

Inhibitory effect of celecoxib in lung carcinoma by regulation of cyclooxygenase-2/cytosolic phospholipase A₂ and peroxisome proliferator-activated receptor gamma

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Abstract Celecoxib is a potent nonsteroid anti-inflammatory drug (NSAID) that has demonstrated great promise in cancer chemoprevention and treatment. The goal of this study was to determine the inhibitory effect and mechanism of celecoxib on Lewis lung carcinoma. The effect of celecoxib on viability of Lewis lung carcinoma cells was assessed with methyl thiazolyl tetrazolium (MTT) assay. Apoptosis and the mitochondrial membrane potential were detected by flow cytometric assay. The protein expression of cytosolic phospholipase A₂ (cPLA₂), cyclooxygenase-2 (COX-2), and peroxisome proliferator-activated receptor gamma (PPAR γ) were determined by Western blot analysis. The concentrations of arachidonic acid (AA) and prostaglandin E₂ (PGE₂) in culture supernatants were measured by the methods of RP-HPLC and PGE₂-specific ELISA, respectively. Celecoxib inhibited the proliferation of Lewis lung carcinoma and induced apoptosis in a dose-dependent manner by breakdown of mitochondrial membrane potential. The protein expressions of cPLA₂ and

PPAR γ were upregulated, but COX-2 protein expression was downregulated in the Lewis lung carcinoma cells exposed to celecoxib. The amount of AA was increased and PGE₂ was decreased in the culture supernatant, respectively. The ratio of AA to PGE₂ was increased in a dose-dependent manner. The major findings in this study are that celecoxib could inhibit the viability of Lewis lung carcinoma cells by interference of the AA pathway and upregulation of PPAR γ simultaneously, which are novel and important since the molecular mechanisms of celecoxib underlying the anti-neoplastic effects remain unclear.

Keywords Celecoxib · Cyclooxygenase-2 · Peroxisome proliferator-activated receptor gamma · Arachidonic acid · Prostaglandin E₂

Introduction

In recent years, arachidonic acid (AA) pathway has emerged as a therapeutic target for the prevention and treatment of various types of cancers [1–3]. Prostaglandins (PGs), most notably prostaglandin E₂ (PGE₂), which is one of the elements in the AA metabolic pathway [4], are involved in the development of cancer [5]. The first step in the formation of PGs is the liberation of AA from membrane-bound phospholipids by the action of phospholipase enzymes, such as cytosolic phospholipase A₂ (cPLA₂). Cyclooxygenases (COXs) then convert AA into endoperoxide intermediates that are ultimately converted by specific synthases to prostanoids, including PGE₂. Two isoforms of COXs have been identified. COX-1 is constitutively expressed in most cell types and mediates physiologic responses, including cytoprotection of the stomach and platelet aggregation regulation. Cyclooxygenase-2

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(COX-2) is highly induced by inflammatory cytokines/chemokines, growth factors, oncogene activation, and tumor promoters. Elevated levels of COX-2 expression have been found in human and animal cancers, and appear to be involved in the development of cancer via PGE₂ production [6–10]. The mechanisms whereby COX-2-derived PGE₂ promotes tumor genesis and progression are not well understood, but may involve promoting cell division [11–13], inhibiting apoptosis [11–13], altering cell adhesion and enhancing metastasis [14, 15], and stimulating neovascularization [16]. Selective COX2 inhibitors have potent anti-tumor activity. Thus, COX-2, the key enzyme in AA pathway, was considered to be an important target for tumor treatment. In addition cPLA₂, another enzyme in AA pathway, also plays a fundamental role in carcinogenesis. Up or downregulated expression of cPLA₂ has been observed in a variety of human cancers and tumor cell lines [17, 18]. Thus, cPLA₂ may be novel targets for chemotherapeutic tumor treatment.

Peroxisome proliferator-activated receptors (PPARs) have recently been reported as potential targets for the treatment of cancer [19, 20]. PPARs are ligand-activated transcription factors, and involved in the regulation of cell differentiation, proliferation, and apoptosis [21]. Among the three subtypes of PPARs (α , β , γ), peroxisome proliferator-activated receptor gamma (PPAR γ) has been the most intensively investigated. PPAR γ plays a role in both adipocyte differentiation and carcinogenesis. PPAR γ is expressed in various cancers. Furthermore, PPAR γ ligands inhibit the proliferation of human breast, prostate, colon, and pituitary cancer cells *in vitro* and *in vivo* through inhibition of cell proliferation, induction of apoptosis and terminal differentiation or through inhibition of angiogenesis [22–24]. Thus, activation of PPAR γ may provide an additional target for prevention of cancer.

