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Regulatory Peptides 114 (2003) 101–107
REGULATORY PEPTIDES
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Inhibition of cytosolic phospholipase A_2 mRNA expression: a novel mechanism for acetylsalicylic acid-mediated growth inhibition and apoptosis in colon cancer cells☆,☆☆

Hong-Gang Yu[a,b], Jie-An Huang[a], Yan-Ning Yang[a], He-Sheng Luo[a], Jie-Ping Yu[a], Juris J. Meier[b], Henning Schrader[b], Andreas Bastian[b], Wolfgang E. Schmidt[b], Frank Schmitz[b,*]

[a] *Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, China*
[b] *Laboratory for Experimental Gastroenterology, Department of Medicine I, St. Josef-Hospital, Ruhr-University Bochum, Gudrunstr. 56, Bochum D-44791, Germany*

Received 30 August 2002; received in revised form 6 March 2003; accepted 7 March 2003

Abstract

Acetylsalicylic acid (ASA) has been confirmed to inhibit proliferation and to induce apoptosis in human colorectal cancer cells in vitro. However, the mechanism by which ASA exhibits antiproliferative and proapoptotic effects in cyclooxygenase 2 (COX-2)-negative cells remains to be further elucidated. In the present study, SW480, a COX-2-negative colon cancer cell line, was treated with various concentrations of ASA (0, 2.5, 5, and 10 mM). The antiproliferative and proapoptotic effects of ASA were confirmed by MTT assay, flow cytometry of propidium iodide (PI)-stained cells, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. After treatment with ASA, intracellular cyclic AMP (cAMP) levels were increased and the production of prostaglandin E_2 (PGE_2) was decreased. RT-PCR analysis revealed that treatment of ASA induced a concentration-dependent downregulation of cytosolic phospholipase A_2 ($cPLA_2$) mRNA expression in SW480 cells and also in two other colorectal cancer cell lines, Colo320 and HT-29 cells. Intracellular calcium levels were unaffected by ASA treatment. Our results indicate that the ASA-induced downregulation of cytosolic phospholipase A_2 mRNA expression might be a novel mechanism for ASA-mediated growth inhibition and apoptosis in colon cancer cells.

Keywords: Phospholipase A_2; Acetylsalicylic acid; Apoptosis

1. Introduction

Despite significant medical advances in diagnosis and therapy, colorectal malignancies remain the worldwide leading cause of cancer-related deaths, underscoring the need for effective chemopreventive strategies. A variety of agents have been reported to protect against colon cancer, among which nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to be efficient [1,3].

Acetylsalicylic acid (ASA) is among the most commonly used NSAID for relieving pain, inflammatory symptoms, and fever. ASA also has an established efficacy in the prevention of myocardial infarction and ischemic stroke, as well as in the treatment of acute myocardial infarction [2]. Recently, ASA has been shown to reduce the risk for colorectal cancer by as much as approximately 40%, a property that is shared by other nonsteroidal anti-inflammatory drugs [4]. Evidence for this effect comes from multiple epidemiological studies, most of which have found that ASA reduces the risk of colorectal adenoma [4] and carcinoma [5], as well as from experimental colon cancer in animal models [6]. However, the molecular mechanisms by which ASA exhibits its anticancer effect are less clear.

Abbreviations: ASA, acetylsalicylic acid; cAMP, cyclic AMP; COX-2, cyclooxygenase-2; $cPLA_2$, cytosolic phospholipase A_2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSAID, nonsteroidal anti-inflammatory drug; PGE_2, prostaglandin E_2; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

☆ Hong-Gang Yu and Jie-An Huang contributed equally to this work.

☆☆ This work was supported by a National Foundation for Natural Science, China grant (no. 39470330) and a Natural Scientific grant (no. SJ-97J083) from Hubei Province, China.

* Corresponding author. Tel.: +49-234-509-2378; fax: +49-234-509-2309.
E-mail address: frank.schmitz@uni-bochum.de (F. Schmitz).

