PGE₂ inhibits apoptosis in human adenocarcinoma Caco-2 cell line through Ras-PI3K association and cAMP-dependent kinase A activation

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Submitted 22 December 2006; accepted in final form 13 July 2007

Leone V, di Palma A, Ricchi P, Acquaviva F, Giannouli M, Di Prisco AM, Iuliano F, Acquaviva AM. PGE₂ inhibits apoptosis in human adenocarcinoma Caco-2 cell line through Ras-PI3K association and cAMP-dependent kinase A activation. Am J Physiol Gastrointest Liver Physiol 293: G673–G681, 2007. First published July 19, 2007; doi:10.1152/ajpgi.00584.2006.—PGE₂ plays a critical role in colorectal carcinogenesis. We have previously shown that COX-2 expression and PGE₂ synthesis are mediated by IGF-II/IGF-I receptor signaling in the Caco-2 cell line and that the pathway of phosphatidylinositol 3-kinase (PI3K)/Akt protects the cell from apoptosis. In the present study, we demonstrate that PGE₂ has the ability to increase Ras and PI3K association and decrease the level of apoptosis in the same experimental system. The effect of PGE₂ on PI3K/Ras association is dependent on the activation of EP4 receptor, the increase of cAMP levels, and the activation of PKA. In fact, treatment of cells with the PKA inhibitor H89 decreases the association of Ras and PI3K and Ras-associated PI3K activity. PKA inhibitor H89 is able to decrease threonine phosphorylation of Akt and to increase serine phosphorylation of Akt by p38 MAPK and counteracts the cytoprotective effect induced by PGE₂. In addition, PGE₂ is able to activate p38 MAPK and the inhibition of p38 MAPK, with SB203580 specific inhibitor or with dominant negative MKK6 kinase, is able to revert the apoptotic effect of H89 and serine phosphorylation of Akt. The effect of PGE₂ on Caco-2 cell survival through PKA activation is mediated and regulated by the balance of threonine/serine phosphorylation of Akt by p38 kinase and PI3K. In conclusion, our data elucidate a novel mechanism for regulation of colon cancer cell survival and provide evidences for new combinatory treatments of colon cancer.

colon cancer; prostaglandin E₂; Akt; protein kinase A; phosphatidylinositol 3-kinase

PROSTAGLANDINS (PGs), one of the major groups of chemical mediators in the mammalian body, are involved in numerous physiological reactions, such as inflammation and cellular differentiation. PGs, especially PGE₂, also have strong cytoprotective effects on the gastric mucosa. Other physiological functions of PGs in gastrointestinal tract include the inhibition of gastric acid secretion, the stimulation of bicarbonate and mucus secretion, the maintenance of mucosal integrity, and increase in DNA synthesis (4, 12, 21, 32). Mounting evidences indicate that, among the metabolites of PGH₂, PGE₂ synthesized by cyclooxygenase-2 (COX-2) plays an important role as an early event in colorectal carcinogenesis. In fact, PGE₂ levels are markedly elevated in tissues of intestinal adenoma and colon cancer (7, 24). Moreover, knockout of the COX-2 gene results in 60% reduction of APC mutation-induced intestinal adenoma in mice (17). Reduced PG biosynthesis through the COX-2 activity is thought to be the molecular basis for the chemopreventive effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on colorectal carcinogenesis in both human and rodents (9, 23). PGE₂ may facilitate colon cancer progression by stimulating cell proliferation and survival, tumor cell invasiveness and production of angiogenic agents in colon cancer cells (2, 18, 26, 31). The molecular mechanism governing the inhibitory effect of PGE₂ on apoptosis in colon cancer cells has, however, yet to be completely elucidated. Although PGE₂ receptors have been pharmacologically subdivided into four mean subtypes (EP1, EP2, EP3, EP4), only EP2 and EP4 receptor have been demonstrated to be involved in the PGE₂ mediated protection of gastric mucosa cells from ethanol-induced apoptosis via cAMP-dependent PKA activation (11). Also, PGE₂-dependent prooncogenic actions have been demonstrated to be mediated through EP2 and EP4 receptor activation and cAMP increase (11, 34). Experimental evidence from our and other laboratories indicates that growth factors and their receptors play a critical role in colorectal carcinogenesis (13, 19, 37). The autocrine insulin-like growth factor-II/insulin-like growth factor-I receptor (IGF-II/IGF-Ir) signal transduction pathway stimulates proliferation and inhibits differentiation of cultured colon cancer cells (38). A large percentage of colon cancers expresses IGF-II and IGF-I receptor (39). A direct correlation between COX-2 and IGF-II expression has been demonstrated, thus suggesting that IGF-II/IGF-Ir-mediated growth and tumor progression may depend at least in part on the upregulation of COX-2 (5). Diverse extracellular stimuli, including growth factors, promote the formation of active GTP-Ras. GTP-Ras directly interacts with the Raf family of serine/threonine kinases and type I phosphatidylinositol 3-kinases (PI3K). Ras, moreover, stimulates cell growth in part by activation of the Raf/ERK pathway. In addition to its role in cell growth, Ras promotes cell survival through activation of Ras/ERK and PI3K/Akt cascades; it has also been demonstrated that ERK activity appears to be required for induction of COX-2 by Ras (33), whereas Akt/PKB activity is required for Ha-Ras-mediated transformation of intestinal epithelial cells (29). PGE₂ can modulate the growth factor-dependent transduction pathways at the early stage of carcinogenesis and adjust the magnitude and duration of growth factor signals. In fact, it has been demonstrated that PGE₂ transactivates EGF receptor (EGFR) and triggers mitogenic signaling in gastric

