Arachidonic acid cascade and epithelial barrier function during Caco-2 cell differentiation

Raquel Martín-Venegas, Sònia Roig-Pérez, Ruth Ferrer, and Juan José Moreno[1]

Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, 08028 Barcelona, Spain

Abstract **The small intestinal epithelium is a highly dynamic system continuously renewed by a process involving cell proliferation and differentiation. The intestinal epithelium constitutes a permeability barrier regulating the vectorial transport of ions, water, and solutes. Morphological changes during cell differentiation, as well as changes in the activity of brush-border enzymes and the expression of transport proteins, are well established. However, little is known about the arachidonic acid (AA) cascade underlying epithelial cell differentiation or its role in the development of epithelial barrier function. The main purpose of this study was to examine the activity of the high-molecular-weight phospholipases A_2 (PLA_2) and cyclooxygenase (COX) pathway during differentiation, with particular emphasis on paracellular permeability. PLA_2 activity, AA release, COX-2 expression, prostaglandin E_2 (PGE_2) production, and paracellular permeability were studied in preconfluent, confluent, and differentiated Caco-2 cell cultures. Our results show that Caco-2 differentiation induces a decrease in both calcium-independent PLA_2 activity and COX-2 expression and, consequently, a decrease in AA release and PGE_2 synthesis in parallel with a reduction in paracellular permeability. Moreover, the addition of PGE_2 to differentiated cells, at concentrations similar to those detected in nondifferentiated cultures, induces the disruption of epithelial barrier function. These results suggest that AA release by calcium-independent PLA_2, COX-2 expression, and subsequent PGE_2 release are important for the maintenance of paracellular permeability in differentiated Caco-2 cells.**—Martín-Venegas, R., S. Roig-Pérez, R. Ferrer, and J. J. Moreno. **Arachidonic acid cascade and epithelial barrier function during Caco-2 cell differentiation.** ***J. Lipid Res.*** **2006.** 47: **1416–1423.**

Supplementary key words intestine • tight junctions • phospholipase A_2 • cyclooxygenase • prostaglandins

The epithelium of the small intestine is a complex structure formed by numerous cell types involved in many homeostatic functions, such as nutrient digestion and absorption, defense from luminal contents, and peptide secretion (1). This epithelium is a highly dynamic system continuously renewed by a process involving cell proliferation and migration from the stem cell compartment, located at the bottom of the crypts, to programmed cell death and the extrusion of terminally differentiated cells from the tip-villus to the intestinal lumen (2, 3). The entire process of proliferation, differentiation, apoptosis, and extrusion occurs over 3 to 5 days depending on the species (3). Cell differentiation of the intestinal epithelium has been the subject of extensive study. The morphological changes during cell differentiation (4), the growth kinetics of epithelial cells (5), and the changes in brush-border enzyme activities during prenatal and postnatal development (6) are well established. However, little is known about the signals underlying cell differentiation along the crypt-villus axis.

The differentiation process of absorptive cells, the most abundant in the intestinal epithelium, can be modeled in vitro using Caco-2 cell cultures. Although cancerous in origin, these cells undergo a gradual differentiation process that takes place spontaneously once confluence has been reached and that is completed after 21–25 days in culture (7). Consequently, Caco-2 cells acquire morphological and functional polarity and show enzymatic activities, as well as protein and mRNA levels, of brush-border membrane enzymes and transporters highly comparable to those of mature enterocytes (7–9). In this sense, Caco-2 cell cultures provide an important tool for studying and obtaining greater insight into the regulation of human intestinal epithelial cellular processes such as enterocytic differentiation (4, 10).

The intestinal epithelium constitutes a permeability barrier that regulates the vectorial transport of ions, solutes, and water. Epithelial cells adhere to each other via three distinct adhesion systems: tight junctions, adherent junctions, and desmosomes. Of these, tight junctions are the most apical components and determine the paracellular permeability across the intestinal epithelium and the interface (fence) between apical and basolateral membrane domains (11). The existence of a relationship be-

Manuscript received 27 December 2005 and in revised form 28 March 2006.

Published, JLR Papers in Press, April 3, 2006.
DOI 10.1194/jlr.M500564-JLR200

Abbreviations: AA, arachidonic acid; BEL, bromoenol lactone; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; TEER, transepithelial electrical resistance.

[1] To whom correspondence should be addressed.
e-mail: jjmoreno@ub.edu

tween cell differentiation and tight junction structure and permeability has been demonstrated in vivo in the small intestine; tight junction strand counts are higher in differentiated tip-villus cells than in nondifferentiated crypt cells (12).

Arachidonic acid (AA) mobilization from cellular phospholipids by phospholipase A_2 (PLA_2) is the rate-limiting step in the AA cascade. PLA_2s are a growing family of enzymes with distinct substrate specificity, cofactor requirements, subcellular localization, and cellular function. Thus, mammalian PLA_2 enzymes have been broadly classified into three groups: low-molecular-weight Ca^{2+}-dependent secretory phospholipase A_2 ($sPLA_2$), high-molecular-weight Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$), and high-molecular-weight Ca^{2+}-independent phospholipase A_2 ($iPLA_2$). After AA release, the fatty acid is oxidized by oxygenases such as cyclooxygenases (COXs), lipoxygenases, or cytochrome P-450 monooxygenases. Thus, AA is the precursor for the further metabolism of many biologically active products, collectively termed eicosanoids (13). This large family of bioactive lipids have pleiotropic actions and modulate diverse physiological responses, including cell growth and differentiation and pathological conditions involving altered cellular proliferation. COX catalyzes the conversion of AA to prostaglandin H_2, the immediate precursor of prostanoids. In eukaryotic cells, two COX isoforms have been identified and cloned (14): COX-1 is encoded by a housekeeping gene and is thought to be involved in the maintenance of physiological functions, whereas the expression of COX-2 is induced by various stimuli, including mitogens (15). The overexpression of COX-2 has been associated with a variety of proliferative diseases such as colorectal cancer (16). In this sense, enhanced prostaglandin E_2 (PGE_2) synthesis has been observed in human colon cancer tissue (17), thus increasing the tumorigenic potential of colonic epithelial cells (18).

Recently, we observed that $cPLA_2$ and $iPLA_2$ were expressed in Caco-2 cells. We also demonstrated that $iPLA_2$ was involved in AA release and the subsequent prostaglandin production induced by serum. Moreover, we reported the first evidence that $iPLA_2$ may be involved in the signaling pathways involved in the control of Caco-2 proliferation (19). The main objective of this study was to examine the activity of high-molecular-weight PLA_2, a key enzyme of the AA cascade, during Caco-2 differentiation. Furthermore, we determined the role of PLA_2s on Caco-2 differentiation, with particular emphasis on the relationship between PLA_2 activities, AA release, COX-2 expression, AA metabolite production, and paracellular permeability.