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doi:10.1016/S0167-0115(03)00084-3

The best-defined molecular targets for ASA and other NSAIDs are cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2). COX-2, the inducible isoform of cyclooxygenase, is considered to play a key role in carcinogenesis [7] and angiogenesis [8]. One putative mechanism for the antitumor properties of ASA is its inhibitory action on COX-1 and COX-2 [1]. However, several lines of evidence suggest that the wide range of antiproliferative potencies of ASA does not correlate with COX-2-inhibitory activities. Some recent reports revealed that NSAIDs induce apoptosis in colon cancer cell lines that lack detectable expression of COX-2 protein [9,10].

Phospholipase A_2 (PLA_2; EC 3.1.1.4) are a family of enzymes that catalyze the hydrolysis of the fatty acyl ester bond at the *sn*-2 position of the glycerophosphocholine molecule, generating free fatty acids, such as arachidonic acid, and lysophosphatidylcholine [11]. Arachidonic acid is further metabolized by COX to produce prostaglandins (PGs) and thromboxanes [12]. Cytosolic phospholipase A2 ($cPLA_2$) is the major intracellular form of PLA_2. In colorectal cancer cells, $cPLA_2$ is increased for as-yet undefined reasons [13,14]. Prostaglandin E_2 (PGE_2), a metabolite of arachidonic acid metabolism, is an important mediator of inflammation and physiological processes. Elevated levels of PGE_2 have also been detected in a variety of common human tumors including colon cancer [15,16].

The aim of the present investigation was to study the effects of ASA on proliferation, apoptosis, $cPLA_2$ mRNA expression, and prostaglandin E_2 release in human colon cancer cell lines. SW480 cells and Colo320, two colon cancer cell lines without a detectable expression of COX-2 proteins, were used in the present study. Furthermore, two well-defined second messengers, intracellular calcium ($[Ca^{+}]_i$) and cyclic AMP (cAMP), were also investigated [17].

2. Materials and methods

2.1. Materials

ASA, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], penicillin, streptomycin, and RNase A were purchased from Sigma (St. Louis, MO). The RNAgents® total RNA isolation system was obtained from Promega (Madison, WI). cAMP[^{125}I] and Prostaglandin E_2[^{125}I] Biotrak Assays were purchased from Amersham. Fura-2/AM was from Molecular Probes. RPMI 1640 media, trypsin/EDTA solution, and fetal bovine serum were from GIBCO BRL.

2.2. Cell culture and culture conditions

Human colon cancer cell lines SW480, Colo320, and HT-29 were cultured in RPMI 1640 media containing 10% fetal bovine serum and 1% antibiotic solution at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Proliferation assay

SW480 or Colo320 cells were plated at a density of 5×10^3 cells/well on a 96-well plate. At 24 h after seeding, ASA (0, 2.5, 5, and 10 mM) was added to the culture medium. Cell viability was assayed colorimetrically on days 1, 3, and 5 of drug treatment using an MTT-based test. In brief, MTT was added to each well at a concentration of 500 μg/ml and plates were incubated for 4 h at 37 °C. After 4 h, media were aspirated and cells were lysed with 400 μl of DMSO. Cells were incubated for a further 10 min at 37 °C with gentle shaking. Absorbance readings at 570 nM were determined using a computer-controlled microplate analyzer.

2.4. Cell cycle analysis

The distribution of the cells in the cell cycle was determined by propidium iodide (PI) staining and FACS analysis. Briefly, SW480 cells suspended on day 3 of ASA treatment were fixed with ice-cold 75% ethanol, washed twice with PBS, and incubated in 20 μg/ml RNase A for 30 min at 37 °C. Subsequently, cells were stained with 50 μg/ml propidium iodide and measured for DNA content by FACScan cytometry (Becton Dickinson, Helmetta, NJ).

2.5. Determination of apoptosis

SW480 cells grown to confluence on coverslips were treated with various concentrations of ASA (0, 2.5, 5, and 10 mM) for 3 days. On day 3, apoptotic cells were identified using the In Situ Cell Death Detection Kit, AP (Roche Molecular Biochemicals) according to the manufacturer's instructions. This assay is based on terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL). Briefly, cells were washed in PBS and fixed in 4% paraformaldehyde for 30 min. Cells were washed again, permeabilized with 0.1% sodium acetate/0.1% Triton X-100, and incubated with fluorescein-tagged nucleotides in the presence of terminal deoxynucleotidyl transferase for 60 min. Cells were washed again and incubated with alkaline phosphatase-conjugated antifluorescein antibodies. Cells were subsequently stained using the alkaline phosphatase substrate mix, NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate; Roche Molecular Biochemicals), and visualized with a Nikon TMS microscope at ×400 magnification. TUNEL staining was quantitated for each sample by calculating the percentage of positively stained cells in 10 randomly chosen fields (200–300 cells/field).

