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Original Article

Celecoxib inhibits endometrial cancer cells through COX-2-dependent and non-dependent pathways

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Abstract: Background: This study aimed to investigate the antitumor effects of the cyclooxygenase-2 (COX-2) selective inhibitor, celecoxib, on endometrial cancer cells and its related mechanisms. Methods: By screening endometrial cancer cell lines with different COX-2 expression levels, we identified the Ishikawa cell line and the AN3 CA cell lines as expressing high and low levels of COX-2, respectively. We examined the effect of celecoxib on tumor cell proliferation, apoptosis, and the cell cycle. Also, its related control proteins were analyzed, and exogenous PGE_2 was added to determine whether celecoxib caused a significant counterreaction effect of on both cell lines. Results: Celecoxib exerted a comparable antitumor effect on Ishikawa and AN3 CA cancer cell growth, and apoptosis. It also arrested the cell cycle at the G_0/G_1 phase with a decreased production of prostaglandin-E_2 (PGE_2) and an increased expression of the peroxisome proliferator-activated receptor γ (PPAR-γ). The anticancer role of celecoxib was more prominent in endometrial cancer cells, which have relatively high levels of COX-2. In addition, exogenous PGE_2 could not reverse the antitumor effects of celecoxib completely in both cancer cell lines. Conclusion: Celecoxib may be involved in anticarcinogenic activities through COX-2-dependent and -independent (PPAR-γ involvement) molecular pathways. These results may provide new biological targets and therapeutic strategies for endometrial cancer treatment.

Keywords: Endometrial cancer, celecoxib, COX-2, Ishikawa cell line, AN3 CA cell line

Introduction

Endometrial cancer is one of the most common cancers of the female reproductive system and remains a major health concern worldwide. It accounts for 7% of all cancers in women and 30% of gynecological malignancies [1-3]. According to its histological features, etiology, clinical prognosis, and molecular features, endometrial cancer can be categorized into two subtypes [4]: Endometrioid endometrial carcinoma (EEC), also known as type I cancer, represents 75% to 80% of all endometrial cancer cases, and is related to unopposed estrogen exposure, complex hyperplasia with atypia. Type II endometrial cancers mainly present as papillary serous (<10%) and clear cell carcinoma (4%), with relatively poor clinical outcomes. Although hysterectomy and bilateral salpingo-oophorectomy are very effective treatments for patients with endometrial cancer, recurrences and metastases occur in those cases that present with advanced disease and lead to a poor prognosis for 15% of patients.

Cyclooxygenase-2 (COX-2) is a kind of rate-limiting enzyme in the conversion from arachidonic acid to prostaglandin. The concentration of COX-2 in tissues has been reported to abnormally increase in many human cancers. It is closely related to tumor cell growth, proliferation, angiogenesis, lymph node metastasis, and other malignant biological behaviors [5-7]. An epidemiological survey of a large number of samples showed that long-term, regular use of non-steroidal anti-inflammatory drugs (NSAIDs) can effectively reduce the incidence rate of many types of tumors, but the molecular mechanisms of the anti-tumor effect of NSAIDs has not been clarified so far [8-10]. Traditionally, NSAIDs have been believed to inhibit the expression and activation of COX-2 and thereby reduce the synthesis of the prostaglandin PGE_2. NSAIDs also exert their anti-tumor functions

through a COX-2-dependent route. However, in some tumor cell lines not expressing COX-2 or expressing low levels, NSAIDs still show tumor inhibition. Additionally, the exogenously increased COX-2 expression or addition of high dose PGE_2 does not completely reverse the tumor inhibiting effect of NSAIDs, which indicates a COX-2-independent route of tumor inhibition [11-13].

In this study, we used the COX-2 selective inhibitor, celecoxib, and, in what might be the first study of its kind, we explore COX-2's its inhibiting effects on human endometrial cancer tumor cells and discuss different control mechanisms, providing new theoretical bases and experimental evidence for the clinical prevention and treatment of endometrial cancer.

Materials and methods

Cell culture

The human endometrial cancer cell lines Ishikawa, AN3 CA (Chinese Science Academy, Shanghai), RL95-2, KLE, and HEC-1A (American Type Culture Collection Center, ATCC), were preserved in Shanghai Jiaotong University, Shanghai No. 1 Hospital Obstetrics and Gynecology Department Institute.