MATERIALS AND METHODS

Materials

Tissue culture supplies, including Transwells, were obtained from Costar (Cambridge, MA). D-[2-^{3}H]mannitol (specific activity, 30 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid ([^{3}H]AA) (200–240 Ci/mmol), and phosphatidylcholine-L-α-1-palmitoyl 2-arachidonyl [arachidonyl-1-^{14}C] (50–60 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). DMEM, heat-inactivated FBS, penicillin G, streptomycin, and trypsin/EDTA were purchased from BioWhittaker Europe (Verviers, Belgium). Bromoenol lactone (BEL) was obtained from Alexis Corp. (San Diego, CA). Nonessential amino acids, L-glutamine, BSA, ethidium bromide, acridine orange, aprotinin, leupeptin, diethyldithiocarbamic acid, PMSF, DTT, calcium ionophore A23187, PMA, and Igepal CA-630 as well as other chemicals were supplied by Sigma Chemical (St. Louis, MO). Polyclonal antiserum directed against COX-2 was acquired from Cayman Chemicals (Ann Arbor, MI). The enhanced chemiluminescence substrate, Supersignal West Dura Extended Duration Substrate, was provided by Pierce (Rockford, IL). All other reagents were of analytical grade.

Cell culture

Caco-2 cells were kindly provided by Dr. David Thwaites (School of Cell and Molecular Biosciences, University of Newcastle upon Tyne, UK). The cells (passages 107–116) were routinely grown in 75 or 150 cm^2 plastic flasks at a density of 5×10^4 cells/cm^2 and cultured in DMEM supplemented with 4.5 g/l D-glucose, 1% (v/v) nonessential amino acids, 2 mM L-glutamine, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a modified atmosphere of 5% CO_2 in air. Cells were harvested with trypsin/EDTA and passed to 60 or 100 mm diameter plastic dishes, 12 mm plastic clusters, or 12 mm polycarbonate filters (Transwells) with a pore size of 0.4 μm. For growth on filters, cells were seeded at a density of 4×10^5 cells/cm^2. Growth medium was replaced twice per week and the day before the experiment. The experiments were performed in cells maintained for 3, 7, or 21 days in culture (preconfluent, confluent, or differentiated cells, respectively). Cell viability was estimated with ethidium bromide/acridine orange staining, as described by Parks et al. (20).

Transmission electron microscopy

Preconfluent, confluent, and differentiated monolayers grown on filters were prepared for transmission electron microscopy observation as described previously (21). Briefly, the filters were fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 h at 4°C and subsequently washed in 0.2 M PBS. The samples were postfixed in OsO_4 (1% in 0.2 M PBS), dehydrated in acetone, and further embedded in Spurr's embedding medium (22). Ultrathin sections (60 nm; Ultracut; Reichert-Jung) were stained with uranyl acetate and lead citrate, according to Reynolds (23), and examined with a Philips EM 301 electron microscope operating at 80 kV. Sample processing and observation were carried out at the Serveis Cientificotècnics of the Universitat de Barcelona.

Sucrase activity

Sucrase, a brush-border marker enzyme of cell differentiation, was assayed in accordance with Dahlqvist (24) in homogenates of cells cultured in clusters. The results were expressed as mU/mg protein.

Paracellular permeability

Paracellular permeability was estimated from transepithelial electrical resistance (TEER) and unidirectional apical-to-basal D-mannitol fluxes in cells maintained on filters. TEER was determined as described by Hidalgo, Raub, and Borchardt (8). Monolayers grown on filters were gently washed by sequential transfer through four beakers containing 500 ml of modified Krebs buffer (room temperature) containing (mM) 137 NaCl, 5.4 KCl, 2.8 $CaCl_2$, 1.0 $MgSO_4$, 0.3 NaH_2PO_4, 10 D-glucose, and

10 HEPES/Tris (pH 7.4). The filters were then placed in culture wells containing 1.5 and 0.7 ml of modified Krebs buffer in the basolateral and apical compartments, respectively, and TEER was determined using a Millicell-ERS voltohmmeter (Millipore, Bedford, MA). Results are expressed as Ω/cm^2 monolayer surface area. The resistance of the supporting membrane in filters was subtracted from all readings before calculations.

Unidirectional D-mannitol fluxes were determined in accordance with Thwaites et al. (25). After TEER determination, apical medium was replaced by the same volume of modified Krebs buffer containing 0.2 μCi/ml D-[2-^{3}H]mannitol, and the cells were incubated for 5 min at 37°C. At the end of the incubation, basal medium was withdrawn and radioactivity was counted in a scintillation counter (1500 Tri-Carb®; Packard, Downers Grove, IL).

Protein determination

Total protein was measured by the Bradford method (26) using the Bio-Rad protein assay (Hercules, CA) with BSA as a standard.

PGE_2 quantification

PGE_2 concentration was determined in the culture medium of monolayers maintained in dishes. To stimulate the cells, the cultures were maintained overnight without FBS and then incubated for 3 h with 10% FBS or with 1 μM PMA. At the end of the incubation, 250 μl aliquots of the incubation medium were acidified with 1 ml of formic acid (1%) and PGE_2 was extracted in ethyl acetate. After discarding the aqueous phase, the organic phase was evaporated under a stream of N_2. The overall recovery for the extraction procedure, established by including [^{3}H]PGE_2 (DuPont-New England Nuclear, Boston, MA), was estimated to be 88 ± 2% (mean ± SEM, n = 4). PGE_2 levels were determined with a PGE_2 monoclonal enzyme immunoassay kit (Cayman Chemicals) according to the manufacturer's protocol. Finally, results are expressed as pg PGE_2/mg protein.

Determination of PLA_2 activity

PLA_2 activity was assayed in cells maintained in clusters and stimulated as described above. At the end of the incubation with FBS or PMA, the medium was replaced by cold lysis buffer (200 mM Tris-HCl, 500 μg/ml aprotinin, 500 μg/ml leupeptin, 500 μg/ml PMSF, 400 μM NaF, and 400 μM Na_3VO_4) and the cells were scrapped off using a rubber policeman and briefly sonicated. PLA_2 activity was determined as described previously (27) using phosphatidylcholine-L-α-1-palmitoyl 2-arachidonyl [arachidonyl-1-^{14}C] as substrate (28). To discriminate between different PLA_2s, 0.5 mM DTT was added to the PLA_2 assay mixture to inactivate sPLA_2 (29), and BEL was added to inactivate iPLA_2 (30). These agents were solubilized in DMSO and diluted in medium. The final concentration of DMSO never exceeded 0.1%.