2.6. Measurement of intracellular calcium

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with the fluorescent probe fura-2 as described by Grynkiewicz et al. [18]. Briefly, SW480 cells were trypsinized, washed, and loaded with 3 μM fura-2/AM by incuba-

Table 1
Nucleotide sequence of the PCR primers and size of the PCR-amplified gene fragment

Gene	Sequence	PCR product (bp)
β-Actin	5′-AGGCATTGTGATGGACTCCG-3′	301
	5′-AGTGATGACCTGGCCGTCAG-3′	
$cPLA_2$	5′-ATGCCCAGACCTAGAATTTA-3′	737
	5′-AGGGGTTTTCTTCATACTTC-3′	

tion in the dark at 37 °C for 30 min. Cuvettes containing a 2-ml suspension of fura-2-loaded SW480 cells were positioned in a Perkin Elmer LS-50B spectrofluorometer. Fluorescence in continuously stirred SW480 cell suspension was recorded before and after the addition of ASA and thapsigargin. The values of $[Ca^{2+}]_i$ (in nanomolar) were calculated from the ratio of the fluorescence intensities at 340 and 380 nm.

2.7. Intracellular cAMP measurement

SW480 cells were treated with various concentrations of ASA (0, 2.5, 5.0, and 10.0 mM) for 3 days. On day 3, 1 ml of methanol was added to stop the reaction and to extract the cAMP. Cell samples were then sonicated and centrifuged for 5 min at 3000 × *g*. The pellet was washed with 1.0 ml of methanol, and the washings were added to the supernatant of each sample. The combined solution was evaporated to dryness, and the residue was dissolved for intracellular cAMP measurement. In the present study, a commercial radioimmunoassay kit (Amersham) was used for the detection of cAMP level.

2.8. PGE_2 measurement

SW480 cells were plated at a density of 5×10^3 cells/well on a 96-well plate. At 24 h after seeding, ASA (0, 2.5, 5, and 10 mM) was added to the culture medium. The culture medium was replaced by fresh medium with or without ASA everyday. On days 1, 3, and 5 of ASA treatment, the content of PGE_2 in the culture medium of SW480 cells was measured using a commercially available radioimmunoassay kit from Amersham at a final sample dilution of 1:500.

2.9. Expression of cytosolic PLA_2 mRNA

After treatment with increasing concentrations of ASA (0, 2.5, 5, and 10 mM) for 6 h, total RNA was extracted from SW480, Colo320, and HT-29 cells using the RNAgents® total RNA isolation system (Promega). First-strand cDNA was synthesized from 1 μg of total RNA using murine reverse transcriptase and the first-strand cDNA synthesis kit from Promega in a total volume of 20 μl. One microliter of the first-strand cDNA was subjected to PCR amplification with 35 cycles consisting of 95 °C for 30 s, 58 °C for 45 s, and 72 °C for 90 s using specific primers for $cPLA_2$ and β-actin (Table 1). The PCR products were visualized in ethidium bromide-stained agarose gels (2%).

2.10. Statistical analysis

Data were expressed as mean ± standard deviation, as described in individual figure legends. Data were analyzed for statistically significant differences with the GraphPad InStat 3.0 software (GraphPad Software, San Diego, CA). A p value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of ASA on proliferation and apoptosis

The effect of ASA on cell proliferation was measured with an MTT-based assay (Fig. 1). SW480 and Colo320