Cell culture and subculture: Dulbecco's modified Eagle's medium (DMEM)-F12 cell culture medium, DMEM high glucose medium, RPMI-1640 medium, and fetal bovine serum (FBS) were purchased from GIBCO. The cell lines were cultured in different media containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37°C, with 5% CO_2 and 100% humidity. The medium was removed when 70% to 80% of the cells fused; the cells were washed with PBS and digested with trypsin for 3 min at room temperature. When the cells dissociated, as observed under a microscope, 2 times the volume of fresh medium was added to terminate the digestion, followed by 1:3 subculture.

Cytometry analysis of cell cycle and apoptosis

For experimental convenience, three concentrations of celecoxib (Sigma-Aldrich, St. Louis, MO, USA), solution were used: 12.5 μmol/L, 50 μmol/L, and 100 μmol/L. Cytometry analysis was performed 48 h after celecoxib treatment. Each experiment was performed in triplicate.

The influence of exogenous PGE_2 on tumor cells

Tumor cells were treated with an IC_{50} concentration of celecoxib and different concentrations of exogenous PGE_2 (rabbit prostaglandins E_2, Merck, Darmstadt, Germany). The cell proliferation rate was determined after incubation for 48 h. The experimental groups used were: the blank control group, the solvent control group with <0.1% DMSO, and the exogenous PGE_2 groups comprising of 5 μmol/L PGE_2 + IC_{50} Celecoxib, 10 μmol/L PGE_2 + IC_{50} Celecoxib, 15 μmol/L PGE_2 + IC_{50} Celecoxib, 20 μmol/L PGE_2 + IC_{50} Celecoxib. Each experiment was performed in triplicate.

Analysis of prostaglandins E_2 (PGE_2) using ELISA

As described above, three concentrations of celecoxib were used: 12.5 μmol/L, 50 μmol/L, and 100 μmol/L. The cell culture sample was collected 48 h after the treatment, centrifuged at 3000 rpm for 20 min, and the upper clear solution was stored at -20°C. We analyzed the samples within 3 days in order to avoid damage due to repeated freezing and thawing. We prepared the standard curve and performed ELISA. Each experiment was performed in triplicate.

Determination of COX-2 mRNA expression levels, and protein content in endometrial cancer cell lines

Real-time polymerase chain reaction (RT-PCR) and western blotting were used to determine the COX-2 mRNA expression levels and the protein content in the endometrial cancer cell lines. The cell lines that showed the highest and lowest expression levels were used for follow-up cell function experiments.

RT-PCR determination of COX-2 mRNA content: RT-PCR was performed as follows, under strict experimental conditions: extraction of total RNA from cells, characterization of the purity and concentration of RNA, storage of RNA at -80°C until use, synthesis of cDNA via reverse transcription, storage of cDNA at -20°C until use (validity of which was for 6 months). *COX-2* and *GAPDH* sequence primers were designed

as described previously [14, 15], and verified with Gene bank. The primers for *COX-2* and *GAPDH* were as follows: COX-2-F: 5'-CATTGACCAGAGCAGGCAGAT-3'; COX-2-R: 5'-CAAGGAGAATGGTGCTCCAACT-3'; GAPDH-F: 5'-GAAGGTCGGAGTCAACGGATT-3'; GAPDH-R: 5'-CGCTCCTGGA AGATGGTGAT-3'. These primers were synthesized by Shanghai Yingjun Biological Engineering Co., Ltd. The PCR reaction system was comprised of a total volume of 25 μL and the reaction conditions were 2 min per cycle, 95°C for 10 s, 60°C for 30 s, and 70°C for 45 s, for 40 cycles overall. Each sample was run in triplicate and the results were averaged.