Incorporation and release of [^{3}H]AA

In cultures maintained in clusters, the medium was removed and replaced by 0.5 ml of DMEM containing 0.1% fatty acid-free BSA and 0.1 μCi of [^{3}H]AA (1 nM). Cells were incubated for 4 h and washed three times with 0.5% BSA-containing medium to remove unincorporated [^{3}H]AA. Cell lipids were then extracted with ethyl acetate (5 ml) acidified with 1% formic acid. The organic phase was evaporated under a N_2 stream, and the residue was resuspended in chloroform. Finally, an aliquot was separated by thin-layer chromatography in a solvent system consisting of chloroform-methanol-glacial acetic acid-water (100:30:35:3, v/v/v/v) to determine [^{3}H]AA incorporated into phospholipids.

To determine [^{3}H]AA release, [^{3}H]AA-labeled monolayers were stimulated with FBS, PMA, or A23187 in the presence or absence of BEL (10 μM). The medium was then removed to determine the amount of radioactivity released from the cells. Cells were solubilized with Triton X-100, and the radioactivity was quantified. The release of [^{3}H]AA was expressed as the percentage of total cell-incorporated radioactivity.

Measurement of the relative amounts of [^{3}H]AA metabolized

To determine the amount of [^{3}H]AA metabolized, cultures were incubated with [^{3}H]AA (0.1 μCi) for 2 h, and cell supernatant was extracted with 5 ml of ethyl acetate acidified with 1% formic acid. The organic phase was evaporated and resuspended in 100 μl of chloroform and purified by ascending chromatography on silica thin-layer plates using as a solvent the mixture diethyl ether-hexane-acetic acid (60:40:1, v/v/v), which resolves AA from different oxidation metabolites. Thin-layer plates were exposed to rhodamine B (0.2%) and activated at 100°C. [^{3}H]AA and [^{3}H]AA metabolites fluoresce under ultraviolet light. Finally, the radioactive spots were quantified by scratching and scintillation counting.

Western blot analysis

Cells grown in plastic dishes were washed twice with ice-cold PBS, scraped off into PBS containing 2 mM sodium EDTA, and pelleted. The pellets were then sonicated in PBS containing 4 mM sodium EDTA, 500 μg/ml aprotinin, 500 μg/ml leupeptin, 500 μg/ml PMSF, and 400 μg/ml diethyldithiocarbamic acid and resuspended in lysis buffer containing 200 mmol/l Tris-HCl, 200 mmol/l NaCl, 2% Igepal CA-630, 400 μM NaF, and 200 μM DTT. Immunoblot analysis for COX-2 was performed as follows: 20 μg of protein from cell lysate was separated by 7.5% SDS-PAGE (31) and blotted for 1 h at a constant voltage of 100 V onto a nitrocellulose membrane (Trans-Blot; 0.4 μm pore size; Bio-Rad) using a MiniProtean II system (Bio-Rad). A prestained SDS-PAGE protein standard (Bio-Rad) was used as a molecular weight marker to check transfer efficiency. Membranes were blocked with 5% nonfat milk powder in PBS-0.1% Tween 20 for 1 h. A rabbit polyclonal antiserum directed against COX-2 was applied at a dilution of 1:2,000 for 1 h. The specificity of antibody used was established previously (32). The blots were washed several times in PBS-0.1% Tween 20 and incubated with goat anti-rabbit antibody at a 1:2,000 dilution for 1 h. For β-actin immunoblotting, stripped membranes were overlaid with monoclonal anti-actin antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by rabbit anti-mouse antibody (1:1,000; Santa Cruz Biotechnology). Antibody binding was visualized by an enhanced chemical luminescence technique using Supersignal West Dura Extended Duration Substrate (Pierce) and Kodak Bio-Max Light-2 film (Rochester, NY).

Data analysis

Results are expressed as means ± SEM. Differences between groups were assessed using either Student's *t*-test or one-way ANOVA followed by the least significant difference test. $P < 0.05$ was considered significant.

RESULTS

PLA_2 activity and AA release during Caco-2 cell differentiation

After 21 days in culture, the cells showed a more developed and organized brush border and higher sucrase

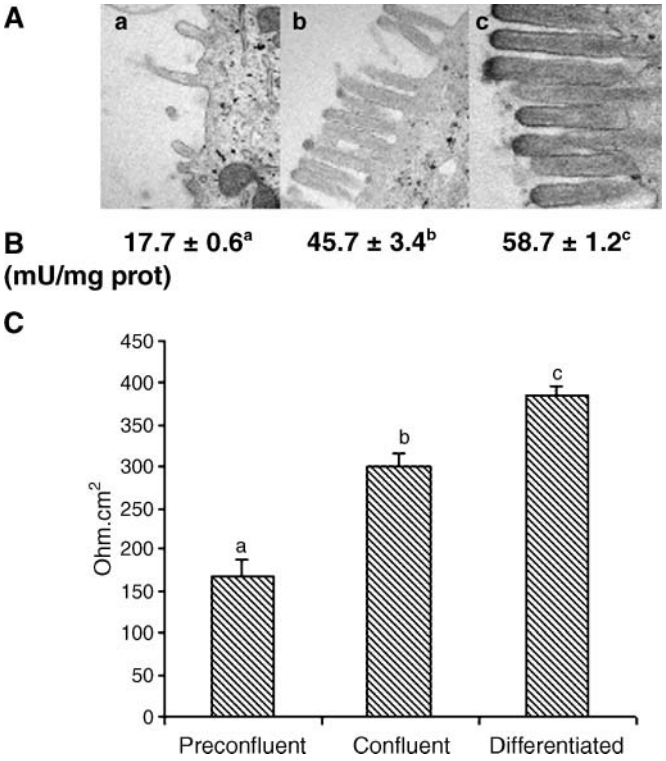

Fig. 1. A: Transmission electron micrographs of Caco-2 cell brush-border membrane from preconfluent (a; 3 days), confluent (b; 7 days), and differentiated (c; 21 days) cultures. B, C: Sucrase activity and transepithelial electrical resistance (TEER) in preconfluent, confluent, and differentiated Caco-2 cell monolayers. Results are expressed as means ± SEM (n = 6). Mean values labeled with different letters are significantly different ($P < 0.05$).

and TEER values (**Fig. 1**) compared with preconfluent and confluent monolayers, thus attaining a small intestine-like phenotype.

