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Aspirin Protects Caco-2 Cells from Apoptosis after Serum Deprivation through the Activation of a Phosphatidylinositol 3-Kinase/AKT/p21$^{Cip/WAF1}$ Pathway

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ABSTRACT

Our previous studies indicated that millimolar doses of aspirin induced growth arrest and resistance to anticancer drug treatment in Caco-2 cells. The present study was designed to better elucidate at the molecular level the effect of aspirin treatment on pathways that regulate cell death during serum withdrawal. Caco-2 cells were cultured under serum deprivation in the presence or absence of aspirin. Effects on cell cycle, phosphatidylinositol 3-kinase (PI3-kinase) and mitogen-activated protein (MAP) kinase pathways were investigated. We found that aspirin, but not the selective cyclooxygenase-2 inhibitor *N*-[2-(cyclohexyloxyl)-4-nitrophenyl]-methane sulfonamide (NS-398); prevented apoptosis and G_2/M transition after prolonged Caco-2 cells serum deprivation. Aspirin-dependent inhibition of apoptosis and G_2/M transition was prevented by treatment with the PI3-kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), but not with the MAP kinase kinase inhibitor 2′-amino-3′-methoxyflavone (PD98059). The effects of aspirin were mediated at molecular levels, through activation of PI3-kinase/AKT pathway and increase in the p21$^{Cip/WAF1}$ level. The ability of aspirin to activate AKT protein was observed also in presence of etoposide cotreatment. Our data indicate a new intracellular target of aspirin with potential clinical impact for treatment schedules involving both anticancer agents and aspirin in malignancies.

Aspirin is one of the most widely used drugs in the world. Recently, it has been suggested to use aspirin to prevent intravascular thrombosis, reduce Alzheimer disease, and prevent colon cancer. The molecular effects of aspirin vary with dose being, at the millimolar range, also independent on its well known ability to inhibit cyclooxygenase (COX) and prostaglandin synthesis. Because high concentrations of aspirin are achievable "in vivo" during treatment of rheumatic disorder and arthritis (Insel, 2001), an expanding literature is evaluating additional, COX-independent, molecular targets for these high doses of aspirin. Aspirin and its deacetylated metabolite salicylate can inhibit the activation of nuclear factor-κB by preventing the phosphorylation and degradation of the inhibitory subunit IκB (Yin et al., 1998); aspirin and salicylate can modulate gene transcription (Xu et al., 1999), the activity of several protein kinases, and other molecular pathways as well (Dong et al., 1997; Pillinger et al., 1998).

Numerous studies "in vivo" and "in vitro" have focused on the property of salicylates, in concentrations between 1 and 5 mM, to inhibit cell proliferation and DNA synthesis in a number of normal and transformed cell types (Aas et al., 1995; Cercek et al., 1997). The molecular mechanisms responsible for these antiproliferative effects have been occasionally investigated. It has been shown in vascular smooth cells that salicylate induced cell cycle arrest mainly by the inhibition of Cdk-2 activity and retinoblastoma protein phosphorylation and the induction of p21$^{Cip/WAF1}$, without identifying the specific pathway involved (Marra et al., 2000). In human embryonic kidney 293 cells, salicylate-induced growth arrest was associated with inhibition of p70s6k and down-regulation of c-myc, Cyclin D1, Cyclin A, and proliferating cell nuclear antigen (Law et al., 2000).

We have previously shown that aspirin treatment inhibited dose dependently the growth and induced differentiation of the human colon adenocarcinoma cell line Caco-2 (Ricchi et

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ABBREVIATIONS: COX, cyclooxygenase; PI3-kinase, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein; FITC, fluorescein isothiocyanate; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; TLC, thin layer chromatography; MEK, mitogen-activated protein kinase kinase; IGF, insulin-like growth factor; NS-398, *N*-[2-(cyclohexyloxyl)-4-nitrophenyl]-methane sulfonamide; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PD98059, 2′-amino-3′-methoxyflavone.

al., 1997). Also, we have recently demonstrated that aspirin treatment at millimolar concentration significantly prevented apoptosis and G_2/M phase of cell cycle accumulation-induced by the topoisomerase inhibitors etoposide and irinotecan (Ricchi et al., 2002). These effects were observed both in Caco-2 cells and in SW 480 cells independently from their COX's profile of expression, thus suggesting that aspirin acted through a COX-independent mechanism (Ricchi et al., 2002).

In the present work, we study the molecular mechanisms responsible for the effect of aspirin on cell cycle and survival. We now report that aspirin treatment counteracts apoptosis and G_2/M phase of cell cycle transition that follows serum-deprivation in Caco-2 cells. The effect of aspirin is sustained at least in part by a dose-dependent activation of the PI3-kinase/AKT signal transduction pathway and by the induction of $p21^{Cip/WAF1}$, whereas is abolished by the addition of the selective PI3-kinase inhibitor LY294002.

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% CO_2 in air in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and buffered with HEPES (20 mM). Caco-2 cells were seeded at 5×10^4 cells/ml and were routinely subcultured when about 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 (aspirin, PD98059, and LY294002) were added at the beginning of the serum withdrawal.

Aspirin (Sigma, Milan, Italy) was dissolved in a 0.1 M Tris-HCl, pH 7.8, solution. The solution was buffered with Tris base to obtain a final pH equal to that of control Dulbecco's modified Eagle's medium and prepared every 2 weeks. LY294002 (Calbiochem, Darmstadt, Germany) was dissolved in dimethyl sulfoxide and prepared as a 16.2 mM stock solution and added at a final concentration of 50 μM. PD98059 (Calbiochem) was dissolved in dimethyl sulfoxide and prepared as an 8 mM stock solution and added at a final concentration of 40 μM.