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epithelial and colon cancer cells, as well as in rat gastric mucosa in vivo (39). Recently, it has been reported that PGE2 and EGFR/Ras/MAPK system synergistically induce the expression of pronoecogenic gene amphiregulin, a member of the EGF family, which stimulates proliferation of colon cancer cells through an autocrine mechanism (25). In this study, we examine the molecular machinery involved in the PGE2-mediated survival of Caco-2 colon cancer cells. Our data show that PGE2 inhibit apoptosis induced by serum deprivation through activation of cAMP-dependent protein kinase A (PKA) and stabilization of Ras-Pi3K association. We also demonstrate that inhibition of PKA decreases the Ras-Pi3K association and the threonine phosphorylation of Akt, increases the activity of p38 MAPK and serine phosphorylation of Akt by p38 MAPK and reverts the cytoprotective effect of PGE2.

In conclusion, PKA is able to stabilize the Ras/Pi3K complex and to balance serine/threonine phosphorylation of Akt by modulating the Pi3K p38 MAPK activity.

MATERIALS AND METHODS

Cell growth and culture. Caco-2 cells were routinely grown in 100-mm plastic dishes at 37°C in a humidified atmosphere of 5% CO2 in air in DMEM supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) and buffered with HEPES (20 mM). Caco-2 cells were seeded at 5 × 10^4 cells/ml and were routinely subcultured when ~80% confluent. The culture medium was changed every other day. Cells were serum withdrawn 3 days after plating at the beginning of the proliferative phase of the growth. Drugs were added for 24 h after 48 h of the serum withdrawal. H89 (Calbiochem, Darmstadt, Germany) was dissolved in DMSO and prepared as an 8 mM stock solution and added at the final concentration of 10 μM. PGE2 (Sigma) was dissolved in ethanol and prepared as a 1 mM stock solution and added at the final concentration of 2.5 μM. LY294002 (Calbiochem, Darmstadt, Germany) was dissolved in DMSO and prepared as a 16.2 mM stock solution and added at the final concentration of 50 μM. PD98059 (Calbiochem, Darmstadt, Germany) was dissolved in DMSO and prepared as an 8 mM stock solution and added at the final concentration of 40 μM. SB203580 p38 inhibitor (Calbiochem, Darmstadt, Germany) was dissolved in DMSO and added at the final concentration of 5 μM. ONO-AE1-329 (an EP4 agonist) was gift kindly provided by Ono Pharmaceutical (Osaka, Japan) and was dissolved in DMSO and prepared as a 10 mM stock solution and added at the final concentration of 1 μM.