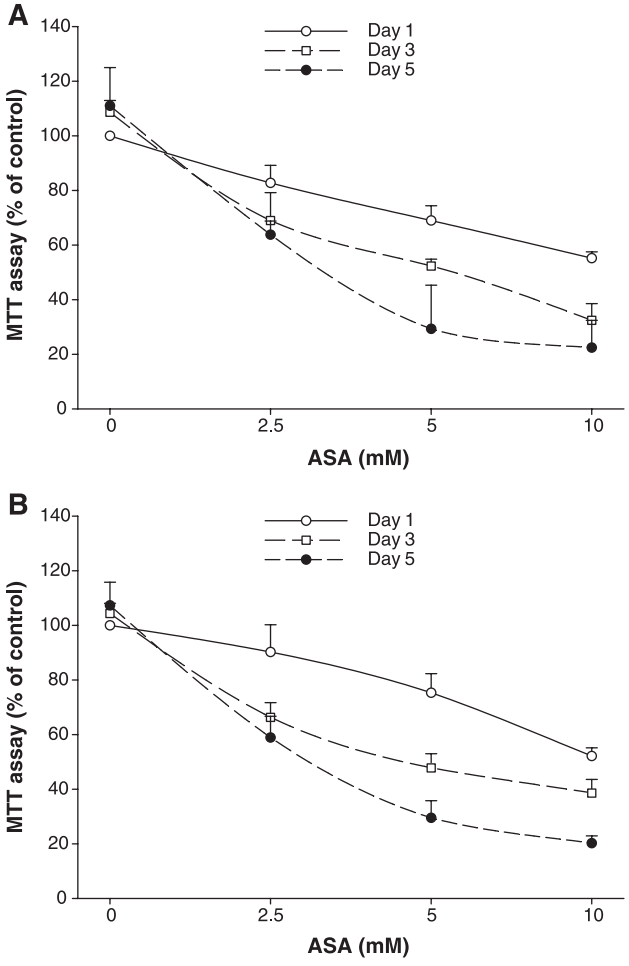

Fig. 1. Time–response characteristics of ASA effects on the proliferation of SW480 (A) and Colo320 cells (B). The cells were treated with various concentrations of ASA (0, 2.5, 5, and 10 mM) for 1, 3, and 5 days, respectively. The antiproliferative effects were measured by MTT assay. The values are expressed as mean ± S.D. from four independent experiments. The value from untreated cells (without ASA) at 1 day was preset as control.

cells were incubated with various concentrations of ASA (0, 2.5, 5, and 10 mM) for 1, 3, and 5 days. Cells treated with vehicle only (1% DMSO) were used as controls. After ASA treatment, growth was inhibited significantly in a time- and concentration-dependent manner. The maximum inhibition (73%) was observed on day 5 of treatment with 10 mM ASA.

To determine the effects of ASA on cell cycle progression and apoptosis, SW480 cells were stained with PI and subjected to FACScan cytometry. As shown in Fig. 2, treatment with various concentrations of ASA significantly ($p<0.01$) increased the cell fraction at the G_0-G_1 phases, and decreased the cell fraction at the S and G_2-M phases in a concentration-dependent manner. The sub-G_1 fraction was significantly ($p<0.01$) increased in a concentration-dependent manner after treatment with ASA (Fig. 2A). Similar results were also obtained from TUNEL staining assay in SW480 cells (Fig. 2B).