Cell Cycle Analysis and Apoptosis Detection. To define the cell cycle distribution and the apoptosis rate, Caco-2 cells were trypsinized, pelleted, fixed, and propidium iodide stained as described previously (Nicoletti et al., 1991). Propidium iodide staining fluorescence of individual cells was analyzed by using a FACSCalibur flow cytometer apparatus (BD Biosciences, San Jose, CA) and the MODFIT analysis software. For each sample, at least 20,000 events were stored.

Apoptosis was additionally evaluated by using annexin V-FITC staining technique. Briefly, trypsinized Caco-2 cells were collected, including floating apoptotic cells and the cells spontaneously detached during washing procedure, and annexin V-FITC stained by using a detection kit from Medical and Biological Laboratories Co., Ltd. (Naka-ku Nagoya, Japan) according to the manufacturer's instructions. Fluorescence analysis was performed by a flow cytometer apparatus (BD Biosciences) and the CELL QUEST analysis software. For each sample, at least 30,000 events were stored. Quadrant settings were based on the negative control. Each experiment was repeated at least three times.

Western Blot Analysis. Rabbit polyclonal anti-human $p21^{Cip/WAF1}$ antibody, mouse monoclonal antibodies against phosphorylated ERK1/2, and rabbit polyclonal against total ERK2 protein were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). (Ser 473) Phosphorylated AKT and total AKT were detected by using rabbit polyclonal antibodies from Cell Signaling Technology Inc. (Beverly, MA). 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% Nonidet P-40, and 0.01% SDS) containing complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Lysates from adherent cells were collected by scraping and centrifuged at 12,000*g* for 15 min at 4°C. Protein concentration in cell lysates was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA), and 50 μg of total protein from each sample was analyzed. Proteins were separated by a 12% SDS-polyacrylamide gel electrophoresis and transferred on nitrocellulose membrane (Hybond-ECL nitrocellulose; Amersham Biosciences Inc., Little Chalfont, Buckinghamshire, UK). After incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Bio-Rad) diluted 1:2000 in PBS, 0.2% Tween, the membranes were washed and protein bands were detected by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). In the case of $p21^{Cip/WAF1}$ Western blot analysis, control for loading and transfer was obtained by probing with anti-α-tubulin (Sigma) at 1:4000 dilution. For quantitation of immunoblots, relative intensities of bands were quantified by densitometry with a desk scanner (Amersham Discovery System) and RFLPrint software (PDI, Huntington Station, New York).

PI3-Kinase Assay. Cells lysates (300 μg) were immunoprecipitated with a pTyr antibody (Santa Cruz Biotechnology, Inc.). Pellets were washed in cold PBS and then in 100 mM Tris-HCl, pH 7.4, supplemented with 500 mM LiCl, 1 mM EDTA, and 0.2 mM $NaVO_4$. Pellets were further resuspended in 30 mM HEPES, pH 7.5, and 6.25 mM $MgCl_2$ and 125 μM cold ATP; the kinase reaction was initiated by addition of 2 μg/μl phosphatidylinositol (Sigma) and 10 μCi/μl $\gamma[^{32}P]$ATP (3000 Ci/mmol) and performed for 15 min at 37°; the reaction was stopped by 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 (TLC). $[^{32}P]$phosphoinositides were visualized by autoradiographs, scraped from TLC, and counts per minute were quantified by a β-counter (Beckman Coulter, Inc., Fullerton, CA).

Statistical Analysis. Statistical comparisons were performed using the Mann-Whitney *U* test. A probability value <0.05 was considered a significant difference.

Results

Effects of Serum Withdrawal and Aspirin Treatment on Caco-2 Cell Survival and Cell Cycle Distribution. To evaluate the effect of serum-withdrawal on Caco-2 cell survival and cell cycle distribution, cells were incubated in serum-free medium 3 days after plating. The effect on apoptosis was first evaluated by analyzing the percentage of sub-G_1 population at flow cytometry. Spontaneous apoptosis of Caco-2 cells at day 4 of culture in complete culture medium was in the average 6% (data not shown); the percentage of apoptotic cells was approximately 8, 20, and 30 after serum deprivation for 48, 96, and 168 h, respectively (Fig. 1). The effect of 2 and 5 mM aspirin treatment on serum withdrawal-induced apoptosis was first evaluated through the analysis of the subdiploid DNA peak at flow cytometry. Aspirin inhibited apoptosis in a statistically significant manner ($*p < 0.05$) at all time points tested compared with untreated cells (Fig. 1A). Aspirin (2 mM) treatment caused a 60% reduction in apoptosis compared with untreated cells with the maximal effect at 96 h of serum deprivation. The percentage of apoptosis increased up to 11% after treatment with 5 mM aspirin but was unmodified during the starvation time. Similar results were obtained when the percentage of apoptotic cells was evaluated by annexin staining (Fig. 1B); however, because of the high sensitiveness of this procedure, higher

levels of apoptosis were observed in untreated cells compared with those obtained at the analysis of subdiploid peak (Fig. 1B).

We also analyzed in the same experimental conditions the distribution of cells in the phases of the cell cycle. We did not find any relevant modification in cell cycle distribution between starved Caco-2 cells and cells cultured in complete medium in the presence or absence of aspirin for 48 h (data not shown). On the contrary, prolonged serum deprivation caused a time-dependent decrease in the proportion of cells in the G_1 phase of cell cycle and increase in the proportion of cells in the G_2/M phase of cell cycle (Fig. 2). Aspirin (5 mM) treatment and to a lesser extent 2 mM aspirin treatment counteracted the G_1 decrease and G_2/M increase in cell cycle distribution induced by prolonged serum starvation leading to a profile of cell cycle similar to that of cells cultured in serum for 168 h (Fig. 2, data point "in serum" versus 96- and 168-h serum withdrawal).