Transfection. Cells were seeded at 5 × 10^4 cells/ml and after 24 h transfected by the Lipofectamine Plus procedure as indicated by the manufacturer ( Gibco-BRL, Life Technologies, Milan, Italy). Stable transfectants were isolated as single clones in the presence of 0.4 mg/ml G418 during the course of all the experiments. The plasmid with dominant negative of MKK6K was kindly provided by Dr. Mario Chiariello, Istituto di Endocrinologia ed Oncologia Sperimentale G. Salvatore, Naples, Italy. The efficiency of transfection was tested by Western Blot with anti-GST antibody.

Apoptosis detection. To define the level of apoptosis, Caco-2 cells were trypsinized, pelleted, fixed, and propidium iodide (PI) stained. PI staining fluorescence of individual cells was analyzed by using a FACSCaliber flow cytometer apparatus (Becton-Dickinson, Mountain View, CA) and the MODFIT analysis software. For each sample, at least 20,000 events were stored. Each experiment was repeated at least three times.

Western blot analysis. Cells were washed in cold PBS and lysed for 10 min at 4°C with 1 ml of lysis buffer (50 mM Tris, pH 7.4, 0.5% NP40, 0.01% SDS) containing complete protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates from adherent cells were obtained by scraping and centrifuging cells at 12,000 g for 15 min at 4°C. The supernatants were collected and protein concentration in cell lysates was determined by Bio-Rad Protein Assay (Bio-Rad, Richmond, CA) and 70 μg of total protein from each sample was analyzed. Proteins were separated by a 12% SDS-PAGE and transferred on nitrocellulose membrane (Hybond-ECL Nitrocellulose, Amersham, Rainham, UK). The membrane was blocked with TBS-Tween 0.1% containing 5% nonfat dry milk for 1 h at room temperature. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4°C. Mouse monoclonal anti-p-ERK1/2 and anti-p38 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:1,000 in PBS-Tween 0.1%, BSA 5%; anti-phosphoserine 473 Akt (Cell Signaling), anti-phosphothreonine 308 (Upstate, Lake Placid, NY), and anti-Akt (Cell Signaling) rabbit polyclonal antibodies were diluted 1:1,000 in 1 × PBS in the presence of 0.1% Tween and 5% BSA. After the incubation, the membranes were washed six times with PBS-Tween 0.1% and were incubated with the corresponding secondary antibody conjugated to horseradish peroxidase (Amersham, Buckinghamshire, UK). After incubation, the blots were washed six times with TBS-Tween 0.1% and were incubated with 50 μl of the peroxidase substrate hydrogen peroxide and 5 mg/ml diaminobenzidine (DAB) in 0.1 M Tris-HCl, pH 7.5, for 5 min. Western blot analysis. Cells were washed in cold PBS and lysed for 10 min at 4°C with 1 ml of lysis buffer (50 mM Tris, pH 7.4, 0.5% NP40, 0.01% SDS) containing complete protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates from adherent cells were.
with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Bio-Rad, Richmond, CA) diluted 1:2,000 in PBS, 0.2% Tween, the membranes were washed, and protein bands were detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Uppsala, Sweden). For quantization of immunoblots, relative intensities of bands were quantified by densitometry with a desk scanner (Pharmacia Discovery System) and RFLPrint software (PDI).

Immunoprecipitation. Fresh cells lysates (300 µg) were incubated overnight with 10 µl of pan-Ras antibody (Oncogene, Boston, MA) or with 5 µl of p38 antibody (Santa Cruz Biotechnology) at 4°C. After the incubation, protein A-G Sepharose was added for 1 h. Pellets were washed in cold lysis buffer 5–6 times and resuspended in Laemmli 2× for Western blot analysis.