Celecoxib was the first COX2-selective nonsteroidal anti-inflammatory drug (NSAID) approved for the treatment of adult arthritis. Celecoxib exerts potent chemopreventive and therapeutic activities against various human cancers, including breast, prostate, colon, cervix, liver, and lung cancer [25–28]. The chemopreventive property of celecoxib is due to its ability to induce apoptosis. Both COX-2-dependent and COX-2-independent mechanisms are involved in celecoxib-induced apoptosis of tumor cells. Although celecoxib proved to be highly effective in reducing lung cancer cell growth, the molecular mechanisms underlying the anti-neoplastic effects remain unclear. In this study, we attempted to elucidate the suppressing mechanism of celecoxib by examining the protein expression of cPLA₂, COX-2, and PPAR γ , as well as the content of AA and PGE₂ in Lewis lung carcinoma cells.

Materials and methods

Chemicals and antibodies

The Lewis cell line was originally obtained from ATCC and stored at -80°C . Celecoxib (purity $\geq 98\%$) was purchased from Sunheat Chemicals, Shanghai, China. cPLA₂ and PPAR γ antibodies, and goat anti-rabbit IgG/HRP antibody were purchased from Santa Cruz, USA, whereas rabbit COX-2 and β -actin antibody were purchased from Cayman USA. Chemical agents for Western blot and 3-(4,5)-dimethylthiazolium(-z-y1)-3,5-di-phenyl-tetrazoliumromide (MTT) were purchased from Sigma, Shanghai, China. Bradford protein assay kit and the enhanced chemistry luminescence (ECL) reagent kit were purchased from Applygen Technologies Inc., Beijing, China. The chemical agents used in high-performance liquid chromatography (HPLC) were purchased from TEDIA Company Inc., USA. PGE₂ ELISA kits were purchased from Scoochow University, Jiangsu, China. The AA standard was purchased from Cayman. Other reagents were obtained from Beijing General Chemical Reagent Factory, Beijing, China.

Cell culture

Lewis lung carcinoma cell line was cultured in Dulbecco's modification of Eagle's medium Dulbecco (DMEM) supplemented with 10% FBS (v/v), 100 U/ml of penicillin, and 100 $\mu\text{g}/\text{ml}$ of streptomycin. Cultures were maintained in a humidified incubator at 37°C in 5% CO₂. DMEM with 0.25% trypsin and 0.02% EDTA was used as cell dissociation buffer in subculture.

Cell treatments

Lewis lung carcinoma cells were plated in six-well plates and cultured in serum-free media for 16–18 h before treatment. Cells were treated with celecoxib (50–200 μM) in serum-free medium for 24 h. At the end of treatment, the medium containing celecoxib was removed and the cells were washed twice with phosphate buffered saline (PBS).

MTT assay

After cultured in serum-free media for 16–18 h in 96-well plates, Lewis lung carcinoma cells were treated with celecoxib at concentrations ranging from 50 to 200 μM . Afterward, 20 μl MTT (5 mg/ml) were added to each well after 12, 24, 48, and 72 h treatment with celecoxib. After 3 h of incubation with MTT, the supernatants were discarded, MTT crystals were dissolved with dimethylsulfoxide and the absorbance was measured at 570 nm (A_{570}).

The ratio of cell proliferation inhibition = (average A_{570} value of control group – average A_{570} value of treatment group/average A_{570} value of control group) \times 100%.

Determination of apoptosis

The induction of apoptosis was determined using the Annexin V-FITC apoptosis detection method. The Lewis lung carcinoma cells were collected, washed with PBS and assayed for the presence of extracellular phosphatidylserine using a conjugated Annexin V-FITC antibody with propidium iodide (PI). The results were performed using a FACS can cytometer.

Measurement of mitochondrial membrane potential

Lewis lung carcinoma cells were treated with different concentrations of celecoxib at different times. Cells were then treated with rhodamine 123 with a final dye concentration of 10 $\mu\text{g/ml}$ and incubated at 37°C in 5% CO_2 for 15 min prior to examination. Mitochondrial membrane potential was determined by flow cytometry. The change of fluorescent intensity of rhodamine 123 indicated the change in mitochondrial membrane potential.