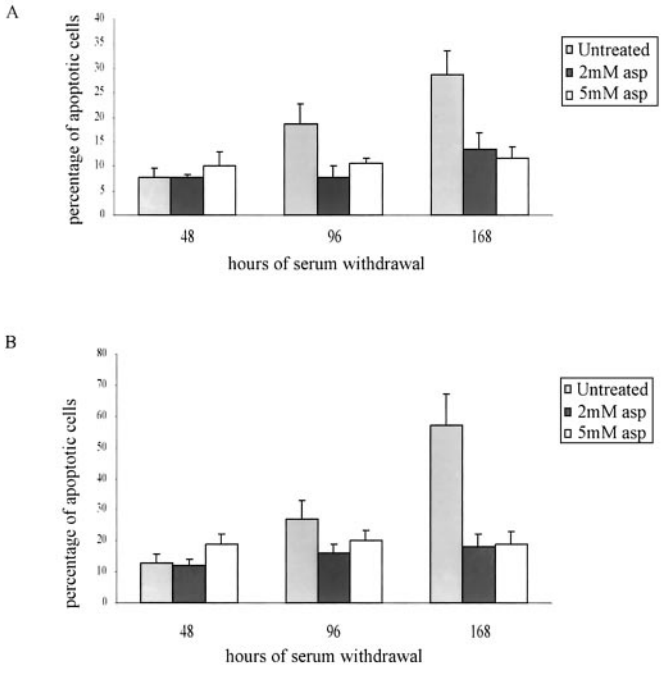

Fig. 1. Effect of 2 and 5 mM aspirin treatment on apoptosis induced by serum deprivation in Caco-2 cells. After 72 h of culture in complete medium, cells were serum deprived in the absence or presence of aspirin. After 48, 96, and 168 h of serum withdrawal, apoptosis was calculated as the percentage of cells showing a subdiploid DNA peak (A) and as the percentage of cells positive at annexin V-FITC staining (B), as described under *Materials and Methods*. Data are expressed as mean ± S.D.

Data from our laboratory indicated that Caco-2 cells expressed COX-2 but not COX-1 (Di Popolo et al., 2000). To evaluate whether the effects of aspirin treatment on Caco-2 cells cycle and survival were dependent on the inhibition of COX-2 activity, we analyzed the effect of NS-398 treatment, a COX-2-selective inhibitor, on survival during serum deprivation. To address this issue, cells were starved in the presence of 10 μm NS-398, a concentration that completely inhibits the biosynthesis of prostaglandin E_2 in Caco-2 cells. NS-398 treatment at this concentration did not cause any significant modifications on apoptosis and cell cycle parameters with respect to starved cells (data not shown). These results, therefore, suggested that aspirin interfered with apoptosis and cell cycle modification induced by serum withdrawal in Caco-2 cells in a COX-independent manner.

Effect of Aspirin on PI3-Kinase and MAP Kinase Activation during Caco-2 Cell Serum Withdrawal. We have previously shown that an autocrine IGF-II/IGF-Ir pathway sustains cells proliferation and survival of Caco-2 colon cancer cells (Zarrilli et al., 1994, 1996). In particular, we and others have demonstrated that the PI3-kinase pathway preferentially, with respect to the MAP kinase pathway, delivered an antiapoptotic and proliferative signal in Caco-2 cells (Di Popolo et al., 2000; Gauthier et al., 2001). Thus, it was of interest to evaluate whether aspirin treatment had any effect on MAP kinase and PI3-kinase pathways during Caco-2 serum deprivation.

To address these questions, we focused on the activation status of ERK1 and ERK2, two major components of the MAP kinases cascade, and of the AKT protein that is a downstream effector of the PI3-kinase. Lysates from cells serum deprived for 96 h in the presence or absence of 2 and 5 mM aspirin were probed for phosphorylated AKT at Ser 473 and phosporylated ERK1/2 kinases. To evaluate the PI3-kinase/AKT-dependent pathway in serum cultured cells and to compare the effects of aspirin in both culture conditions, lysates of cells cultured in complete medium and treated for 96 h with 2 and 5 mM aspirin were also collected. The bands were quantified and normalized to total AKT and ERK2 protein kinases, respectively.

As shown in Fig. 3, aspirin treatment dose dependently induced activation of AKT protein and ERK1/2 protein with respect to untreated cells. The relative densitometric analysis showed 1.5- and 2.7-fold increase for AKT protein phos-

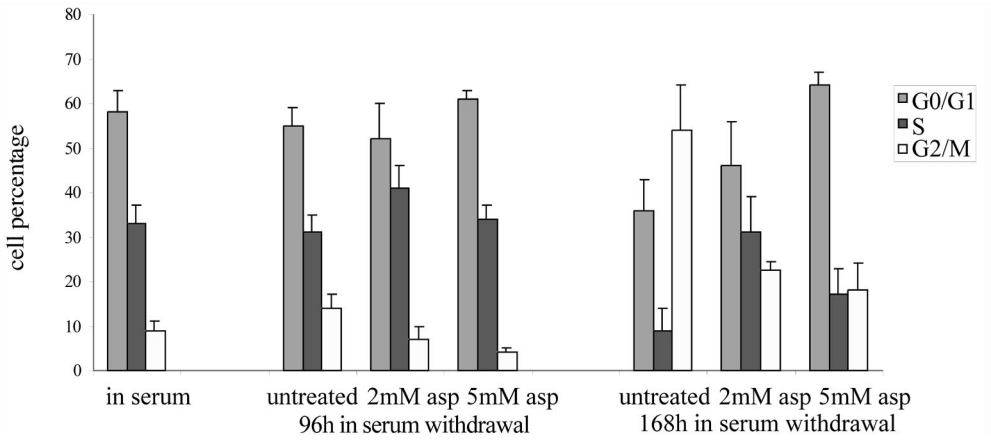

Fig. 2. Effect of 2 and 5 mM aspirin treatment on cell cycle distribution observed after Caco-2 cells serum withdrawal. After 72 h of culture in complete medium, cells were serum deprived in the absence or presence of aspirin for 96 and 168 h. DNA cell cycle analysis was performed by propidium iodide labeling as described under *Materials and Methods*. A data point representative for Caco-2 cells grown in complete medium for 168 h is also shown. Data points represent the mean of triplicate experiments ± S.D.