In a previous study, we observed that growing 3T6 fibroblasts release large amounts of prostanoids after stimulation by different means until the cells reach confluence (33). Similarly, preconfluent Caco-2 cultures stimulated with FBS (10%) or PMA (1 μM) produced significant amounts of PGE_2, which was reduced significantly after cell-cell interaction at confluence (**Fig. 2**). Interestingly, an additional reduction in PGE_2 release was observed in differentiated Caco-2 cell cultures only in response to PMA (Fig. 2). These results suggest that differentiation of Caco-2 cells may be related to changes in the AA cascade. To further investigate this possibility, PLA_2 activity was determined in control cells and after FBS or PMA stimulation at different growth/differentiation stages. The results show that total PLA_2 activity decreased markedly when Caco-2 cultures reached confluence, with a further decrease observed after differentiation (**Table 1**). In the presence of DTT, an inactivator of the $sPLA_2$ enzyme (34), no modifications were observed in PLA_2 activity in any of the culture conditions tested. However, PLA_2 activity in the presence of BEL, an irreversible $iPLA_2$ inhibitor (30), showed a decrease that was more pronounced in preconfluent cultures. These data suggest that preconfluent and confluent Caco-2 cultures mainly contain $cPLA_2$ and $iPLA_2$ activities, whereas differentiated cultures mainly contain $cPLA_2$ activity. Therefore, the changes observed can be correlated with a pronounced decrease in $iPLA_2$ activity in confluence, followed by a slight decrease until differentiation. These changes in PLA_2 activity appear to be essential for AA mobilization and show good correlation with [^{3}H]AA release (**Table 2**). Thus, [^{3}H]AA release induced by FBS, PMA, or A23187 in preconfluent cultures was higher than in differentiated cultures. Inhibition of $iPLA_2$ with BEL reduced [^{3}H]AA release in preconfluent cultures but had a minor effect on differentiated monolayers. Therefore, $iPLA_2$ activity is involved in AA mo-

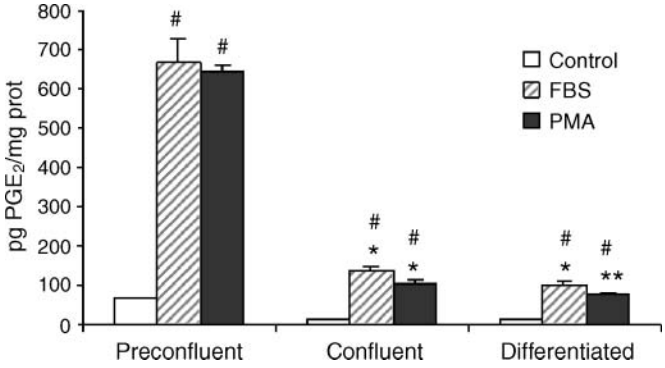

Fig. 2. Prostaglandin E_2 (PGE_2) concentration in the culture medium of preconfluent, confluent, and differentiated Caco-2 cell cultures. The cells were maintained overnight in DMEM without FBS and then stimulated for 3 h with 10% FBS or PMA (1 μM). PGE_2 in cell supernatant was determined by enzyme immunoassay. Results are expressed as means ± SEM (n = 6). # Significant difference ($P < 0.05$) with respect to control conditions. * Significant difference ($P < 0.05$) with respect to preconfluent conditions. ** Significant difference ($P < 0.05$) with respect to confluent cultures.

TABLE 1. PLA_2 activity in preconfluent, confluent, and differentiated Caco-2 cells

Treatment	No DTT or BEL	+DTT	+BEL
Control			
Preconfluent	83 ± 1.6 a	79 ± 1.3 a	62 ± 0.8 d
Confluent	65 ± 1.5 b	61 ± 1.2 b	57 ± 1.2 e
Differentiated	55 ± 1.0 c	51 ± 1.3 c	53 ± 1.1 c
FBS			
Preconfluent	158 ± 3.3 a,*	146 ± 2.6 a,*	67 ± 2.3 d
Confluent	91 ± 2.2 b,*	87 ± 2.1 b,*	65 ± 2.1 e,*
Differentiated	68 ± 2.0 c,*	65 ± 1.6 c,*	53 ± 1.6 f
PMA			
Preconfluent	167 ± 2.9 a,*	151 ± 3.0 a,*	71 ± 1.7 d,*
Confluent	102 ± 2.1 b,*	94 ± 1.6 b,*	75 ± 1.8 e,*
Differentiated	71 ± 1.4 c,*	64 ± 1.8 c,*	54 ± 1.2 f

BEL, bromoenol lactone; PLA_2, phospholipase A_2. Cells were maintained without FBS overnight and then incubated for 3 h with 10% FBS or PMA (1 μM). The cells were then harvested and frozen for the subsequent measurement of PLA_2 activity (pmol/mg protein/min) in the absence or presence of DTT (0.5 mM) or BEL (10 μM). Results are expressed as means ± SEM (n = 6). Different lowercase letters denote significant differences within control, FBS, or PMA conditions. Asterisks denote differences with respect to control values ($P < 0.05$).

TABLE 2. [^{3}H]AA release from preconfluent, confluent, and differentiated Caco-2 cells

Treatment	Preconfluent	Confluent	Differentiated
Control	5.4 ± 0.2	6.6 ± 0.4	7.2 ± 0.7
FBS	22.1 ± 0.7 a	20.5 ± 0.8 a	16.4 ± 0.8 c
FBS + BEL	12.4 ± 0.8 b	13.5 ± 0.9 b	14.4 ± 1.0 b,c
PMA	17.6 ± 0.6 a	11.5 ± 0.9 c	11.9 ± 1.1 c
PMA + BEL	11.5 ± 0.3 b	11.7 ± 0.3 b,c	11.5 ± 0.1 b,c
A23187	21.3 ± 1.0 a	18.6 ± 0.7 a	16.3 ± 0.1 b
A23187 + BEL	19.6 ± 1.1 a	17.4 ± 0.6 a	15.3 ± 0.2 c

[^{3}H]arachidonic acid (AA) release (% from total cell-incorporated radioactivity) was determined in cells labeled with [^{3}H]AA and stimulated for 3 h with FBS (10%), PMA (1 μM), or calcium ionophore A23187 (1 μM) in the presence or absence of BEL (10 μM). Results are expressed as means ± SEM (n = 6). Different lowercase letters denote significant differences within FBS, PMA, or A23187 treatments or within preconfluent, confluent, or differentiated conditions ($P < 0.05$).

bilization in nondifferentiated cultures but does not appear to be involved in differentiated Caco-2 cultures.