Preparation of cell extracts and Western blot analysis

After the treatment, the cells were collected, washed with PBS and suspended in the cell lysis buffer at 4°C for 2 h, vortexed every 30 min, and then centrifuged at 12,000 $\times g$ for 15 min. The supernatant was collected as total protein extract. Protein concentration was determined using the Bradford protein assay kit according to the manufacturer's instructions. The immunoblotting analysis was performed by the separation of 40 μg protein on a 5–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunostaining and Western blot assay. After blocking nonspecific binding by non-fat milk, the blot was incubated overnight with rabbit anti-cPLA₂, COX-2, PPAR γ , and β -actin antibody (1:1,000 dilutions) in TBST at 4°C. The HRP-conjugated goat anti-rabbit antibody (1:2,000 dilutions) was used as the second antibody. Protein bands were visualized by ECL detection system.

Measurement of arachidonic acid production

The culture supernatants were collected and centrifuged to remove floating cells. Before measurement of AA production, the AA in 1 ml culture supernatants was derivatized by *p*-bromoacetophenone for detection under the ultraviolet light [20]. Subsequently, the amount of AA was determined by the Agilent 1100 HPLC system. The reverse phase-HPLC (RP-HPLC) measurements were carried out using

chromatographic column: Vydac-C18 (4.6 \times 250 mm, 5 μm). The mobile phase was a mixture of acetonitrile and water (81:19) and the flow rate was 1.0 ml/min. The detection wavelength was 292 nm and the column temperature was 55°C.

Measurement of PGE₂ production

The culture supernatants were collected and centrifuged to remove floating cells. Each sample (100 μl) was used for the following examination. PGE₂ level was measured using PGE₂ ELISA kit according to the manufacturer's instructions.

Statistical analysis

The data reported as mean \pm SEM ($n = 3/\text{group}$). Statistical analysis was performed by the unpaired Student's test. $P < 0.05$ was considered to have statistical significance.

Results

The inhibitory effect of celecoxib on Lewis lung carcinoma cell viability

To examine if celecoxib inhibited Lewis lung carcinoma cell growth, the cells were treated with various concentration of celecoxib for 24–72 h. As shown in Fig. 1, celecoxib exposure significantly reduced the viability of Lewis lung carcinoma cells in a dose- and time-dependent manner. IC₅₀ values of 72 h were 134.06 \pm 2.97 μM .

Celecoxib-induced apoptosis in Lewis lung carcinoma cells

Lewis lung carcinoma cells were treated with 100–200 μM concentration of celecoxib for 24 h. Apoptosis was

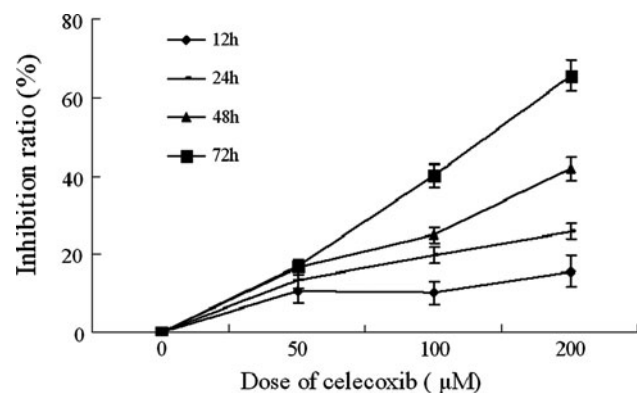


Fig. 1 Time- and dose-dependent growth inhibition induced by celecoxib in Lewis cell lines. The results are depicted as the mean \pm SEM of three independent experiments

detected by annexin V-PI staining and flow cytometric analysis. As demonstrated in Fig. 2a, Lewis cells were viable in Q3 quadrant without celecoxib treatment. Celecoxib treatment, however, increased the number of late apoptotic cells in Q2 quadrant. Celecoxib significantly increased the proportion of apoptotic cells in a dose-dependent manner. This suggested that apoptotic cell death event contributes to the growth inhibitory effect of celecoxib.