Western blot and determination of COX-2 protein content: We extracted total protein and determined the total protein concentration (using the Bradford method), based on which, we prepared sample solutions with equal concentrations using a loading buffer. We then performed a western blot, following these steps: preparation of gel, loading, electrophoresis, membrane transfer, and incubation with rabbit anti-human β-actin polyclonal antibody, diluted 1:2000 and rabbit anti-human COX-2 polyclonal antibody (Abcam, Cambridge, UK), diluted 1:1000. The system was incubated overnight with a well-sealed PVDF membrane at 4°C, after which, the membrane was washed. Development and identification were performed after incubation for 2 h at room temperature. The developed X-ray films with the protein bands were scanned in order to quantify the levels of the proteins.

CCK-8 experiments

Based on results from RT-PCR and western blot, the Ishikawa endometrial cancer cell line showed a high expression of COX-2, but the AN3 CA cell line showed a low expression of COX-2. These cell lines were further used for cell function experiments. We dissolved 7.6 mg celecoxib in 2 mL DMSO under sterile conditions to obtain a 10 mmol/L reserved solution, and we stored at -20°C. The experimental concentrations were prepared in advance and the final concentration of the DMSO solution used in all experiments was <0.1%. The experimental groups used were the blank control group, the sample without celecoxib or DMSO; the solvent control group, the <0.1% DMSO group; and the celecoxib solutions, 12.5 μmol/L, 25 μmol/L, 50 μmol/L, 75 μmol/L, and 100 μmol/L.

We used Ishikawa and AN3 CA tumor cells in the logarithmic growth phase, adjusted to 5×10^4 mL^{-1}, and then we added 100 μL tumor cells to each well of a 96-well plate, with 6 wells per sample. We added celecoxib to each well, as per the groups and re-incubated the cells. Every 24 h, we added 200 μL of fresh medium containing 15 μL CCK-8 reagent to each well and incubated the cells for another 1.5 h. We measured the absorbance at 450 nm using an enzyme microplate reader. We prepared the dose-reaction curve and used SPSS statistical software 17.0 for the Probit analysis. We calculated the half-maximal inhibitory concentration (IC_{50}) of celecoxib for the Ishikawa and AN3 CA cells after 48 h. Each experiment was performed in triplicate.

The influence of celecoxib treatment on cell cycle and apoptosis-related proteins

Forty-eight hours post treatment of tumor cells with IC_{50} celecoxib, western blot was used to determine the expression levels of the cell cycle and apoptosis-related proteins. The antibodies used were: 1:2000 diluted rabbit anti-human β-actin polyclonal antibody, 1:3000 diluted rabbit anti-human PPAR-γ (Cell Signaling, USA) monoclonal antibody, 1:200 diluted mice anti-human cytochrome C monoclonal antibody, 1:3000 diluted mice anti-human cleaved caspase-3 monoclonal antibody, 1:3000 diluted rabbit anti-human cleaved caspase-9 monoclonal antibody, 1:2000 diluted rabbit anti-human PPAR monoclonal antibody, 1:1000 diluted rabbit anti-human cyclinD1 monoclonal antibody, 1:1500 diluted mice anti-human cyclin E monoclonal antibody, 1:400 diluted rabbit anti-human P^{21Waf1} monoclonal antibody, and 1:400 diluted rabbit anti-human P^{27Kip1} monoclonal antibody. All antibodies were incubated at 4°C overnight. Each experiment was performed in triplicate.

Statistical analysis

SPSS version 16.0 software was used for the statistical analysis. The results are shown as the mean ± standard deviation. One-way ANOVA was used to compare differences between groups. The differences were considered significant when $P<0.05$.

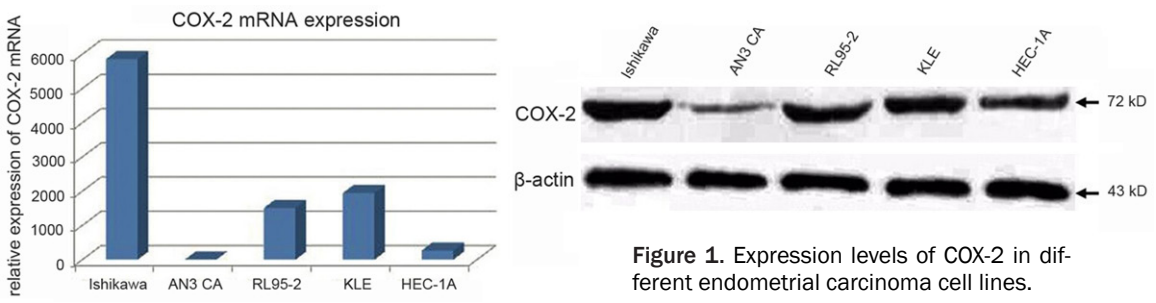

Figure 1. Expression levels of COX-2 in different endometrial carcinoma cell lines.