Caco-2 cell differentiation could depend upon the culture conditions, such as cell support. Thus, we compared [^{3}H]AA release using Caco-2 cultures on plastic clusters or polycarbonate filters. Preconfluent, confluent, and differentiated Caco-2 cells cultured on filters released 6.1, 6.9, and 6.3% in control conditions and 19.8, 18.3, and 14.85% in the presence of FBS, respectively. Because these results were similar to [^{3}H]AA release in cell cultures maintained on plastic (Table 2), we suggest that the different cell support used did not affect the variables determined in this study.

On the other hand, the impairment of [^{3}H]AA release in differentiated Caco-2 might be the consequence of a minor [^{3}H]AA incorporation. However, this possibility can be rejected because similar [^{3}H]AA incorporation to Caco-2 phospholipids was observed in the three culture conditions (**Table 3**).

TABLE 3. [^{3}H]AA incorporation and metabolization by preconfluent, confluent, and differentiated Caco-2 cells

Cells	[^{3}H]AA Incorporated to Membrane Phospholipids
	Dpm/μg protein
Preconfluent	1,720 ± 19
Confluent	1,693 ± 12
Differentiated	1,682 ± 25

Treatment	[^{3}H]AA Metabolized		
	Preconfluent	Confluent	Differentiated
	dpm/μg protein		
Control	203 ± 8.6 a	32 ± 2.1 d	27 ± 1.3 d
FBS	1,049 ± 24.0 b	218 ± 3.0 e	156 ± 3.5 f
PMA	1,302 ± 16.7 c	220 ± 5.8 e	162 ± 4.6 f

[^{3}H]AA incorporation into cell lipids was determined in preconfluent, confluent, and differentiated Caco-2 cultures incubated with 0.1 μCi of [^{3}H]AA for 4 h. [^{3}H]AA metabolism was determined in Caco-2 cultures incubated with FBS (10%) or PMA (1 μM) for 3 h. [^{3}H]AA was added, and cells were incubated for another 2 h. The medium was then removed, and [^{3}H]AA and [^{3}H]AA metabolites were separated by thin-layer chromatography and quantified by lipid scintillation spectroscopy. Different lowercase letters denote significant differences ($P < 0.05$).

COX-2 expression and AA metabolism during Caco-2 cell differentiation

The formation and release of prostanoids from Caco-2 cells is a multistep process requiring AA release and the subsequent enzymatic conversion of AA by COXs. To investigate the possibility that COX-2 expression induced by FBS or PMA decreased as cells progressed to confluence and/or differentiation, COX-2 expression was studied in these experimental conditions. **Figure 3** shows a significant decrease in COX-2 expression induced by FBS, from preconfluent to confluent cells, and a further decrease to differentiated cells. PMA-stimulated cells only showed a decrease from confluent to differentiated cells, one less pronounced than that detected in FBS-stimulated cells. On the other hand, we added [^{3}H]AA to preconfluent, confluent, and differentiated Caco-2 cells and measured [^{3}H]AA metabolism. The results show a significant decrease of [^{3}H]AA metabolism in confluent cultures and a further impairment in differentiated cells (Table 3). Together, these results suggest that the reduction in COX-2 expression observed in differentiated Caco-2 cells acts as a rate-limiting step in AA metabolism.

Relationship between PGE_2 levels and paracellular permeability in Caco-2 cultures

The data suggest that during differentiation of Caco-2 cells, AA release, PLA_2 activity, and COX-2 mass are decreased, leading to a decrease of PGE_2 synthesis. Taking into account that there were marked differences in PGE_2 concentrations in our experimental conditions (differentiated Caco-2 cultures presenting the lowest PGE_2 levels), we examined the effects of PGE_2-supplemented culture medium on paracellular permeability. The exogenous addition of PGE_2 to differentiated cells, at a PGE_2/protein

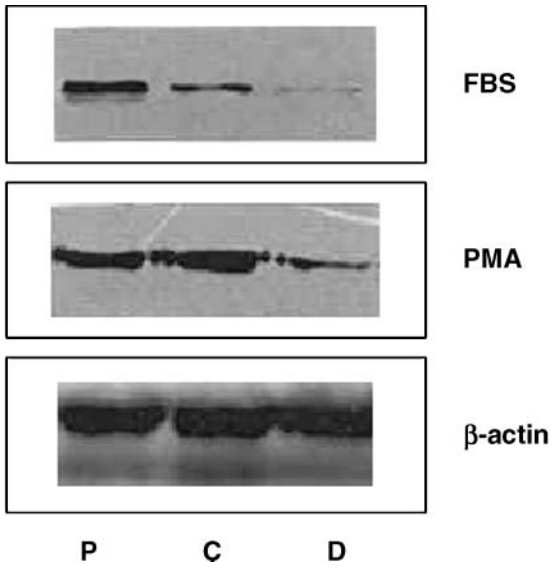

Fig. 3. Cyclooxygenase-2 (COX-2) levels in preconfluent (P), confluent (C), and differentiated (D) Caco-2 cell cultures. Monolayers were stimulated for 3 h with 10% FBS or PMA (1 μM) and scraped off to determine COX-2 mass. Results are representative of three Western blots. COX-2 values were normalized to β-actin expression.