The alteration of mitochondrial membrane potential is a vital event in apoptosis. The status of mitochondrial membrane potential was assessed by rhodamine 123 staining. The results showed that mitochondrial membrane potential of Lewis lung carcinoma cells was decreased after treatment with 100 and 200 μM celecoxib, which occurred in a dose-dependent manner (Fig. 2b).

The effect of celecoxib on cPLA₂, COX-2, and PPAR γ

It is well known that both cPLA₂ and COX-2 are involved in PEG₂ biosynthesis. High levels of cPLA₂ and COX-2 expression are predicted to facilitate maximal PEG₂ production. Increased PG biosynthesis is associated with the expression and activity of cPLA₂ and COX-2. Meanwhile, pharmacological activators of PPAR γ inhibited growth of non-small-cell lung cancer (NSCLC) cell lines in vitro and in xenograft models. To explore the pharmaceutical effect of celecoxib, we observe the protein expression of cPLA₂, COX-2, and PPAR γ in the present study. As shown in Fig. 3, the expressions of cPLA₂ (Fig. 3a) and PPAR γ (Fig. 3c) were upregulated, but COX-2 (Fig. 3b) was downregulated in Lewis cells treated with celecoxib at 50–200 μM for 24 h.

The effect of celecoxib on AA level

It has been indicated that the activation of cPLA₂ results in the accumulation of intracellular AA. Elevated intracellular AA altered mitochondrial membrane permeability and caused cytochrome *C* to be released finally induced the cell apoptosis. According to the standard curve of AA concentration versus area under curve (AUC) (Fig. 4a), the linear range was from 7.8125 to 250 μM , and the linear correlation appeared to be satisfactory ($R > 0.99$). The concentration of AA was elevated in the Lewis cells after incubation with celecoxib at a dose of 50–200 μM for 24 h (Fig. 4b).

The effect of celecoxib on PGE₂ level

COX-2 inhibitors have been demonstrated to interfere with tumorigenesis and apoptosis through decreased PEG₂ production. In the present study, PGE₂ production was

Fig. 2 Celecoxib-induced apoptosis in Lewis lung carcinoma cells. **a** Flow cytometry analysis with annexin V-FITC/PI double staining. Cells were incubated either in the absence of celecoxib, or in the presence of 100 or 200 μM of celecoxib C for 24 h undamaged cells were stained with negative annexin V-FITC/PI (bottom left quadrant). After incubation with celecoxib for 24 h, a significant number of apoptotic cells were stained with positive annexin V-FITC and negative PI (bottom right quadrant). Data are presented as means \pm SD from triplicates and three independent experiments. Two other independent experiments produced similar results. **b** Flow cytometry assay of mitochondrial membrane potential changes with rhodamine 123 dye in Lewis cells under exposure to 100, 200 μM celecoxib for 24 h. The change of fluorescent intensity of rhodamine 123 indicated the change in mitochondrial membrane potential. Data are presented as means \pm SD from triplicates and three independent experiments. Two other independent experiments produced similar results

measured in culture supernatants and a linear correlation (Fig. 5a, $R > 0.99$) was obtained. The PGE₂ production in Lewis cells was significantly decreased when the cells were treated with 50–200 μM celecoxib (Fig. 5b).

The effect of celecoxib on the ratio of AA to PGE₂

Since the production of AA and PGE₂ play a key role in the tumorigenesis, the ratio of AA to PGE₂ concentration was calculated after transforming the concentration unit of PGE₂ into μM . The ratio was increased by celecoxib treatment in a dose-dependent manner in Lewis cells (Fig. 6).

Discussion

Over the last few years, epidemiological studies have indicated that celecoxib treatment reduces the risk of tumorigenesis, including breast, prostate, lung, and liver cancer. Experimental researches, both in vitro and in vivo, have generally supported these epidemiologic studies. Celecoxib has been shown to have anti-tumor effect in human tumor xenograft models of colon [25, 27], breast [26], prostate [28], and Lewis lung carcinoma [25]. The in vitro anti-proliferative effect of celecoxib has been attributed to the induction of apoptosis [29]. Our results are in agreement with these reported findings and showed that treatment with celecoxib significantly inhibited the proliferation of Lewis cells in a dose- and time-dependent manner. Although the mechanism underlying anti-cancer activity of celecoxib was not entirely understood, a large number of studies have shown that celecoxib prevented carcinogenesis via COX-2-dependent or -independent pathway.