phorylation status after aspirin treatment at 2 and 5 mM, respectively (Fig. 3A) and 2.7-fold increase for ERK1/2 protein phosphorylation status after aspirin treatment at 5 mM in serum-deprived cells (Fig. 3B). On the other hand, serum deprivation slightly reduced the activation status of Akt with respect to serum-cultured cells (Fig. 3A), but aspirin treatment, although to a lesser extent than in serum withdrawal, still induced phosphorylation of Akt (Fig. 3A).

To directly demonstrate the activation of PI3-kinase by aspirin treatment in serum-starved cells, we analyzed the PI3-kinase activity in the pTyr immunoprecipitate from 96-h serum-deprived Caco-2 cells in the absence (control) or presence of 2 and 5 mM aspirin. As shown in Fig. 4, aspirin treatment dose dependently increased the levels of three phosphate phosphatidylinositols.

Because the molecular effects of aspirin have been observed after prolonged exposure to drug, we asked whether these effects could be related to a direct activation of PI3-kinase by aspirin. To address this issue, we evaluated the effect on AKT and ERK phosphorylation status after aspirin acute stimulation. After 24 h of serum deprivation, Caco-2 cells were treated with aspirin at 5 mM and cell lysates were collected after 5, 10, 15, and 30 min of drug addition. As shown in Fig. 5, 5 mM aspirin treatment transiently induced activation of AKT and ERK1/2 proteins with respect to serum-deprived cells after 10 min of exposure.

To further evaluate the relative involvement of ERK1/2 and PI3-kinase pathways in Caco-2 cells survival, we analyzed also the effects, during serum deprivation, of PD98059 or LY294002 inhibitor treatments alone and in combination with 2 and 5 mM aspirin (Fig. 6 , A and B; Table 1).

Treatment with the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 alone did not cause any relevant change in activation status of AKT protein, slightly decreased ERK1/2 phosphorylation, but reduced apoptosis with respect to control cells (Fig. 6; Table 1). The aspirin-induced activation of AKT persisted in the presence of PD98059 (Fig. 6A) but PD98059 did not counteract 5 mM aspirin-induced activation of ERK1/2 kinases (Fig. 6B), thus suggesting that ERK1/2 kinases were activated by aspirin independently from MAP kinases. On the contrary, the PI3-kinase inhibitor LY294002 alone reduced basal activation of AKT (Fig. 6A) and further increased apoptosis from 19 to 35% with respect to control cells (Table 1). Furthermore, LY294002 in cotreatment with aspirin completely reduced AKT activation (Fig. 6A) and partially reduced ERK1/2 activation induced by aspirin (Fig. 6B), whereas increased apoptosis levels up to 40 and 45% in the presence of aspirin at 2 and 5 mM, respectively (Table 1). These data suggest that in Caco-2 cells ERK1/2 kinase pathway could be, at least in part, under PI3-kinase pathway control. Similar data were obtained in aspirin-treated cells for 168h (data not shown).

Because treatments with aspirin, through the activation of

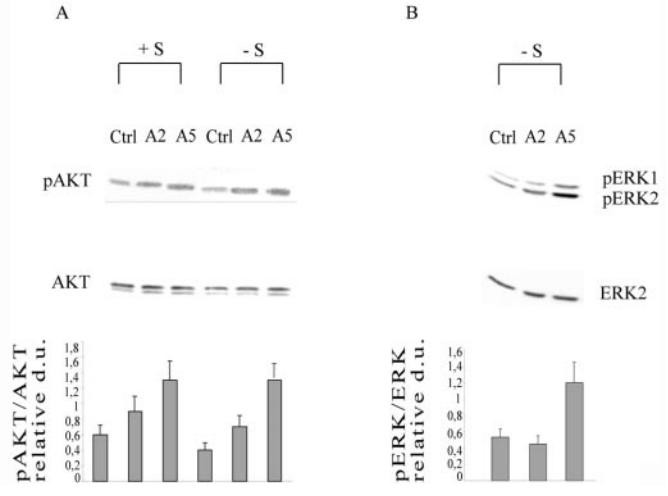

Fig. 3. Effect of 2 and 5 mM aspirin treatment on (Ser 437) phosphorylated AKT and total AKT (A) and phosphorylated ERK1/2 and total ERK2 (B) expression in Caco-2 cells. Western immunoblot analysis was performed on protein lysates from cells cultured in the presence (+S) and in absence (−S) of serum for 96 h (ctrl), after treatment with 2 mM (A2) or 5 mM aspirin for 96 h (A5). The immunoblots were stripped and reblotted with antibodies against total AKT or ERK2 protein. The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