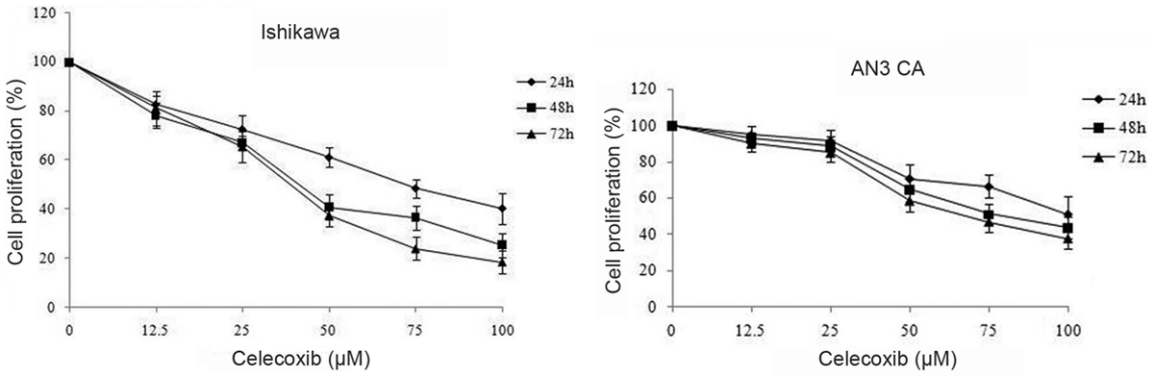

Figure 2. Survival rates of Ishikawa and AN3 CA cells treated with celecoxib for 24, 48 and 72 hrs.

Results

The Ishikawa cells showed a high expression and the AN3 CA cells showed a low expression of COX-2

The expression levels of COX-2 mRNA and proteins were determined in the endometrial cancer cell lines Ishikawa, AN3 CA, RL95-2, KLE, and HEC-1A. The results show that the expression level of COX-2 was the highest in Ishikawa and the lowest in the AN3 CA cell lines (**Figure 1**).

The inhibition effect of celecoxib on the proliferation of Ishikawa and AN3 CA cell

Following the celecoxib treatment, the proliferation of Ishikawa endometrial cancer cells was significantly inhibited ($P<0.05$) (**Figure 2**, Supplementary Data 1). On the other hand, treating AN3 CA cells with low concentration celecoxib (12.5 μM) did not show a tumor inhibiting effect ($P>0.05$), but high concentration celecoxib (50 μM, 100 μM) significantly inhibited the proliferation of AN3 CA tumor cells ($P<0.05$) (**Figure 2**, Supplementary Data 2). After 48 h, the IC_{50} of celecoxib was determined to be 40.69 μM for the Ishikawa endometrial cancer cells and 81.8 μM for the AN3 CA cells.

Celecoxib-induced apoptosis in the Ishikawa and AN3 CA cells

Forty-eight hours post celecoxib treatment, the rate of apoptosis in the Ishikawa cells was significantly increased ($P<0.01$) (**Figure 3**). Similarly, the AN3 CA cells treated with low concentration celecoxib (12.5 μM) showed an apoptosis rate of 6.01%, which was higher than the rate of 3.78% in the control group. However, the difference was not statistically significant ($P=0.065$). The high concentration groups (50 μM, 100 μM) showed apoptosis rates of 10.3% and 17.06%, respectively ($P<0.01$) (**Figure 3**).

Celecoxib induced a cell cycle block in the Ishikawa and AN3 CA cells

Forty-eight hours post celecoxib treatment, the cell cycle was arrested at the G_0/G_1 phase in both the Ishikawa and AN3 CA cells (**Figure 4**) ($P<0.05$).

