAIDs and FAS inhibitors exert their effects as anticarcinogenic drug. The inhibition of lipid body generation may affect the subcellular compartmentalization of COX-2 and in consequence inhibit the enhanced prostaglandin synthesis that is related to the pathogenesis of colon cancer.

Besides of its function as organelles involved in the generation of eicosanoids, lipid bodies constitute sites of compartmentalization

of several signaling-relevant proteins, which may have functions beyond AA metabolism. Indeed, proteins with well-established roles in oncogenic cell transformation, tumorigenesis, and metastasis, including PI3K, extracellular signal-regulated kinase 1 (ERK1), ERK2, p38, PKC, and caveolin, were shown to localize in lipid bodies in a variety of cell types (8, 9, 15–17, 52). In addition, lipid body-specific protein ADRP has been recently described as a potential diagnostic and prognostic biomarker for renal cell and hepatocellular carcinoma (53, 54). In conclusion, our findings place lipid bodies as dynamic and functional active organelles centrally involved in PGE₂ synthesis in colon cancer cells that might potentially have implications with the pathogenesis of adenocarcinoma of colon. Thus, the evaluation of lipid bodies in human tissues could potentially be used as diagnostic and/or prognostic index in colon cancer or even as potential target to generate new drugs for cancer treatment.

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