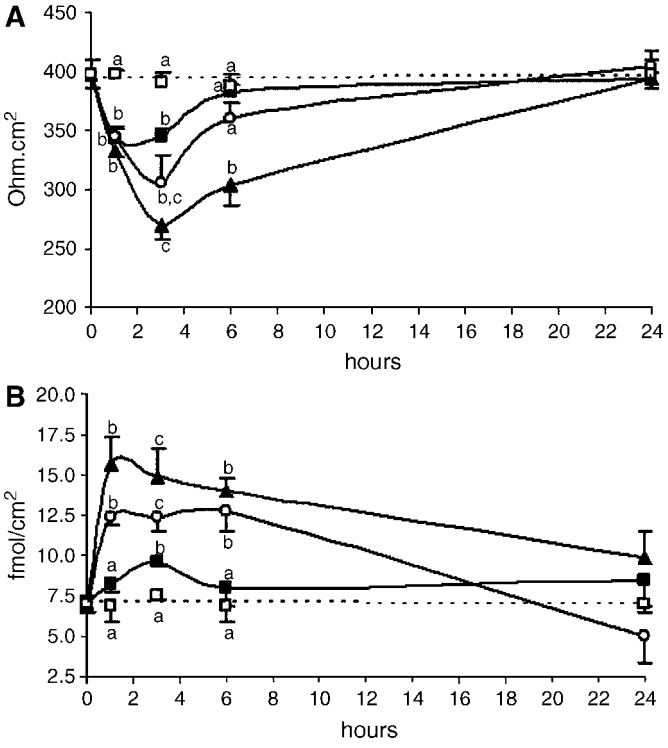

Fig. 4. TEER (A) and D-mannitol fluxes (B) in differentiated Caco-2 cells (open squares, control) incubated for 0–24 h with 10 (closed squares), 100 (open circles), or 1,000 (closed triangles) pg/ml PGE_2 in the apical and basolateral compartments. Ten and 100 pg/ml correspond to the PGE_2 concentrations found in confluent and preconfluent cultures, respectively. Results are expressed as means ± SEM (n = 4). Mean values labeled with different letters, within each incubation period, are significantly different ($P < 0.05$).

ratio detected in confluent or preconfluent cultures, decreased TEER and increased D-mannitol fluxes (**Fig. 4A, B**). These effects were dose-dependent and maximal after 1–3 h of incubation with this mediator. After 6 h of incubation, TEER recovery was almost complete, whereas D-mannitol fluxes needed a longer period to reach control values. The highest PGE_2 concentration tested (1,000 pg/ml), which would be more characteristic of an inflammatory process, induced the highest enhancement of paracellular permeability, which was completely recovered only after 24 h of incubation. Thus, PGE_2 appears to be involved in the regulation of paracellular permeability during Caco-2 cell differentiation. These data suggest that Caco-2 differentiation is related to significant changes in the AA cascade that are involved in the development of typical TEER and the paracellular permeability of differentiated epithelium.

DISCUSSION

As intestinal epithelial cells progress from the crypt-villus unit, they enter a terminal differentiation program that involves, in the case of absorptive cells, the cessation of proliferation and the polarization of morphological and functional features (6). In intestinal Caco-2 cells, this process is completed after 21 days in culture (8). In this study, we observed that Caco-2 cell differentiation is accompanied by marked changes in the AA cascade and that these events can be correlated with the development of epithelial barrier function.

Cell differentiation is characterized by the remodeling of phospholipid fatty acid composition, leading to an increase in polyunsaturated fatty acids, such as AA, and a decrease in monounsaturated and saturated fatty acids. These changes have been described as a general feature of intestinal cell differentiation (35, 36), recently confirmed in Caco-2 cells (37). Apparently, the fact that differentiated Caco-2 cells have more AA esterified to phospholipids than nondifferentiated cells but produce less PGE_2 remains something of a paradox. Our findings show that the impairment of PGE_2 synthesis during Caco-2 differentiation resulted from profound changes in the AA cascade, such as the decrease in PLA_2 activity and COX-2 expression and the consequent impairment of AA metabolism and release to PGE_2. Upon differentiation, which occurred 21 days postconfluence, the ability of Caco-2 cells to release [^{3}H]AA from their membranes decreased after stimulation with FBS, PMA, and A23187. The mechanism underlying this effect is not clear, although the results obtained with DTT and BEL indicate that BEL-sensitive $iPLA_2$ activity may be responsible for the impairment of AA release, whereas $cPLA_2$ activity was present in both nondifferentiated and differentiated Caco-2 cultures. In contrast, Bailleux et al. (38) reported that the postconfluence decrease in [^{3}H]AA release depends on $cPLA_2$ activity in the case of Madin-Darby canine kidney type II and Caco-2 cultures.

Thus, our results and previous findings demonstrating that $iPLA_2$ participates in the signaling pathways involved in the control of Caco-2 proliferation (18) suggest that $iPLA_2$ is involved in PGE_2 release in proliferative Caco-2 cells, whereas differentiation appears to depress this specific PLA_2 activity. Interestingly, the impairment of AA release in differentiated Caco-2 cells was observed in cultures triggered with both pharmacological agents (PMA and the calcium ionophore A23187) and receptor-mediated stimuli such as FBS growth factors. In this situation, AA release appeared as the main rate-limiting step in PGE_2 synthesis, which decreased in parallel with AA release in differentiated Caco-2 cells compared with nondifferentiated cells. Thus, our experimental results indicate that $cPLA_2$ is primarily responsible for AA release in differentiated Caco-2 cells, whereas $iPLA_2$ together with $cPLA_2$ may be involved in AA release in nondifferentiated cultures. $iPLA_2$ has been suggested to be involved in phospholipid remodeling, signal transduction, and apoptosis (for review, see 39), although, to our knowledge, this is the first report showing changes in BEL-sensitive $iPLA_2$ activity during epithelial differentiation. As mentioned above, nondifferentiated Caco-2 cells proliferate more rapidly than differentiated cells, whereas proliferating cells contain substantially less AA in their phospholipids than their differentiated counterparts (37). This has been attributed to

preferential β-oxidation of AA (40), although differentiation-associated decreases in PLA_2 activity could also be involved.

On the other hand, COX-2 was highly expressed in nondifferentiated cultures and strongly decreased with differentiation, suggesting that this downregulation is involved in Caco-2 cell differentiation. A similar modulating effect of COX-2 has been described for mucociliary differentiation in human tracheobronchial epithelial cells (41) and for adipose tissue differentiation (42). On the basis of our results, we propose that the impairment of $iPLA_2$ activity and COX-2 expression may be involved in the mechanisms that induce the impairment of [^{3}H]AA metabolism and consequently the decrease of PGE_2 release during Caco-2 differentiation.

Interactions of Caco-2 cells with the extracellular environment regulate a variety of cellular functions, such as cell differentiation. Furthermore, Caco-2 cells integrate a complex array of signals during this process. Butyrate and vitamin D_3 are examples of compounds that affect Caco-2 differentiation through overlapping or complementary pathways (43, 44). Our results suggest that prostaglandins such as PGE_2 could also be involved in Caco-2 cell differentiation. Thus, impairment of the AA cascade with a subsequent decrease in PGE_2 release could be necessary to complete Caco-2 differentiation, or at least to complete the development of the epithelial barrier function characteristic of differentiated Caco-2 cultures. In this way, Li et al. (45) observed that PGE_2 inhibits the expression of differentiation-related genes and regulates chondrocyte maturation.