The influence of celecoxib on the PGE_2 concentration in tumor cells

Forty-eight hours post celecoxib treatment, the PGE_2 content was significantly decreased in

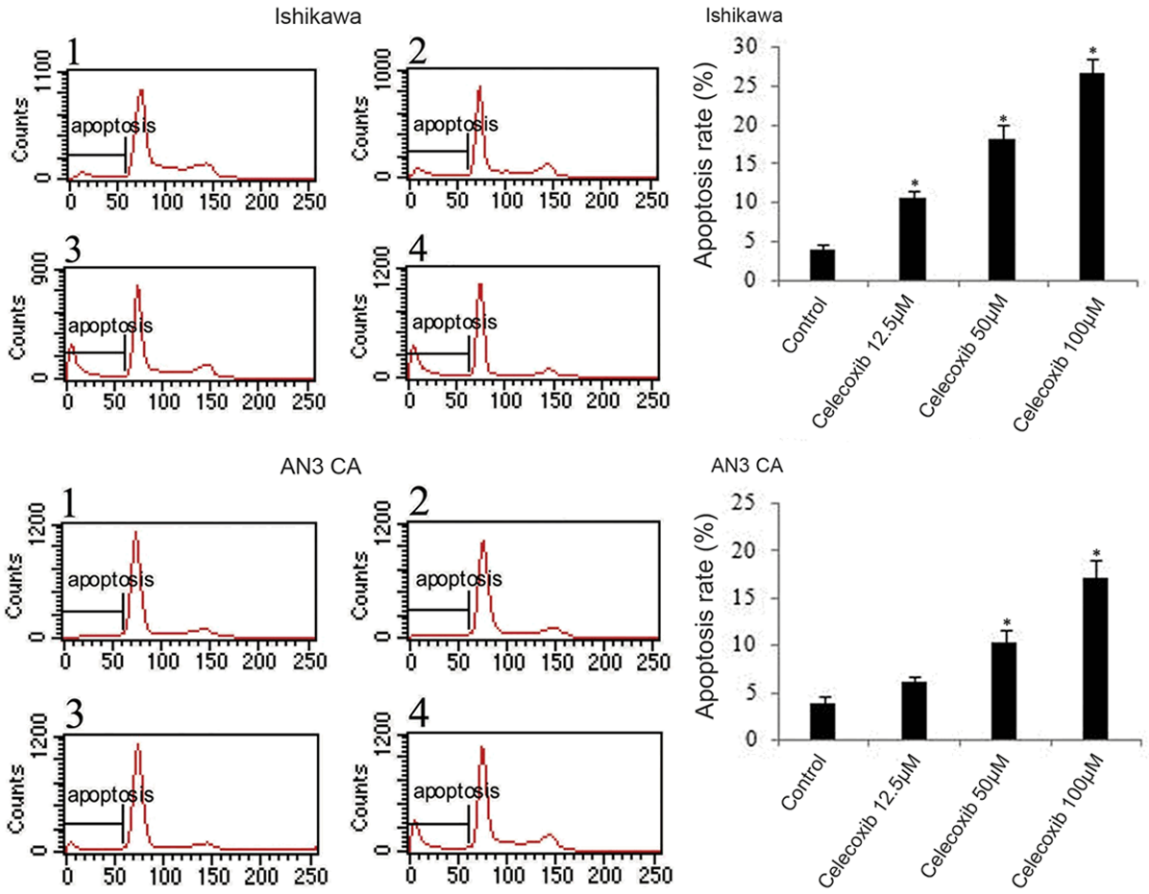

Figure 3. Apoptosis rates in Ishikawa and AN3 CA cells treated with celecoxib for 48 h. 1: Control; 2: Celecoxib 12.5 μM; 3: Celecoxib 50 μM; 4: Celecoxib 100 μM. *P<0.01 vs. control.

Ishikawa endometrial cancer cells ($P<0.01$, **Figure 5**). After treatment with a low concentration (12.5 μM) of celecoxib, the AN3 CA cells did not show any significant decrease in PGE_2 content (P>0.05). However, a high concentration of celecoxib (50 μM, 100 μM) effectively inhibited the secretion of PGE_2 and reduced its concentration ($P<0.01$, **Figure 5**). With the same dose, celecoxib showed a higher inhibition on PGE_2 in the Ishikawa cells than in the AN3 CA cells.