PI3K assay. Fresh cells lysates (300 µg) were incubated overnight with 10 µl of pan-Ras antibody (Oncogene) or 3 µl of PI3K p85 antibody (Upstate Biotechnology) at 4°C. After the incubation, protein A-G Sepharose was added for 1 h. Pellets were washed in cold lysis buffer, then in 100 mM Tris-HCl (pH 7.4) supplemented with 500 mM LiCl, 1 mM EDTA, and 0.2 mM NaVO4. Pellets were further resuspended in 30 mM HEPES (pH 7.5) and 6.25 mM MgCl2 and 125 µM cold ATP, the kinase reaction was initiated by addition of 2 µg/µl phosphatidylinositol (Sigma, Milan, Italy) and 10 µCi γ32P-ATP (3,000 Ci/mmol) and performed for 15 min at 37°C; the reaction was stopped by the addition of 5 M HCl and 0.5 M EDTA and methanol-chloroform (1:1). After mixing vigorously and centrifuging to separate the phases, the organic layer was collected and separated by thin-layer chromatography. [32P]phosphoinositides were visualized by autoradiography. The autoradiographs were quantified by a PhosphorImager using the program ImageQuant (Amersham Pharmacia Biotech).

Statistical analysis. Statistical comparisons were performed by the Mann-Whitney U-test. A P value < 0.05 was considered a significant difference.

RESULTS

PGE2 through CAMP/PKA inhibits apoptosis in Caco-2 cells and stabilizes the association of Ras with PI3K. We have previously demonstrated that IGF-II peptides were secreted at high levels in the medium collected from actively proliferating Caco-2 cells after 48 h of serum deprivation and that IGF-II through the IGF-1 receptor pathway upregulated COX-2 mRNA expression and PGE2 synthesis (5, 37). Our data, also, demonstrated that IGF-II/IGF-Ir reduced apoptosis through the activation of PI3K pathway and the inhibition of this pathway induced apoptosis and decreased PGE2 levels (5, 22).

To understand how PGE2 can cooperate to induce proliferation and inhibit apoptosis in Caco-2 cells, we analyzed the level of PGE2 produced in Caco-2 cells after 72 or 168 h of serum deprivation. The intracellular PGE2 concentrations were 326 and 125 pg/mg after 72 and 168 h of serum deprivation, respectively, whereas no detectable amounts of PGE2 were found in the medium at the same time points (data not shown). Also, the addition of exogenous PGE2 was able to induce by twofold the proliferation of Caco-2 cells that have been serum deprived for 72 and 168 h (data not shown). To demonstrate that exogenous PGE2 was able to inhibit apoptosis induced by serum deprivation and that the effect was mediated by the PGE2 cooperation with the autocrine loop of IGF-II on the
activation of PI3K and MAPK, we analyzed the effect of exogenous PGE2 addition on apoptosis and MAPK and PI3K kinase pathways activation in Caco-2 cells after 72 h of serum deprivation. As shown in Fig. 1A, the addition of exogenous PGE2 at 2.5 μM decreased by 50% the levels of apoptosis, whereas the treatment with PI3K inhibitor (LY) or with competitive inhibitor of EP4 PG receptor (ONO-AEI-329) was able to revert the cytoprotective effect of PGE2. The treatment with MAPK inhibitor was not able to revert the effect of PGE2. In these experimental conditions, PGE2 increased twofold the levels of Ser-p-Akt, whereas this increase was reverted by PI3K inhibitor (LY) or EP4 inhibitor (ONO-AEI-329) (Fig. 1B). To demonstrate that the effect of PGE2 was mediated by receptors coupling G(s) adenylate cyclase, we analyzed the effects on serine Akt phosphorylation following treatment with 10 μM forskolin or forskolin plus H89. We obtained the same effect of PGE2, thus suggesting that the PGE2 effect might be mediated by cAMP-dependent PKA activation (data not shown). Concordantly, exogenously added PGE2 increased 2.5-fold the levels of pERK-1/2, whereas the effect was abolished by the addition of EP4 inhibitor and inhibited by 50% in the presence of PI3K or MAPK inhibitors (Fig. 1B).