Maintenance of barrier function in the intestinal epithelium is crucial for normal physiological homeostasis and is a characteristic of differentiated epithelia. For this reason, we were interested to determine whether prostanoids contribute to the regulation of this important function. Paracellular permeability was assessed in differentiated cultures by measuring TEER and D-mannitol fluxes. Our results show that the exogenous addition of PGE_2, in a concentration range similar to that found in nondifferentiated cultures, induced a decrease in TEER while conversely promoting permeability to D-mannitol. These results agree with those reported previously by Ma and Pedram (46), who observed that prostaglandins increase endothelial paracellular permeability, and by Resta-Lenert and Barrett (47), who proposed that COX-2 upregulation and prostaglandin release induced by enteroinvasive bacteria can modulate epithelial barrier function in Caco-2 cell cultures.

Increased levels of PGE_2 were described in the mucosa of patients with ulcerative colitis and Crohn's disease (48, 49), pathologies characterized by a defect in epithelial barrier function (50). The precise mechanism by which prostanoids modify this function, however, remains unclear. Increasing PGE_2 levels in differentiated Caco-2 cultures to the levels found in nondifferentiated Caco-2 cultures is sufficient to lessen the characteristic paracellular permeability noted in differentiated epithelium. To our knowledge, this is the first report showing the contribution of the AA cascade in Caco-2 differentiation, the role of BEL-sensitive $iPLA_2$ and COX-2 expression in AA release, and the role of PGE_2 production in the maintenance of the characteristic paracellular permeability occurring in differentiated Caco-2 cell cultures.

This study was supported by Grants BFI2001-3397, BFU2004-04960/BFI, and BFU2005-05899/BFI from the Ministerio de Educación y Ciencia and Grants 2001SGR00134 and 2005SGR0632 from the Generalitat de Catalunya. The authors are grateful for the valuable help of the Serveis Cientificotècnics of the Universitat de Barcelona. R.M-V. and S.R-P. are recipients of Recerca i Docència (Universitat de Barcelona) and Formació d'Investigadors (Generalitat de Catalunya) grants, respectively.

REFERENCES

1. Podolsky, D. K., and M. W. Babyatsky. 1995. Growth and development of the gastrointestinal tract. *In* Textbook of Gastroenterology. T. Yamada, editor. Lippincott, Philadelphia, PA. 546–577.
2. Potten, C. S. 1997. Epithelial cell growth and differentiation. II. Intestinal apoptosis. *Am. J. Physiol.* **273:** G253–G257.
3. Karam, S. M. 1999. Lineage commitment and maturation of epithelial cells in the gut. *Front. Biosci.* **4:** D286–D298.
4. Pageot, L. P., N. Perreault, N. Basora, C. Francoeur, P. Magny, and J. F. Beaulieu. 2000. Human cell models to study small intestinal functions: recapitulation of the crypt-villus axis. *Microsc. Res. Tech.* **49:** 394–406.
5. Cheng, H., and M. Bjerknes. 1985. Whole population cell kinetics and postnatal development of the mouse intestinal epithelium. *Anat. Rec.* **211:** 420–426.
6. Traber, P. G., and D. G. Silberg. 1996. Intestine-specific gene transcription. *Annu. Rev. Physiol.* **58:** 275–297.
7. Jumarie, C., and C. Malo. 1991. Caco-2 cells cultured in serum-free medium as a model for the study of enterocytic differentiation in vitro. *J. Cell. Physiol.* **149:** 24–33.
8. Hidalgo, I. J., T. J. Raub, and R. T. Borchardt. 1989. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology.* **96:** 736–749.
9. Grasset, E., M. Pinto, E. Dussaulx, A. Zweibaum, and J. F. Desjeux. 1984. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. *Am. J. Physiol.* **247:** C260–C267.
10. Vachon, P. H., and J. F. Beaulieu. 1991. Transient mosaic patterns of morphological and functional differentiation in the Caco-2 cell line. *Gastroenterology.* **103:** 414–423.
11. Mitic, L. L., and J. M. Anderson. 1998. Molecular architecture of tight junctions. *Annu. Rev. Physiol.* **60:** 121–142.
12. Madara, J. L., J. S. Trier, and M. R. Neutra. 1980. Structural changes in the plasma membrane accompanying differentiation of epithelial cells in human and monkey small intestine. *Gastroenterology.* **78:** 963–975.
13. Needleman, P., J. Turk, B. A. Jakschik, A. R. Morrison, and J. B. Lefkowich. 1986. Arachidonic acid metabolism. *Annu. Rev. Biochem.* **55:** 69–102.
14. Meade, E. A., W. L. Smith, and D. L. DeWitt. 1993. Expression of the murine prostaglandin (PGH) synthase-1 and PGH synthase-2 isozymes in COS-1 cells. *J. Lipid Mediat.* **6:** 119–129.
15. Herschman, H. R. 1994. Regulation of prostaglandin synthase-1 and prostaglandin synthase-2. *Cancer Metastasis Rev.* **13:** 241–256.
16. Dubois, R. N., S. B. Abramson, L. Crofford, R. A. Gupta, L. S. Simon, L. B. Van de Putte, and P. E. Lipsky. 1998. Cyclooxygenase in biology and disease. *FASEB J.* **12:** 1063–1073.
17. Rigas, B., I. S. Goldman, and L. Levine. 1993. Altered eicosanoid levels in human colon cancer. *J. Lab. Clin. Med.* **122:** 518–523.
18. Sheng, H., J. Shao, J. D. Morrow, R. D. Beauchamp, and R. N. Dubois. 1998. Modulation of apoptosis and Bcl-2 expression by prostaglandin E_2 in human colon cancer cells. *Cancer Res.* **18:** 362–366.
19. Sanchez, T., and J. J. Moreno. 2002. Calcium-independent phospholipase A_2 through arachidonic acid mobilization is involved in Caco-2 cell growth. *J. Cell. Physiol.* **193:** 293–298.