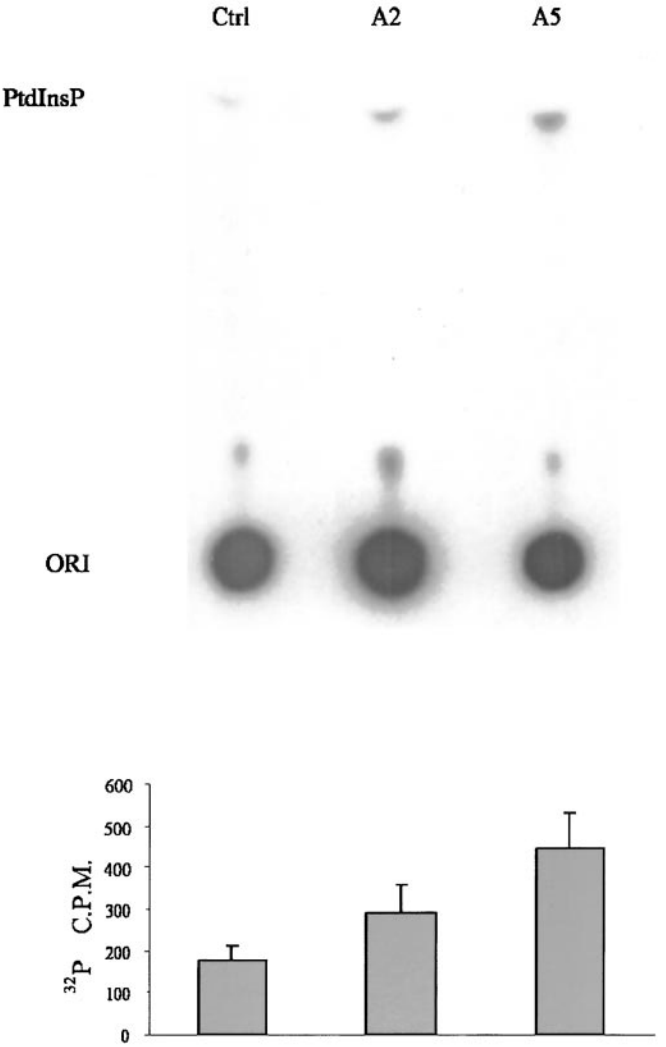

Fig. 4. Effect of 2 and 5 mM aspirin treatment on PI3-kinase activity in 96-h serum-starved cells. Cells extracts were immunoprecipitated with a pTyr monoclonal antibody, and the precipitate assayed for the presence of a PI3-kinase activity using phosphatidylinositol as a substrate as described under *Materials and Methods*. TLC analysis of the PI3-kinase reaction shown corresponds to a representative experiment that was repeated two additional times. PtdInsP, phosphatidylinositol 3-phosphate; ORI, origin. Histograms with S.D. bars represent the counts per minute of [^{32}P]phosphoinositol 3-phosphate of the three experiments.

AKT protein via PI3-kinase pathway stimulation, and with PD98059, by decreasing MAP kinase activity, both reduce apoptosis, the above-mentioned findings support the hypothesis that PI3-kinase inhibition and MAP kinase activation are involved in apoptotic response to serum deprivation in Caco-2 cells.

We have previously shown that in Caco-2 cells cotreated with aspirin and topoisomerase inhibitors, apoptosis and overall toxicity induced by topoisomerase inhibitors were reduced (Ricchi et al., 2002). To further correlate this cytoprotective effect of aspirin with the activation status of AKT, we assayed AKT activation status after 17 μM Vp-16 and 5 mM aspirin cotreatment, an experimental condition where maximal effect on overall viability have been observed (Ricchi et al., 2002). As shown in Fig. 7, phosphorylation status of AKT protein was 2-fold increased both in cells treated with 5 mM aspirin and cells cotreated with aspirin at 5 mM and Vp-16, compared with untreated cells, whereas phosphorylation status of AKT protein was unmodified after Vp-16 treatment compared with untreated cells.

Finally, in the search for substrates of Akt that could be relevant to the survival-promoting effects of Akt in Caco-2 cells, we also evaluated the effects of aspirin treatment on phosphorylation of (Ser 136) Bad in the same experimental system. No effects were detected on phosphorylation status of Bad at Ser 136 both after acute (5–30 min) and 96-h aspirin treatments (data not shown). On the contrary, according to

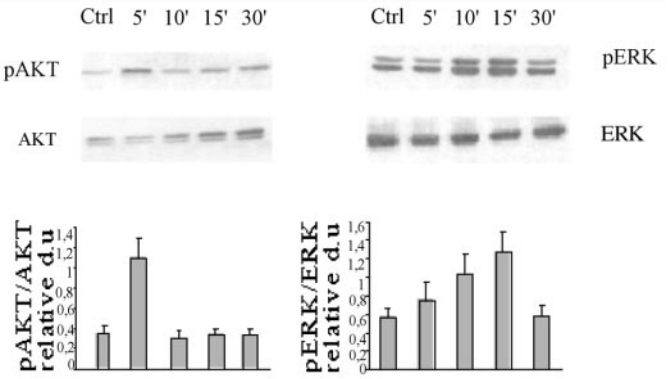

Fig. 5. Effect of 5 mM aspirin treatment on (Ser 437) phosphorylated AKT and total AKT (A) and phosphorylated ERK1/2 and total ERK2 (B) expression in Caco-2 cells. Western immunoblot analysis was performed on protein lysates from cells cultured in the absence of serum for 24 h (ctrl) and from cell treated with 5 mM aspirin for 5 min (5′), 10 min (10′), 15 min (15′), or 30 min (30′). The immunoblots were stripped and reblotted with antibodies against total AKT or ERK2 protein. The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