COX-2 expression in healthy tissues is very low but it is overexpressed in various cancer tissues. Increased COX-2 expression has been demonstrated to contribute to

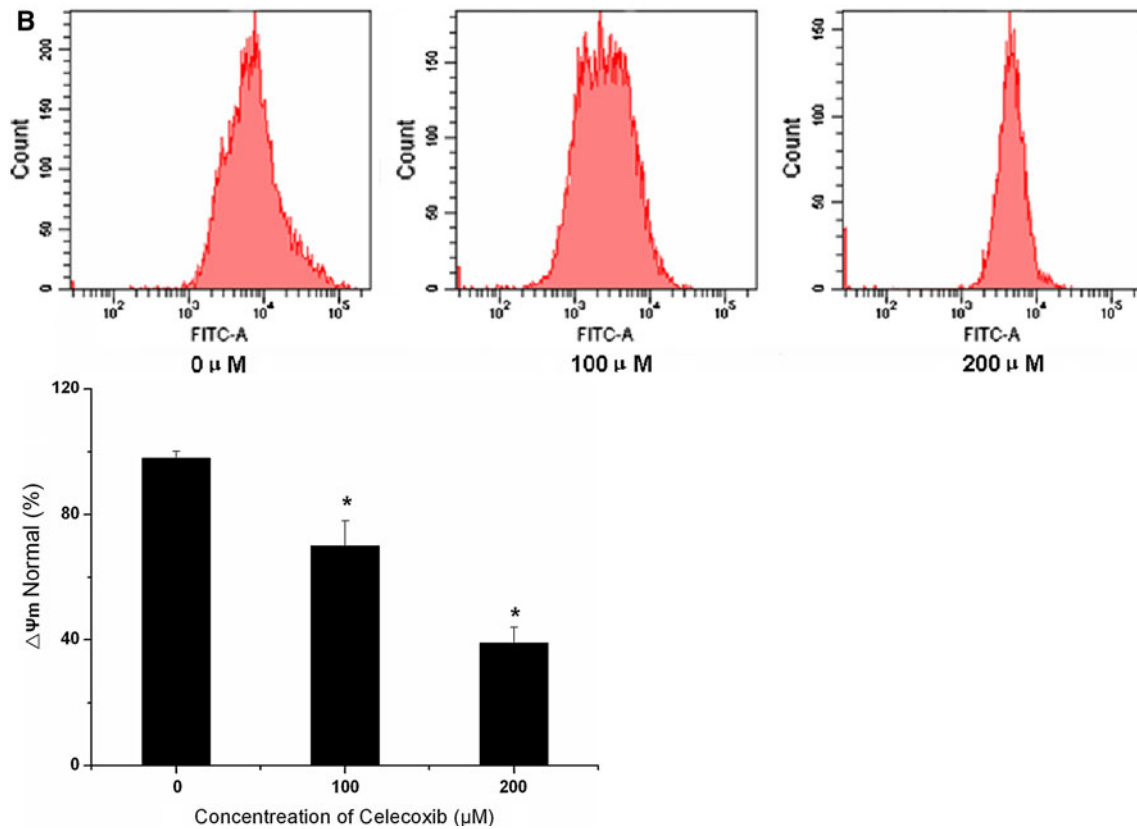
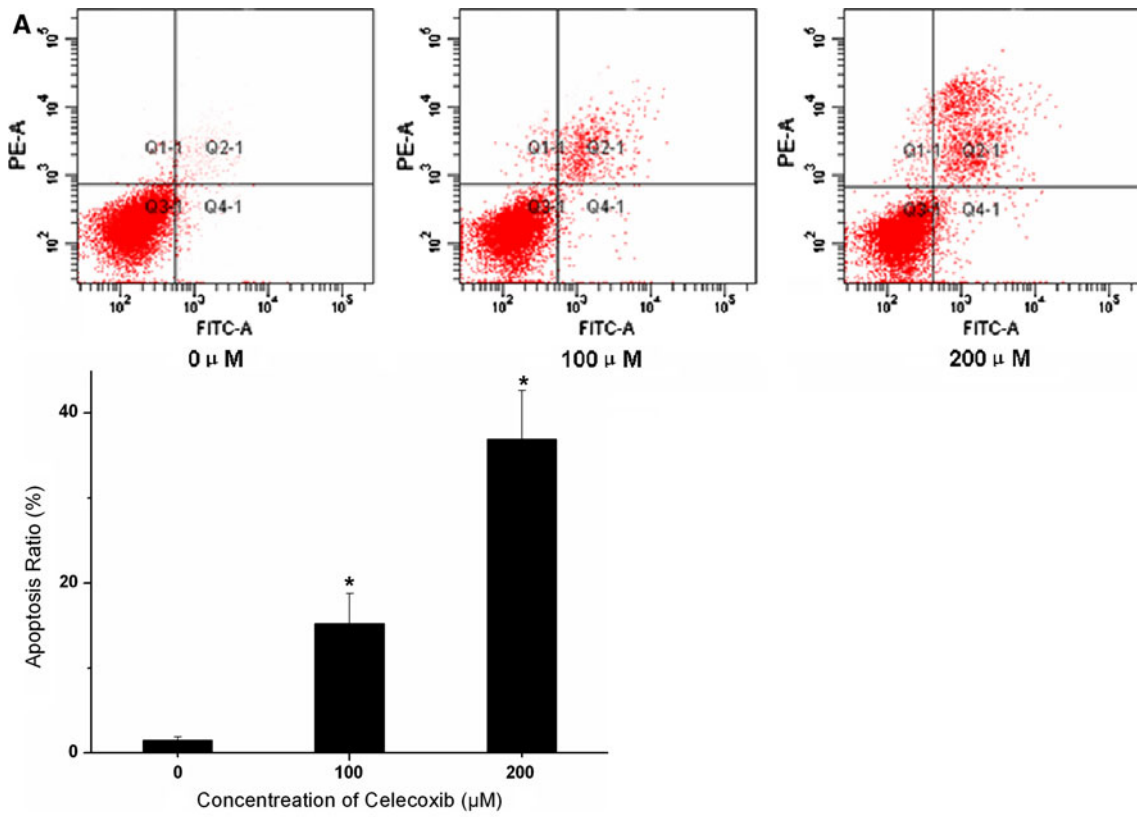