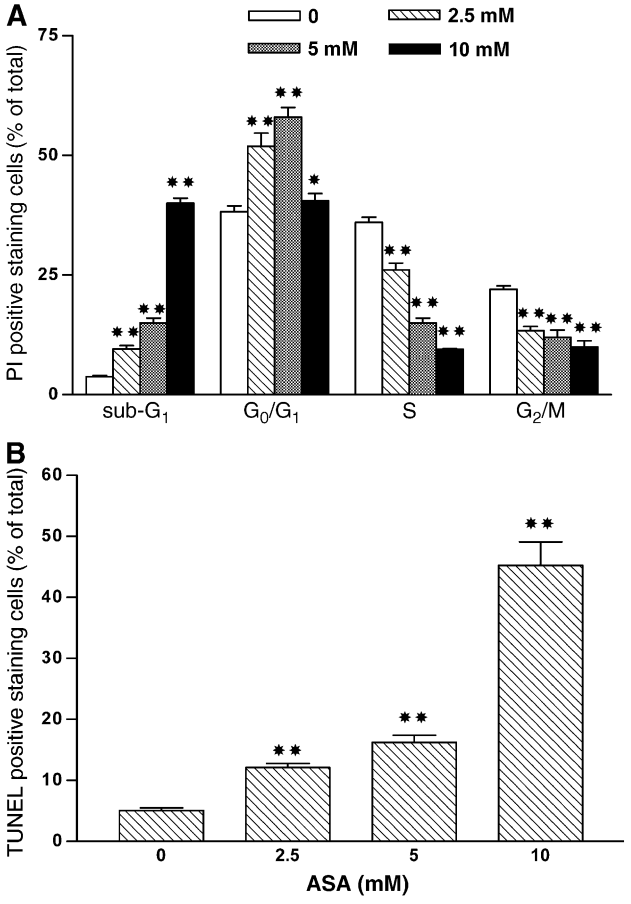

Fig. 2. (A) The cell cycle distribution of SW480 cells treated with or without ASA. Nuclear DNA contents were measured on day 3 by flow cytometry in SW480 cells. Data are expressed as mean ± S.D. from four independent experiments. (B) The effect of ASA on cell apoptosis in SW480 cells. Apoptotic cells were stained on day 3 using a TUNEL assay in SW480 cells, as described in Materials and methods. The percentage of positively staining cells in 10 random fields was calculated. Data are expressed as mean ± S.D. from four independent experiments. *$p<0.05$ and **$p<0.01$ vs. group without treatment with ASA.

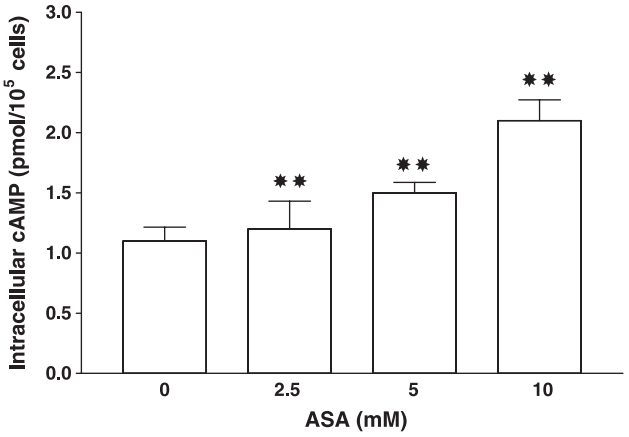

Fig. 3. Effect of ASA on intracellular cAMP in SW480 cells. The cells were treated with various concentrations of ASA (0, 2.5, 5, and 10 mM) for 3 days, respectively. Intracellular cAMP levels of SW480 cells were measured on day 3 using a commercial radioimmunoassay kit (Amersham). Data are expressed as mean ± S.D. from three independent experiments. *$p<0.05$ and **$p<0.01$ vs. group without treatment with ASA.

3.2. Effect of ASA on intracellular cAMP level and calcium concentration

The basal content of intracellular cAMP was 1.06 ± 0.10 pmol/10^5 cells. After treatment with ASA, a concentration-dependent elevation of intracellular cAMP levels was detected on day 3 (Fig. 3).

SW480 cells showed a basal $[Ca^{2+}]_i$ concentration of 90–110 nM, which was stable and without spontaneous oscillations. The addition of thapsigargin induced a significant increase of $[Ca^{2+}]_i$ concentration. No influence on basal $[Ca^{2+}]_i$ level and thapsigargin-induced increase of $[Ca^{2+}]_i$ concentration was observed after treatment with ASA.

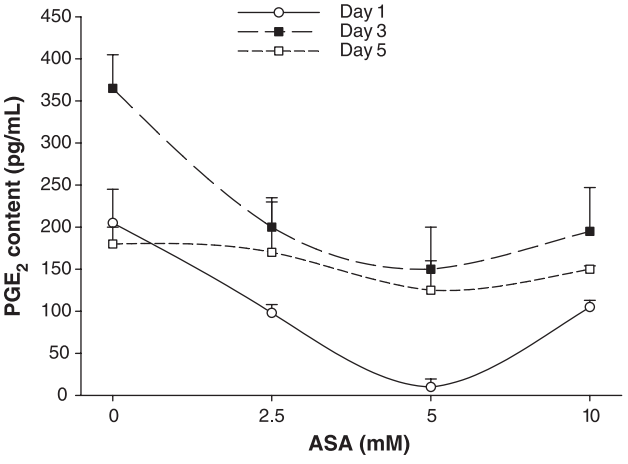

Fig. 4. Effect of ASA on PGE_2 production of SW480 cells. The cells were treated with various concentrations of ASA (0, 2.5, 5, and 10 mM) for 1, 3, and 5 days, respectively. The PGE_2 levels in the culture medium of SW480 cells were measured using a commercial radioimmunoassay kit (Amersham). The values are expressed as mean ± S.D. from three independent experiments.