Since it is known that the effects of PGE2 through EP4 receptor were mediated by cAMP-dependent PKA, we decided to analyze the effect of PKA on PI3K and Akt activation. To analyze the total PI3K activity and Ras-associated PI3K activity, we immunoprecipitated the lysates of cells treated with PGE2 or PGE2 plus PKA inhibitor (H89) with p85 and pan-Ras antibody. As shown in Fig. 2A, exogenous PGE2 slightly increased the association of p85 with Ras, whereas basal and PGE2-induced levels of p85 regulatory subunit of PI3K associated with Ras decreased by fourfold by treatment with PKA inhibitor H89 (Fig. 2A). On the other hand, the total PI3K activity increased following treatment with PGE2 alone or in combination with H89 PKA inhibitor, whereas PI3K activity associated with Ras increased following PGE2 treatment and decreased in the presence of H89 (Fig. 2B). Moreover, the ratio of Ras-associated PI3K activity increased by 10.220.32.246 on October 5, 2017 http://ajpgi.physiology.org/ Downloaded from

Fig. 3. Effect of PGE2 treatments, alone and with H89, on Akt and ERK1/2 phosphorylation in Caco-2 cells. Caco-2 cells were cultured in the absence of serum for 48 h and then treated with 2.5 μM PGE2, alone or in combination with H89 at 10 μM for 24 h. Western immunoblot analysis using anti-(Ser 473) phosphorylated Akt and pERK1/2 antibody was performed on protein lysates from cells cultured in the absence of serum for 48 h and incubated for 24 h with the above-indicated drugs. The immunoblots were stripped and rebotted with antibodies against total Akt and ERK2 protein. Representative autoradiographs are shown. Histograms represent the mean densitometric analysis of 3 separate experiments ± SD.

Fig. 4. Effect of PGE2 treatments, alone and with H89 on apoptosis of Caco-2 cells. Apoptosis analysis was performed on cells cultured in the absence of serum for 48 h, following treatment with PGE2, alone and with H89. Apoptosis was calculated as the percentage of cells showing a subdiploid DNA peak, as described in MATERIALS AND METHODS. Data are expressed as means ± SD.

Fig. 5. Effect of PGE2 treatments, alone and with H89 on glycogen synthase kinase (GSK) phosphorylation. Caco-2 cells were cultured in the absence of serum for 48 h and then treated with 2.5 μM PGE2, alone or in combination with H89 at 10 μM for 24 h. Western immunoblot analysis using anti-phosphorylated GSK antibody was performed on protein lysates from cells cultured in the absence of serum for 48 h and incubated for 24 h with the above-indicated drugs. The immunoblots were stripped and rebotted with antibodies against total GSK protein. Representative autoradiographs are shown. Histograms represent the densitometric analysis of the representative autoradiographs.
Inhibition of PKA-mediated Ras/PI3K association induces inhibition of Akt function through unbalance between serine and threonine phosphorylation. To understand why in the presence of PKA inhibitor there was a strong serine activation of Akt, but this activation was not correlate with cytoprotective effect on apoptosis, we evaluated the activation of glycogen synthase kinase 3 (GSK3) as Akt target. We analyzed the level of GSKo/β phosphorylated in the cells after treatment with PGE2 and PGE2 plus H89. As shown in Fig. 5, PGE2 was able to double the phosphorylation of GSKo/β, whereas the presence of H89 was able to decrease or abolish the PGE2 effect on the phosphorylation of GSKo/β and to increase the serine phosphorylation of Akt (Fig. 6). To understand why the strong serine activation of Akt did not correlate with the activity of Akt and with induction of apoptosis, we analyzed the level of threonine phosphorylation in the presence of H89. As shown in Fig. 6, PGE2 increased by 1.5-fold the level of phosphoserine Akt and by 2.5- to 3-fold the level of phosphothreonine Akt. On the contrary, H89 and PGE2 plus H89 decreased the level of threonine phosphorylated Akt by 80% but increased by 2.5-fold the level of serine phosphorylated Akt. PGE2 can mediate several effects through cAMP or p38 MAPK and some studies report that p38 MAPK is able to phosphorylate in serine Akt (8). To demonstrate that the increase of serine phosphorylation by H89 was mediated by p38 activation, we analyzed the level of serine phosphorylation in Akt in the presence of H89 and SB203580, specific inhibitor of p38-MAPK. As shown in Fig. 7, the inhibitor of p38-MAPK alone and in presence of PGE2 did not have any effect on serine phosphorylation of Akt and on apoptosis levels but was able to revert the increase of phosphoserine Akt and apoptosis mediated by H89.