Fig. 3 The protein expression of cPLA₂, COX-2, and PPAR_γ in Lewis cell treated by different dose of celecoxib. Expression and relative quantification of protein levels expressed relative to the control. Values are the means \pm SE ($n = 3$). * $P < 0.05$ versus controls without insulin stimulation

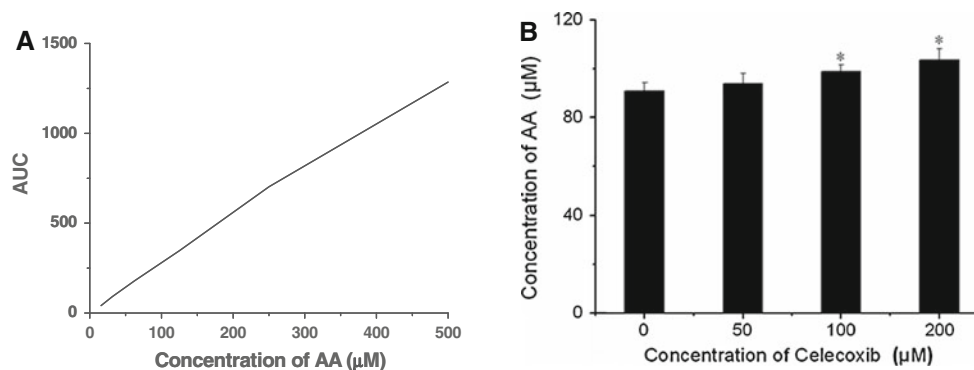
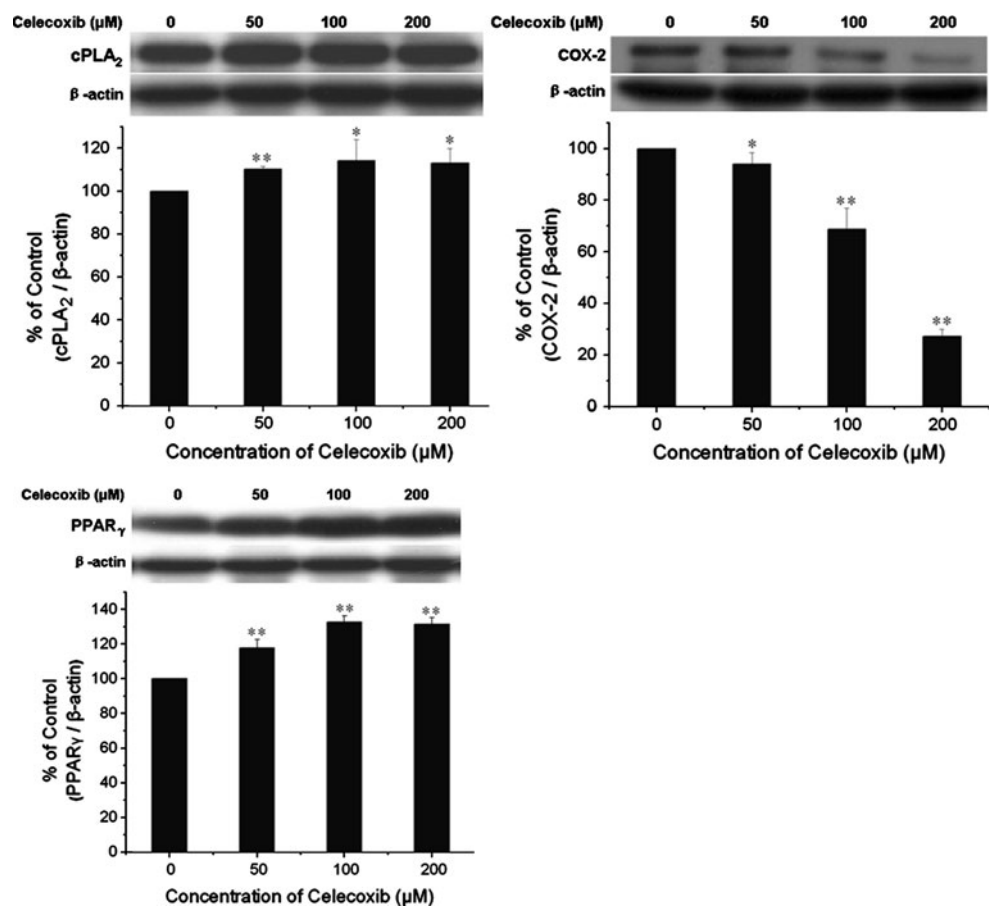


Fig. 4 The effect of celecoxib on AA production in the Lewis cells. The results are depicted as the mean \pm SEM of three independent experiments. * $P < 0.05$ versus control, ** $P < 0.01$ versus control (a standard curve and b result of assay)

carcinogenesis through inhibition of apoptosis, increase in angiogenesis and invasiveness, and modulation of inflammation/immune suppression, which may be linked to increased synthesis of PGE₂. COX-2 inhibitors have been demonstrated to interfere with tumorigenesis and apoptosis through decreased PEG₂ production. In the present study, celecoxib inhibited COX-2 protein expression, decreased the PEG₂ content, and induced apoptosis in Lewis lung cancer cells. It is well known that both cPLA₂ and COX-2 are involved in PEG₂ biosynthesis. High levels of cPLA₂

and COX-2 expression are predicted to facilitate maximal PEG₂ production. Increased PG biosynthesis is associated with the expression and activity of cPLA₂ and COX-2 in A549 cells [30]. The combination of celecoxib with docetaxel significantly decreased the expression of cPLA₂ and the levels of PGE₂, but had no effect on the expression of COX-1 or COX-2 in lung tumor tissues [31]. In disagreement with this report, the present study showed celecoxib upregulated cPLA₂ protein expression and downregulated COX-2 expression. It has been indicated

Fig. 5 The effect of celecoxib on PGE₂ production (by ELISA) in the Lewis cells. The results are depicted as the mean \pm SEM of three independent experiments. * $P < 0.05$ versus control, ** $P < 0.01$ versus control (a standard curve and b result of assay)