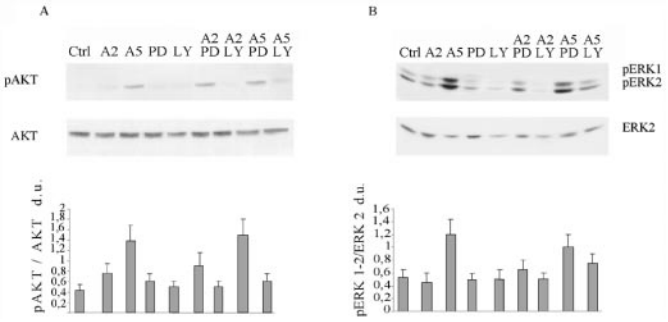

Fig. 6. Effect of PD98059 and LY294002 treatments alone and in combination with aspirin on (Ser 437) phosphorylated AKT and total AKT (A) and phosphorylated ERK1/2 and total ERK2 (B) expression in Caco-2 cells. Western immunoblot analysis was performed on protein lysates from control cells cultured in the absence of serum for 96 h (Ctrl), from cells treated with 2 mM aspirin (A2), 5 mM aspirin (A5), 40 μM PD98059 (PD), 50 μM LY294002 (LY), or from cells cotreated with 2 mM aspirin and 40 μM PD98059 (A2PD), 2 mM aspirin and 50 μM LY294002 (A2LY), 5 mM aspirin and 40 μM PD98059 (A5PD), or 5 mM aspirin and 50 μM LY294002 (A5LY). The immunoblots were stripped and reblotted with antibodies against total AKT or ERK2 proteins. The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

TABLE 1

Effect of PD98059 and LY294002 treatments, alone or in combination with aspirin, for 96 h on apoptosis during Caco-2 cell starvation

Apoptosis was calculated as the percentage of cells showing a subdiploid DNA peak as described under *Materials and Methods*. Data are expressed as mean ± S.D.

Treatment	Aspirin		
	0	2 mM	5 mM
		%	
No Serum	19±4	8±2	11±2
PD98059	8±2	7±3	10±3
LY294002	35±5	40±5	45±5

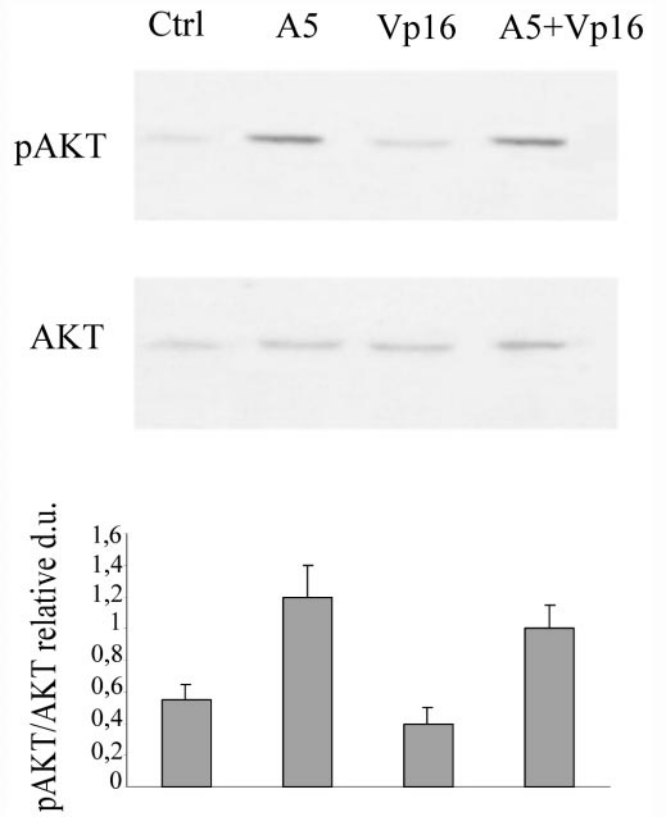

Fig. 7. Effect of aspirin and Vp-16 treatments on the phosphorylation status of AKT protein in Caco-2 cells. Experiments were performed in complete medium. Vp-16 at the concentration of 17 μM or aspirin at the concentration of 5 mM were added alone or in combination for 48 h at day 4 of culture. Western immunoblot analysis of (Ser 437) phosphorylated AKT (top) and total AKT (bottom). The immunoblots were stripped and reblotted with antibody against total AKT protein. Protein lysates were from untreated cells (Ctrl), or cells treated with aspirin at 5 mM (A5), Vp-16 for 48 h at 17 μM (Vp 16), or 17 μM Vp-16 and 5 mM aspirin (A5 + Vp16). The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

our previous data (Ricchi et al., 2002), we found that aspirin long-term treatment caused a dose-dependent increase in the bcl-2 levels (data not shown).

Together, these results strongly suggest that the aspirin cytoprotective effect resides in the ability to activate AKT protein and to deliver antiapoptotic signals in the presence of different apoptotic stimuli.

Effect of Aspirin Treatment on p21$^{Cip/WAF1}$ Expression in Caco-2 Cells. As reported previously, treatment with aspirin dose dependently inhibited growth (Ricchi et al., 1997). Furthermore, aspirin (2–5 mM) did not significantly affect Caco-2 cells viability and counteracted G_2/M phase transition induced by topoisomerase inhibitors (Ricchi et al., 2002). Our data mentioned above on cell cycle further reinforced the idea that aspirin treatment could also regulate some of the proteins involved in control of cell cycle checkpoints. Furthermore, it was previously shown that p21$^{Cip/WAF1}$, but not p27 levels, were elevated in terminal differentiated and nondividing Caco-2 cells (Evers et al., 1996; Gartel et al., 1996) and were almost undetectable in Caco-2 proliferating cells (Zarrilli et al., 1999). We therefore evaluated the effect of aspirin treatment on p21$^{Cip/WAF1}$ expression. Because it has been reported that induction of p21$^{Cip/WAF1}$ could be a downstream event of the activation of the AKT/PKB and ERK1/2 kinase pathways (Olson et al., 1998; Lawlor and Rotwein, 2000a,b), we also studied whether PD98059 or LY294002 treatment, alone or in combination with 2 and 5 mM aspirin for 96 h could modify p21$^{Cip/WAF1}$ expression in Caco-2 serum-starved cells. As shown in Fig. 8, p21$^{Cip/WAF1}$ levels increased by approximately 2-fold and 4-fold in cells treated with aspirin at 2 and 5 mM, compared with untreated cells. PD98059 alone, or in combination with 2 and 5 mM aspirin, increased p21$^{Cip/WAF1}$ levels by 2.7-, 5-, and 4.4-fold, respectively, compared with untreated cells. On the contrary, LY294002 alone did not affect basal level of p21$^{Cip/WAF1}$, whereas in combination with aspirin completely suppressed the increase of p21$^{Cip/WAF1}$ expression induced by aspirin. The above-mentioned data therefore demonstrated that the induction of p21$^{Cip/WAF1}$ in Caco-2 serum-starved cells was a downstream event of the activation of the PI3-kinase survival pathway.