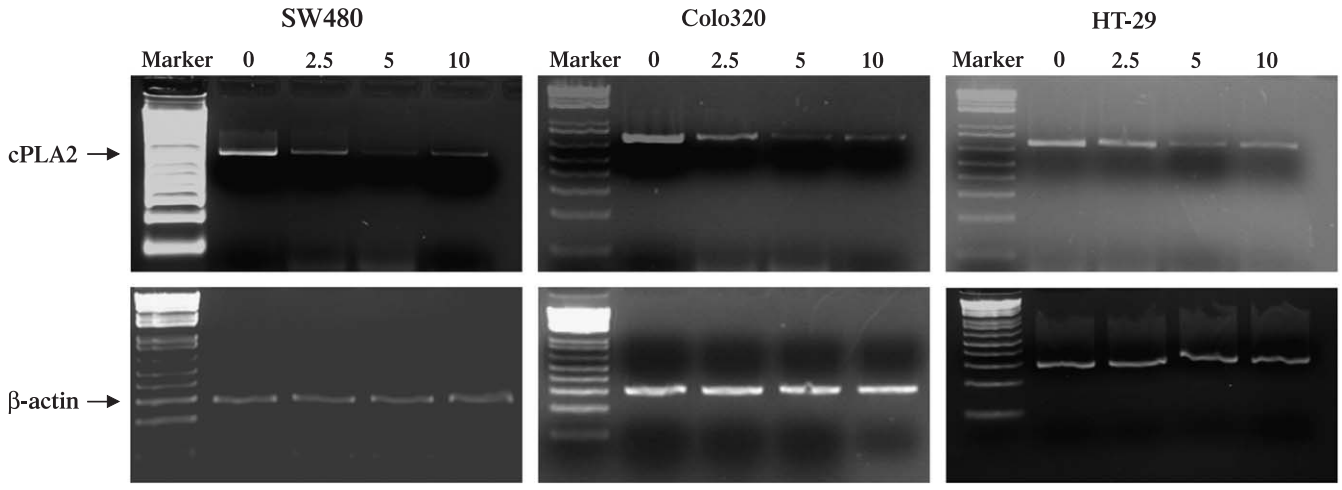

Fig. 5. Effect of ASA on the $cPLA_2$ mRNA expression in colon cancer cells. SW480, Colo320, and HT-29 cells (75–80% confluent) were treated with increasing concentrations of ASA (0, 2.5, 5, and 10 mM) for 6 h. After the incubation period, total cellular RNA was isolated. One microgram of the total RNA was subjected to RT-PCR analysis. The size of the amplified $cPLA_2$ mRNA was 737 bp. RT-PCR-amplified β-actin fragment (301 bp) was used to assess the amount of total RNA for each sample.

3.3. Effect of ASA on PGE_2 production and $cPLA_2$ mRNA expression

The basal PGE_2 level in the culture medium of SW480 cells was 204 ± 26 pg/ml. Treatment of SW480 cells with ASA significantly decreased the level of PGE_2 in the medium. The most pronounced inhibition of PGE_2 production to 11.4 ± 5.0 pg/ml was induced on day 1 of treatment with 5 mM ASA (Fig. 4).

Cytosolic PLA_2 mRNA levels were investigated using a semiquantitative RT-PCR. In SW480 cells, ASA greatly decreased the intensity of the amplified $cPLA_2$ transcript in a concentration-dependent manner while housekeeping gene expression levels (β-actin) were unaffected. Similar results were observed both in Colo320 and HT-29 cells (Fig. 5).

4. Discussion

The current investigation demonstrates that ASA exhibits antiproliferative and proapoptotic effects in human colon cancer cells, which do not express detectable endogenous COX-2 protein levels. ASA treatment causes a cell cycle arrest at the G_0–G_1 phases with a significant cell fraction, which remains at the G_1 phase. This effect was observed after 3 days of treatment with ASA and lasted up to 5 days. Cell cycle progression is tightly regulated by multiple factors including cyclins and cyclin-dependent kinases (CDKs) [19–21]. Earlier reports indicate that ASA may affect cell cycle progression by direct or indirect inhibition of cyclin D1 or by upregulation of p21(Waf1) and p27(Kip1) at the G_1 phase [22,23]. However, the mechanism by which ASA blocks cell cycle progression at the (G_0) G_1 phase remains to be elucidated. One other interesting putative target of ASA in colon cancer may be the reduction of β-catenins, which are also known to regulate the levels of cyclin D1 [24].