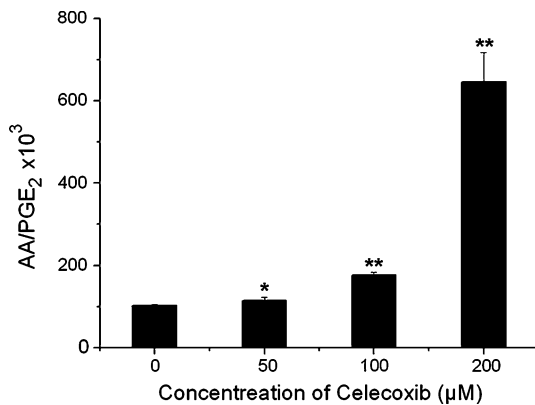
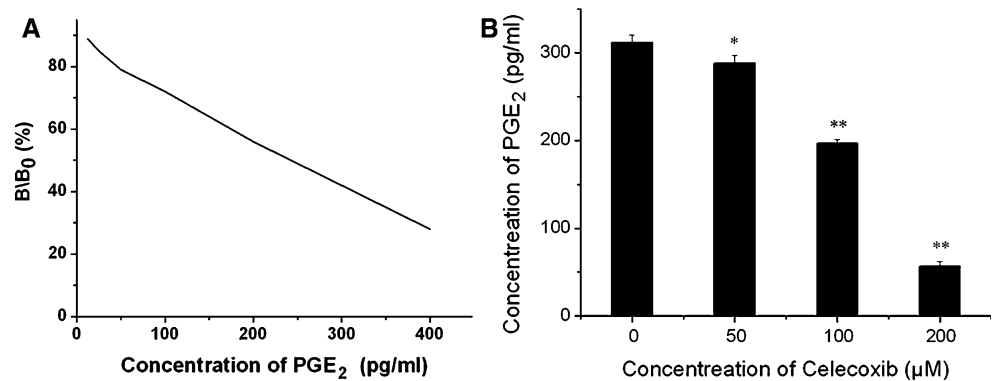


Fig. 6 The effect of celecoxib on ratio of AA and PGE₂ levels in the Lewis cells. The results are depicted as the mean \pm SEM of three independent experiments. * $P < 0.05$ versus control, ** $P < 0.01$ versus control

that the activation of cPLA₂ results in the accumulation of intracellular AA. Elevated intracellular AA can alter mitochondrial membrane permeability and cause cytochrome C to be released, thus leading to apoptosis [32]. Data from our studies showed treatment with celecoxib significantly increased AA content and decreased mitochondrial membrane potential of Lewis lung cells. Moreover, celecoxib increased the ratio of AA and PGE₂ by elevating AA and decreasing PGE₂ level in the supernatant of Lewis cell. This suggested that the decreased cellular PGE₂ and increased AA levels induced by celecoxib might be involved in the inhibition of cell proliferation and induction of apoptosis through downregulation of COX-2 and upregulation of cPLA₂.

Like COX-2, PPAR γ is upregulated in lung cancer cells. Several studies have demonstrated that PPAR γ expression was much higher in the lung tumor tissues when compared to normal lung tissues. In samples from human lung tumors, well-differentiated adenocarcinomas exhibited a greater frequency of PPAR γ positive cells than poorly differentiated samples [33]. Pharmacological activators of PPAR γ inhibited growth of NSCLC cell lines in vitro and in xenograft models [34]. A recent study has demonstrated

that pharmacological activators of PPAR γ also decrease PGE₂ production in NSCLC [35]. Specific activators of PPAR γ , such as anti-diabetic thiazolidinediones, may have both chemopreventive and chemotherapeutic effects in the treatment of NSCLC. Except for thiazolidinediones, polyunsaturated fatty acid, and NSAIDs are also PPAR γ ligands. Polyunsaturated fatty acids such as AA are natural ligands of PPARs [36]. NSAIDs can directly bind to PPAR γ and activate its transcriptional regulatory activities [37]. The AA and NSAIDs have also potent tumor modulating effects on several cancers. In the present study, celecoxib upregulated expression of PPAR γ and inhibited growth of Lewis cancer cells in a dose-dependent manner. Whether increased expression of PPAR γ occurred directly or indirectly due to elevated AA level is not clear yet.

In summary, the present study demonstrated that celecoxib inhibited growth and induced apoptosis of Lewis lung cancer cells, which was accompanied by breakdown of mitochondrial membrane potential. Celecoxib treatment increased AA level and decreased PEG production through upregulation and downregulation of cPLA₂ and COX-2 proteins, respectively. Celecoxib also increased expression of PPAR γ protein. This suggested that the mechanism by which celecoxib induces apoptosis in lung cancer is through interfering with the AA metabolic pathways and stimulating the activity of PPAR γ .

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