The influence of exogenous PGE_2 on the cell proliferation of Ishikawa and AN3 CA cells

IC_{50} celecoxib and PGE_2 at different doses were used to treat Ishikawa and AN3 CA endometrial cancer cells. The results showed that PGE_2, to a certain extent, could reverse the tumor inhibiting effect of celecoxib but could not completely offset the anti-tumor effect ($P<0.05$, **Figure 6**).

The influence of celecoxib on the expression of apoptosis control proteins in Ishikawa and AN3 CA

IC_{50} celecoxib was used to treat Ishikawa and AN3 CA cells for 48 h. The relative concentrations of apoptosis-related proteins such as cytochrome C, cleaved caspase-3, cleaved caspase-9, and PARP were significantly increased ($P<0.05$, **Figure 7**). Meanwhile, the concentration of the PPAR-γ protein was significantly increased, but the expression of the cleaved PARP (89 kDa) protein showed no significant change.

The influence of celecoxib on the expression of cell cycle-associated proteins in the Ishikawa and AN3 CA cells

IC_{50} celecoxib was used to treat Ishikawa and AN3 CA cells for 48 h. The relative concentrations of cell cycle-associated proteins such as

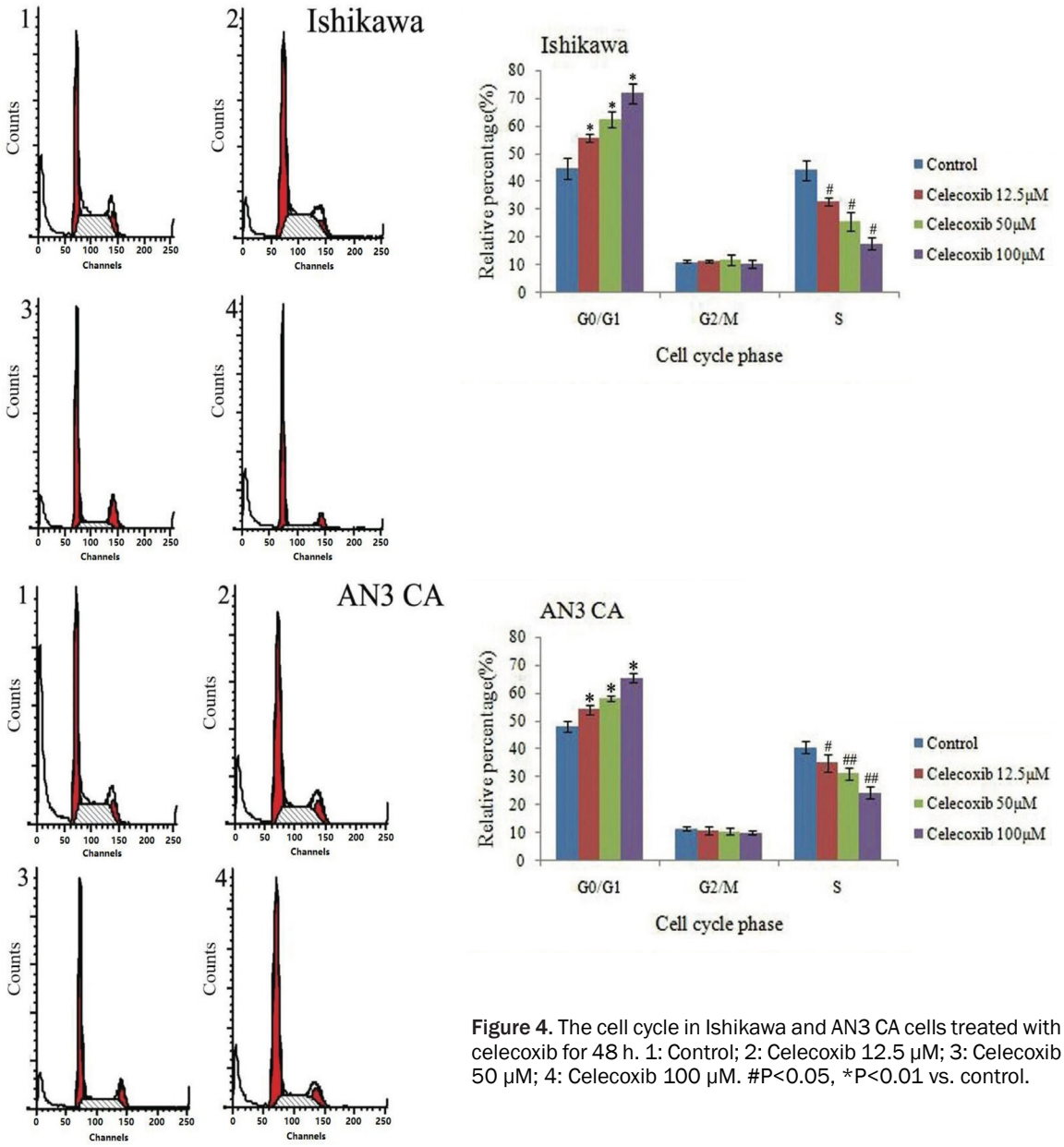

Figure 4. The cell cycle in Ishikawa and AN3 CA cells treated with celecoxib for 48 h. 1: Control; 2: Celecoxib 12.5 μM; 3: Celecoxib 50 μM; 4: Celecoxib 100 μM. #P<0.05, *P<0.01 vs. control.

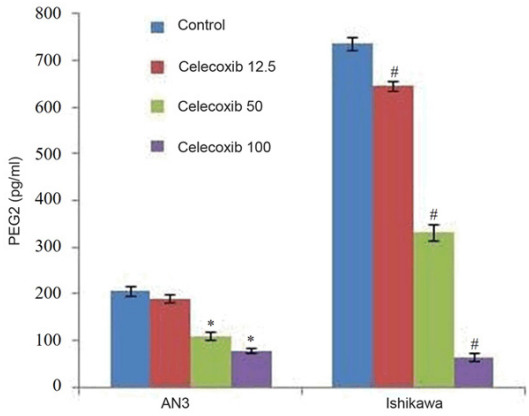

Figure 5. Levels of PGE2 in Ishikawa and AN3 CA cells treated with celecoxib for 48 h. #P<0.01, *P<0.01 vs. control.

cyclinD1 and cyclin E were significantly decreased. The expression of P^{27Kip1} was increased (P<0.05, **Figure 8**) but the expression of the P^{21Waf1} protein showed no significant change.

Discussion

So far, the precise molecular mechanisms underlying the anti-tumor effects of NSAIDs have not been fully clarified. Shaik *et al.* [16] generated A549 mice bearing human lung tumors and showed that tumor incidence and growth can be inhibited by celecoxib treatment, but the expression of COX-1 or COX-2 in the tumor is not affected. Additionally, the expression and activity of peroxisome proliferator-acti-