20. Parks, D. R., V. M. Bryan, V. T. Oi, and L. A. Herzenberg. 1979. Antigen-specific identification cloning of hybridomas with a fluorescence-activated cell sorter. *Proc. Natl. Acad. Sci. USA.* **76:** 1962–1969.
21. Mitjans, M., G. Barniol, and R. Ferrer. 1997. Mucosal surface area in chicken small intestine during development. *Cell Tissue Res.* **290:** 71–78.
22. Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26:** 36–43.
23. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17:** 208–212.
24. Dahlqvist, A. 1964. Method for assay of intestinal disaccharidases. *Anal. Biochem.* **7:** 18–25.
25. Thwaites, D. T., G. T. A. McEwan, C. D. A. Brown, B. H. Hirst, and N. Simmons. 1993. Na^+-independent, H^+-coupled transepithelial β-alanine absorption by human intestinal Caco-2 cell monolayers. *J. Biol. Chem.* **268:** 18438–18441.
26. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
27. Lloret, S., J. Martinez, and J. J. Moreno. 1995. Influence of calcium on arachidonic acid mobilization by murine resident peritoneal macrophages. *Arch. Biochem. Biophys.* **323:** 251–257.
28. Flesch, I., and E. Ferber. 1986. Effect of cellular fatty acid composition on the phospholipase A_2 activity of bone marrow-derived macrophages, and their ability to induce lucigenin-dependent chemiluminescence. *Biochim. Biophys. Acta.* **889:** 6–14.
29. Clark, J. D., N. Milona, and J. L. Knopf. 1990. Purification of a 110-kilodalton cytosolic phospholipase A_2 from the human monocytic cell line U937. *Proc. Natl. Acad. Sci. USA.* **87:** 7708–7712.
30. Hazen, S. L., L. A. Zupan, R. H. Weiss, D. P. Getman, and R. W. Gross. 1991. Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipase A_2. Mechanism-based discrimination between calcium-dependent and -independent phospholipase A_2. *J. Biol. Chem.* **266:** 7227–7232.
31. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
32. Martinez, J., T. Sanchez, and J. J. Moreno. 1997. Prostaglandin H synthase-2-mediated conversion of arachidonic acid in controlling 3T6 fibroblast growth. *Am. J. Physiol.* **42:** C1466–C1471.
33. Sanchez, T., and J. J. Moreno. 2001. Role of phospholipase A_2 in growth-dependent changes in prostaglandin release from 3T6 fibroblasts. *J. Cell. Physiol.* **189:** 237–243.
34. Street, I. P., H. K. Lin, F. Laliberte, F. Ghomashchi, Z. Wang, H. Perrier, N. M. Tremblay, Z. Huang, P. K. Weech, and M. H. Gelb. 1993. Slow- and tight-binding inhibitors of the 85 kDa human phospholipase A_2. *Biochemistry.* **32:** 5935–5941.
35. Alessandri, J. M., J. L. Joannic, and G. A. Durand. 1993. Polyunsaturated fatty acids as differentiation markers of rat jejunal epithelial cells: a modeling approach. *J. Nutr. Biochem.* **4:** 97–104.
36. Reynier, M., H. Sari, M. d'Angleberms, E. A. Kye, and L. Pasero. 1991. Differences in lipid characteristics of undifferentiated and enterocytic-differentiated HT29 human colonic cells. *Cancer Res.* **51:** 1270–1277.
37. Jindrichova, S., O. Novakova, J. Bryndova, E. Turzicka, V. Lisa, F. Novak, and J. Pacha. 2003. Corticosteroid effect on Caco-2 cell lipids depends on cell differentiation. *J. Steroid Biochem. Mol. Biol.* **87:** 157–165.
38. Bailleux, A., D. Wendam, F. Audubert, A. M. Jouniaux, K. Koumanov, G. Trugnan, and J. Masliah. 2004. Cytosolic phospholipase A2-p11 interaction controls arachidonic acid release as a function of epithelial cell confluence. *Biochem. J.* **378:** 307–315.
39. Winstead, M. V., J. Balsinde, and E. A. Dennis. 2000. Calcium independent phospholipase A_2: structure and function. *Biochim. Biophys. Acta.* **1488:** 28–39.
40. Barbieri, B., G. Alvelius, and N. Papadogiannakis. 1998. Lower arachidonic acid content and preferential beta-oxidation of arachidonic acid over palmitic acid in tumor cell lines as compared to normal lymphoid cells. *Biochem. Mol. Biol. Int.* **45:** 1105–1112.
41. Hill, E. M., T. Eling, and P. Nettesheim. 1998. Changes in expression of 15-lipoxygenase and prostaglandin-H synthase during differentiation of human tracheobronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **18:** 662–669.
42. Yan, H., A. Kermouni, M. Abdel-Hafez, and D. C. N. Lau. 2003. Role of cyclooxygenase COX-1 and COX-2 in modulating adipogenesis in 3T3-L1 cells. *J. Lipid Res.* **44:** 424–429.
43. Scaglione-Sewell, B. A., M. Bissonnette, S. Skarosi, C. Abraham, and T. A. Brasitus. 2000. A vitamin D_3 analog induces a G_1 phase arrest in Caco-2 cells by inhibiting cdk2 and cdk6: roles of cyclin E, $p21^{waf1}$, and $p27^{kip1}$. *Endocrinology.* **141:** 3931–3939.
44. Levy, P., H. Robin, F. Bertrand, M. Kornprobst, and J. Capeau. 2003. Butyrate-treated colonic Caco-2 cells exhibit defective integrin-mediated signaling together with increased apoptosis and differentiation. *J. Cell. Physiol.* **197:** 336–347.
45. Li, T. F., M. J. Zuscik, A. M. Ionescu, X. Zhang, R. N. Rosier, E. M. Schwarz, H. Drissi, and R. J. O'Keefe. 2004. PGE_2 inhibits chondrocyte differentiation through PKA and PKC signalling. *Exp. Cell Res.* **300:** 159–169.
46. Ma, T. Y., and A. Pedram. 1996. 16,16-Dimethyl prostaglandin E2 modulation of endothelial monolayer paracellular barrier function. *J. Cell. Physiol.* **167:** 204–212.
47. Resta-Lenert, S., and K. E. Barrett. 2002. Enteroinvasive bacteria alter barrier and transport properties of human intestinal epithelium: role of iNOS and COX-2. *Gastroenterology.* **122:** 1070–1087.
48. Andus, T., V. Gross, I. Casar, D. Krumm, J. Hosp, M. David, and J. Schölmerich. 1991. Activation of monocytes during inflammatory bowel disease. *Pathobiology.* **59:** 166–170.
49. Singer, I. I., D. W. Kawka, S. Schloemann, T. Tessner, T. Riehl, and W. F. Stenson. 1998. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. *Gastroenterology.* **115:** 297–306.
50. Schmitz, H., C. Barmeyer, M. Fromm, N. Runkel, H. D. Foss, C. J. Bentze, E. O. Riecken, and J. D. Schulzke. 1999. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterology.* **116:** 301–309.