Then, we examined whether these treatments had any impact on cell cycle parameters under the same experimental conditions. Table 2 shows the effect of PD98059 and LY294002, alone or in combination with aspirin, on cell cycle distribution of Caco-2 cells. PD98059 alone and in the presence of aspirin increased the percentage of cells in G_1 and S phases and almost suppressed the percentage of cells in G_2/M phase of cell cycle compared with control cells. Interestingly, LY294002 alone increased the percentage of cells in G_1 phase and reduced the percentage of cells in G_2/M phase compared with control cells; on the contrary, LY294002 in cotreatment with aspirin at 2 and 5 mM increased the percentage of cells in G_2/M phase from 7 to 15%($*p < 0.05$) and from 4 to 32% ($*p < 0.02$) with respect to aspirin-treated cells.

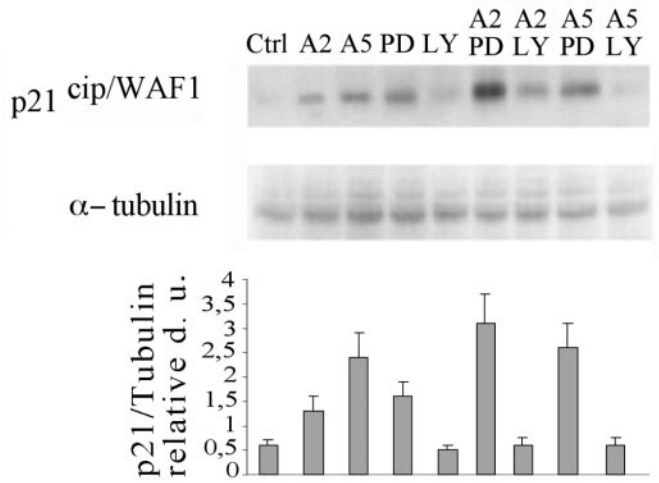

Fig. 8. Effect of 40 μM PD98059 or 50 μM LY294002 treatments, alone or in combination with 2 and 5 mM aspirin on p21$^{Cip/WAF1}$ (top) and α-tubulin (bottom) expression in serum-starved cells. Western immunoblot analysis was performed on protein lysates from control cells (Ctrl), cell treated with 2 mM aspirin (A2), 5 mM aspirin (A5), 40 μM PD98059 (PD), 50 μM LY294002 (LY), 2 mM aspirin and 40 μM PD98059 (A2PD), 5 mM aspirin and 50 μM LY294002 (A2LY), 5 mM aspirin and 40 μM PD98059 (A5PD), or 5 mM aspirin and 50 μM LY294002 (A5LY). The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

Discussion

Mounting evidence suggests that aspirin and salicylate at millimolar concentration have numerous COX-independent effects and are able to interfere with several intracellular molecular pathways (Dong et al., 1997; Pillinger et al., 1998; Xu et al., 1999). Several studies have evaluated the apoptotic effect of aspirin on colon cancer cells by demonstrating the involvement of specific death pathways (Stark et al., 2001; Yu et al., 2002). Salicylate treatments at these concentrations also inhibit proliferation of numerous normal and transformed cells (Aas et al., 1995; Cercek et al., 1997) and are associated with modulation of several cell cycle-related proteins (Marra et al., 2000; Law et al., 2000), neuroprotection (Grilli et al., 1996; Vartiainen et al., 2002) and glucose metabolism control (Hundal et al., 2002). Accordingly, we have previously shown that aspirin treatment inhibited proliferation and slightly increased apoptosis with respect to the baseline level in Caco-2 cells (Ricchi et al., 1997, 2002), but determined resistance to anticancer drugs (Ricchi et al., 2002).

In this study, we tried to better elucidate the molecular mechanisms that link the aspirin-dependent effect on cell cycle to the resistance to other apoptotic stimuli. We used serum deprivation-induced apoptosis in Caco-2 cells as a model system to evaluate aspirin interference with death/

TABLE 2

Effect of PD98059 and LY294002 treatments, alone or in combination for 96 h with aspirin, on cell cycle distribution during Caco-2 cell starvation

DNA cell cycle analysis was performed by propidium iodide labeling as described under *Materials and Methods*. Data are expressed as mean ± S.D.

Treatment	% of Cell Cycle Distribution		
	G_0/G_1	S	G_2/M
Control	56 ± 5	32 ± 5	12 ± 3
2 mM aspirin	53 ± 8	41 ± 5	7 ± 3
5 mM aspirin	61 ± 3	35 ± 3	4 ± 1
PD	58 ± 6	41 ± 8	1 ± 0
PD + 2 mM aspirin	59 ± 7	40 ± 7	1 ± 0
PD + 5 mM aspirin	56 ± 5	39 ± 8	5 ± 3
LY	72 ± 8	25 ± 7	3 ± 2
LY + 2 mM aspirin	60 ± 7	23 ± 7	15 ± 3
LY + 5 mM aspirin	54 ± 9	14 ± 5	32 ± 6

PD, PD98059; LY, LY294002.