To further demonstrate that the treatments with PGE2 plus PKA and p38 inhibitors did not affect cell viability, we performed these treatments at different time points. As shown in Fig. 8, we obtained the same effects at Akt phosphorylation.
after 30 min or 1 h of treatment with PGE2 and PGE2 plus SB. To evaluate the effect of PGE2 on p38 activation, we analyzed the level of phosphorylated p38 after treatment with PGE2. As shown in Fig. 9A, PGE2 induced a 2.5-fold increase of p38 phosphorylation. In addition, to demonstrate that the effect of inhibition of p38 MAPK by pharmacological antagonist was specific, we performed the same experiments in cells transfected with purified recombinant full-length human MKK6 kinase mutated in S207/T211 and with an amino-terminal GST tag. The stable clones were treated with PGE2 and PGE2 plus H89. As shown in Fig. 9B, the effect of PGE2 on Akt phosphorylation was lower in transfected cells than in untransfected cells and PGE2 plus H89 in transfected cells showed 50% of inhibition of serine phosphorylation of Akt respect to untransfected cells. To evaluate the p38 MAPK activity and the levels of p38-associated phosphoserine Akt, we immunoprecipitated cell lysates with p38 antibody and we analyzed through Western blot analysis the level of Akt and phosphoserine Akt. As shown in Fig. 10, the level of phosphoserine Akt associated with p38 MAPK following treatment with H89 or H89 plus PGE2 increased with respect to control and PGE2 alone. Figure 11 shows how PGE2 may modulate the PI3K and p38 pathway through cAMP PKA activation.