An increase of $cPLA_2$ expression has been described in a variety of tumors of the gastrointestinal tract and it has been postulated to be involved in the pathogenesis of cancer [13,14,25–28]. Our results demonstrate that ASA significantly decreases $cPLA_2$ mRNA expression in a concentration-dependent manner. The same inhibitory effect of ASA on $cPLA_2$ mRNA expression has also been observed in two other colorectal cancer cell lines: Colo320 (COX-2-negative) and HT-29 (COX-2-positive) cells. This indicates that the inhibition of $cPLA_2$ expression by ASA is not restricted to one colorectal cell line. Surprisingly, increasing the concentration of ASA to 10 mM does not further inhibit the $cPLA_2$ mRNA expression but induces a slight rebound. The potential mechanism is that 10 mM ASA might initiate some self-protective signals in the colon cancer cells. One recent study [29] showed that 10 mM ASA stimulated the maximal activation of NFκB in SW480 cells. Meanwhile, NFκB has been suggested to regulate the α_2-macroglobulin-induced $cPLA_2$ synthesis in macrophages [30]. Hereby, we speculate that 10 mM ASA-induced NFκB activation might be involved in the rebound of $cPLA_2$ transcription. Further experiments are needed to confirm this hypothesis.

To further explore the antiproliferative effects of ASA on prostaglandin metabolism, we also examined the production of PGE_2. PGE_2 has been considered to exhibit growth-promoting and antiapoptotic effects in some colorectal cancer cells [31]. In the cited study, ASA was found to decrease the production of PGE_2 with a maximal inhibition induced by 5 mM ASA treatment. This is consistent with our findings on ASA-mediated inhibition of $cPLA_2$ expression. ASA-mediated inhibition of PGE_2 production further supports the effects of ASA on $cPLA_2$ mRNA expression in

SW480 cells ($p < 0.01$). In an in vivo model of inflammation, it has been demonstrated recently that increased prostaglandin production is a two-component response: (a) increased COX-2 expression, and (b) increased supply of arachidonic acid [32]. Since SW480 cells only express COX-1, it might be speculated that ASA treatment suppresses PGE_2 synthesis predominantly by depriving COX-1 of its substrate, arachidonic acid, by drastically suppressing $cPLA_2$ mRNA expression, which has also been previously suggested by Yuan et al. [33]. However, our data cannot rule out that the effect of ASA on the proliferation and apoptosis of SW480 cells is COX-independent. One previous study showed that ASA can inhibit COX-1 but does not necessarily reduce tumor numbers in $Apc^{Min/+}$ mice, suggesting that COX-1 involvement in the pathogenesis of colorectal cancer may be minimal [34]. It is still unclear why PGE_2 release by SW480 cells reaches the maximum on day 3 of culture and returns to steady levels on day 5. One putative explanation is that SW480 cells were seeded at low density (5×10^3 cells/well) in 96-well plates where the cells grow fast and reach 70% confluency within 3 days of culture. Therefore, the release of PGE_2 is elevated along with increasing cell numbers. On day 5, however, cells are fully confluent and kept in quiescence while the release of PGE_2 is decreased.

The second messengers cAMP and $[Ca^{2+}]_i$ have been implicated in the modulation of proliferation and apoptosis [35,36]. In the present study, our results show that ASA does not affect the mobilization of $[Ca^{2+}]_i$ but elevates intracellular cAMP levels approximately twofold in SW480 cells. Until now, there is no evidence for a direct regulation of intracellular cAMP levels by ASA. Hereby, the mechanism responsible for elevated intracellular cAMP still needs to be clarified.

In conclusion, our results support the hypothesis that ASA might inhibit growth and stimulate apoptosis through the inhibition of $cPLA_2$ expression and decreased PGE_2 production. This novel finding provides new insights into the chemopreventive effects of ASA against colorectal cancer.

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