survival pathways. Our data show that in Caco-2 cells apoptosis induced by serum deprivation is a late event accompanied with increase in G_2/M cell cycle phase. These findings are in agreement with those obtained in different experimental systems in which PI3-kinase activity was found to be required for growth factor-dependent survival and differentiation (Kennedy et al., 1997; Eves et al., 1998) and in which MAP kinase cascade was responsible for cell cycle progression (Aliaga et al., 1999; Abbott and Holt, 1999). Our data show that aspirin treatment in Caco-2 cells interferes with serum deprivation-induced apoptosis through PI3-kinase and ERK kinase pathways. We also show that the effect of aspirin on survival is mediated through the activation of the PI3-kinase. In fact, aspirin treatment in the presence of the specific PI3-kinase inhibitor LY294002 is able to revert the effect of aspirin on Caco-2 cells survival being even more toxic than LY294002 alone. On the other hand, the inhibition of MEK activity by PD98059 alone or in cotreatment with aspirin does not have the same effect of LY294002 and maintain the cytoprotective effect of aspirin. Further studies are needed to clarify at molecular level the role of PI3-kinase pathway in the observed aspirin-dependent activation of ERK kinase.

That aspirin treatment has a direct effect on PI3-kinase and ERK kinase pathways is demonstrated by our data showing a transient activation of these pathways by acute aspirin treatment. However, because the majority of our data are obtained with aspirin long-term treatment, we cannot exclude that aspirin might have also an indirect effect through the IGFII/IGF-Ir autocrine loop that sustains Caco-2 cells survival (Zarrilli et al., 1994, 1996). Therefore, further studies are needed also to elucidate at molecular levels the site(s) of aspirin interference with the IGFII/IGF-Ir-dependent PI3-kinase survival pathway and the mechanism(s) responsible for PI3-kinase activation.

Our data show that aspirin not only prevents apoptosis but also the increase in G_2 cell cycle after Caco-2 cells serum deprivation and dose dependently induces $p21^{Cip/WAF1}$ expression. Mounting evidence indicates that $p21^{Cip/WAF1}$ is a downstream target of the PI3-kinase/AKT pathway. In fact, it has been shown in muscle cells that IGFI/IGF-I receptor activation regulates survival and differentiation through the stimulation of PI3-kinase that in turn activates AKT, which stimulates the expression of $p21^{Cip/WAF1}$ (Lawlor and Rotwein, 2000a,b).

However, although it has been shown that AKT stimulates $p21^{Cip/WAF1}$ expression, other reports indicate that the activated AKT phosphorylates $p21^{Cip/WAF1}$ to prevent $p21^{Cip/WAF1}$ nuclear localization and the $p21^{Cip/WAF1}$-dependent cell cycle arrest, as well as Mdm2, to prevent Mdm2 nuclear localization and Mdm2-dependent degradation of p53, thus determining cell cycle progression (Zhou et al., 2001a,b).

The data presented herein indicate that aspirin activates a survival pathway that is responsible for the induction of $p21^{Cip/WAF1}$ also in Caco-2 cells and that there is a strict correlation between $p21^{Cip/WAF1}$ expression and its inhibitory function toward cell cycle progression. In fact, as well as restoring apoptosis induced by serum deprivation, treatment with LY294002 in the presence of aspirin leads to $p21^{Cip/WAF1}$ suppression and G_2/M accumulation. Thus, the aspirin-dependent activation of AKT/PKB protein and the induction of $p21^{Cip/WAF1}$ expression provides a mechanism for its ability to prevent apoptosis and G_2/M phase accumulation induced by prolonged serum deprivation and Etoposide treatment and indicates a novel tool by which aspirin may control cell cycle without resulting in a toxic treatment in Caco-2 cells. In accordance with our data, the activation of a similar pattern has been recently shown by Yu and collaborators, who demonstrated that the overexpression of the receptor tyrosine kinase p185ERbB2 increased $p21^{Cip/WAF1}$ expression, conferred resistance to taxol-induced apoptosis, and prevented cell entrance to G_2/M phase (Yu et al., 1998).

On the other hand, we found also that inhibition of the MEK pathway led to induction of $p21^{Cip/WAF1}$ expression and G_2/M cell cycle phase reduction; similarly, the highest increase of G_2/M phase was observed during 5 mM aspirin and LY294002 cotreatment in presence of activation of ERK1/2 kinases and $p21^{Cip/WAF1}$ suppression. Although the mechanism by which PD98059 treatment increases $p21^{Cip/WAF1}$ level remains unclear, the above-mentioned data both confirm the protective effect of PD98059 against apoptotic stimuli (Cho et al., 2002; Cuda et al., 2002) and strongly suggest that the apoptotic response to serum deprivation in Caco-2 cells involves also the activation of MAP kinase pathway and $p21^{Cip/WAF1}$ suppression both responsible for G_2/M accumulation. These finding are in agreement with other reports showing that $p21^{Cip/WAF1}$ expression could play a role in enhancing cell survival (Cheng et al., 2000), protecting various cell types from apoptosis after anticancer drug treatment (Waldman et al., 1997). Further studies are needed to clarify the effect of aspirin treatment on $p21^{Cip/WAF1}$ expression and its phosphorylation status on the control of cell cycle check points in Caco-2 cells and the mechanism by which the coordinated activity of PI3-kinase and MAP kinase pathways regulates its expression and the survival signaling.

Additional studies are needed to determine the ability of aspirin to activate PI3-kinase pathway in different tumor cell lines and provide evidence that this mechanism occurs also in vivo. In fact, it has been recently recognized that PI3-kinase signaling in the liver is essential for normal carbohydrate and lipid metabolism in living animals (Miyake et al., 2002). Because it has been demonstrated that high dose of aspirin could improve glucose metabolism in humans (Hundal et al., 2002), the data of this report may encourage further evaluation of the in vivo mechanism for aspirin use in therapeutic regimens.

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