DISCUSSION AND CONCLUSIONS

Upregulation of COX-2 and PG-dependent effects is an essential part of colon carcinogenesis. Emerging clinical and experimental evidence now supports a potent antineoplastic efficacy of NSAIDs in colon cancer (1). NSAIDs induce apoptosis in intestinal epithelial cells through COX-2 inhibition, although some reports have proposed that COX-independent events might mediate the effects of NSAIDs (10, 36, 40). Among the COX-dependent events, production of PGs is likely to be a critical element in mediating the functions of COX-2 in carcinogenesis, as demonstrated by studies in genetically engineered mutant mice (39). Mice lacking EP2 and EP4 PGE2 receptor have a markedly decreased colon cancer incidence in chemically induced or genetic models (34). Furthermore, production of PGE2 is increased in human and experimental colon cancers (22). Growth factors can inhibit apoptosis induced by a variety of stimuli through Ras signaling, and protection against apoptotic stimuli by Ras is thought to contribute to tumor development. Several studies, moreover, have shown that PI3K, a direct downstream effector of Ras, plays a crucial role in Ha-Ras-mediated transformation of intestinal epithelial cells (40).
Our previous data demonstrated that IGF-II/IGF-Ir is able to upregulate COX-2 expression and PGE2 synthesis through the PI3K activation pathway (5). It has been demonstrated that PGE2 transactivates EGFR and triggers mitogenic signaling in gastric epithelial and colon cancer cells as well as in rat gastric mucosa in vivo (38). In addition, the COX-2/PGE2 signaling pathway and the receptor tyrosine kinase-dependent signaling system promote the growth of colon cancer in a synergistic fashion (22). In this study we demonstrate that PGE2 is able to inhibit apoptosis induced by serum deprivation through PI3K activation and thus the inhibition, at different levels, of PI3K pathway is able to revert this protective mechanism. Cumulative evidence suggests that COX-2 is activated through a Ras-dependent pathway (30, 35, 40). In addition, the inhibition of COX-2 activity suppresses the growth of xenograft of Ras-transformed rat intestinal epithelial cells in nude mice (28). Also, PGE2 synergistically enhance Ras-induced transcription of the prooncogenic gene amphiregulin (22), whose overexpression in colorectal tumor has been well investigated (3, 16). These findings suggest that COX-2-generated PGs may contribute to Ras-mediated transformation of intestinal epithelial cells through different mechanisms. It has clearly been demonstrated that EP2 and EP4, the major PGE2 receptor on intestinal epithelial cells, mediate their activity through increase in cAMP production, and it has been demonstrated that increased cellular cAMP stimulates the expression of COX-2 and vascular endothelial growth factor in the stroma (3). In addition, one possible mechanism for COX-2 inhibition of apoptosis is the induction of cAMP-dependent cellular inhibitor of apoptosis (IAP2) and the induction of amphiregulin transcription via the cAMP/PKA pathway mediated by PGE2 signaling (15). The observation that cAMP suppresses apoptosis in transformed colon epithelial cells suggests that this second messenger might play a role in the formation of colorectal adenoma and cancer. Whether the antiapoptotic function of cAMP agonists, including PGE2, can interfere with activation of proliferative signals has not been elucidated yet. We demonstrate that exogenous PGE2 is able to induce proliferation in colon carcinoma Caco-2 cell line and protect them by serum deprivation-induced apoptosis, increasing Ras/PI3K association and the activation of p-Akt and MAPK. Also, the effect of PGE2 is mediated by PKA. In fact, H89, a PKA-selective inhibitor, is able to decrease the association of Ras with PI3K and to revert the cytoprotective effect. Recent studies suggest that cAMP and PKA participate in the regulation of growth factor signaling by interfering with activation of the Raf1 Ras-mediated pathway or by regulating several phosphatases that act to dephosphorylate various components of the Ras-ERK pathway (20). In our experimental system, the inhibition of PKA in the presence of PGE2 decreases cell proliferation and MAPK activation but strongly increases activation of Akt. However, the activation of Akt does not correlate with cell survival and with serine 9 phosphorylation of GSK, the substrate of Akt. We demonstrate that inhibition of PKA is able to reduce the Akt-threonine phosphorylation and, conversely, to increase the Akt-serine phosphorylation and this mechanism may modulate the Akt activity. We show that PGE2 plus H89
treatment activates serine Akt phosphorylation and induce apoptosis. The proapoptotic effect of H89 might be dependent on the unbalance between the presences of higher serine than threonine phosphorylation. It has been demonstrated that PGE₂ inhibits leukotriene synthesis through inhibition of translocation of 5-lipoxygenase. The PKA inhibitor allows p38 phosphorylation and 5-lipoxygenase translocation and activity in thapsigargin-stimulated neutrophils (6). Also, it has been demonstrated that cAMP, through ERK1/2 and p38 MAPK, promotes CREB-dependent induction of cellular inhibitor apoptosis protein 2 (14). The data presented herein demonstrate that treatment of Caco-2 cells with p38 kinase inhibitor or transfection with MKK6 kinase mutated in S270/TR211 decreases the serine phosphorylation of Akt in the presence of H89. The effect of H89 is decreased only by 50% in the presence of dominant negative MKK6K, and this may be related to p38 activation by MKK3 kinase. On the basis of the above findings, it is possible to postulate that PKA inhibitor-dependent apoptosis may be mediated through p38 kinase activation. In support of this hypothesis, it has been recently shown that p38 activation is able to increase the levels of phosphoryserine Akt-2 and that modulation of PI3K pathways in myogenesis regulate p38 activity (8). We also demonstrate that the H89-dependent increase of Akt serine phosphorylation is associated to p38 MAPK. Additional experiments will be necessary to confirm the hypothesis that Ras/PI3K association may modulate the activation of p38 kinase in colon cancer cells. In conclusion, our study suggests that, through PKA, PGE₂ can enhance or stabilize Ras/PI3K association and modulate the Akt phosphorylation and, thus, the cell survival, and this may be one mechanism responsible for the involvement of COX-2 in Ras-mediated transformation (4, 30, 40) and PGE₂ in Ras-mediated activation of peroxisome proliferator-activated receptor-γ (27).

ACKNOWLEDGMENTS
We thank Maria Grazia Catenacci for the artwork and Rita Cerillo for technical assistance.

GRANTS
This work was supported in part by a grant from Ministero dell’Università e della Ricerca Scientifica e Tecnologica, Italy.

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