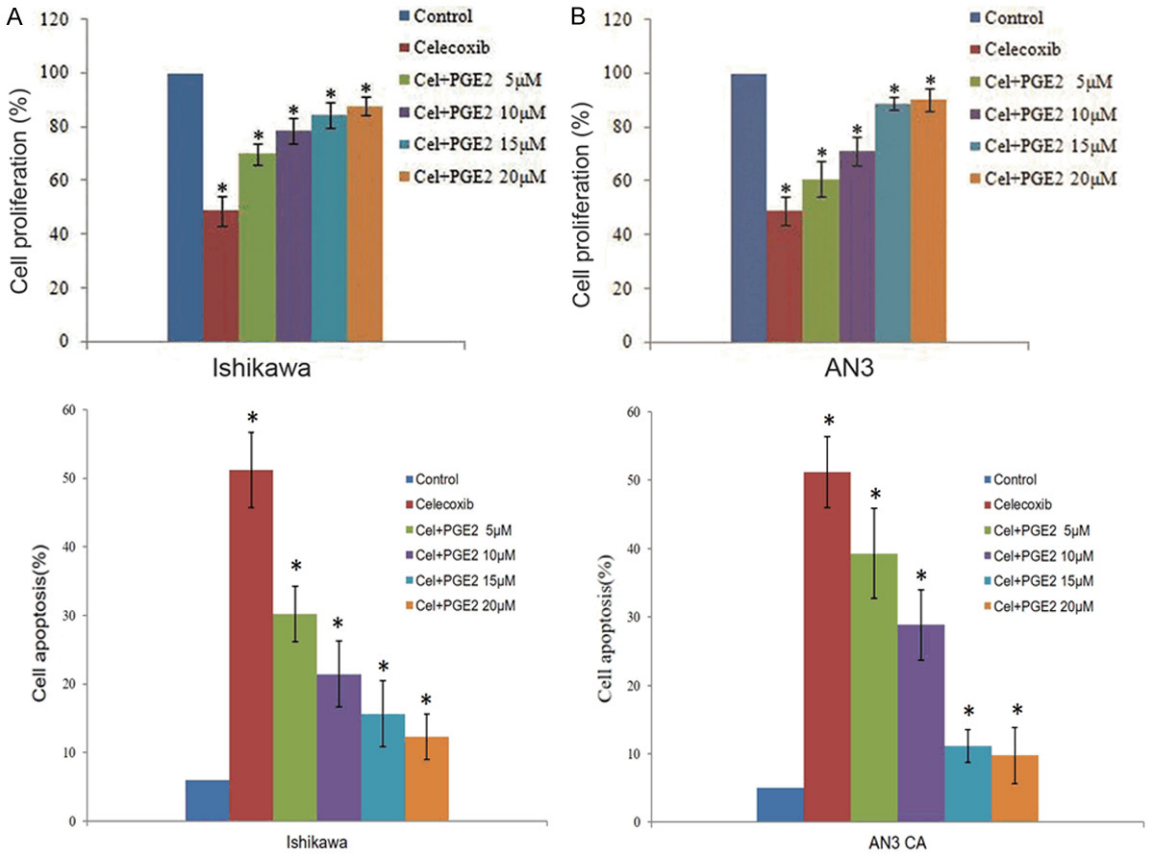

Figure 6. A, B. Reversal of celecoxib activity by exogenous PGE_2 in Ishikawa and AN3 CA cells. *P<0.05 vs. control.

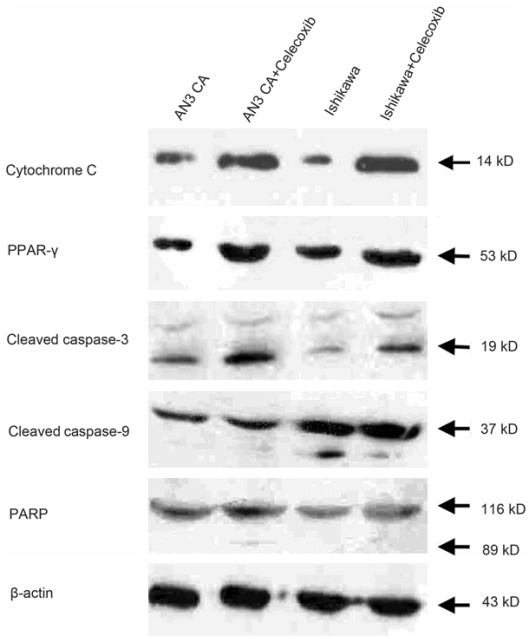

Figure 7. The expressions of apoptotic-associated proteins in Ishikawa and AN3 CA cells treated with IC_{50} celecoxib for 48 h.

vated receptor gamma (PPAR-γ) is significantly increased. The study conducted by Nikitakis *et al.* [17] showed that NSAIDs (sulindac sulfide) can effectively inhibit the proliferation of oral squamous cell carcinoma (SCC), induce apoptosis, significantly increase the expression level of PPAR-γ, and exert an synergistic effect on its expression. The tumor inhibiting effect of sulindac sulfide can also be significantly reversed. The above results show that, except for the classic COX-2-dependent route, NSAIDs also have a COX-2-independent mechanism of tumor inhibition. Since COX-2 itself does not show any signal transduction kinase function, its possible signal transduction is routed via the catalysis of various downstream prostaglandins which combine with corresponding acceptors and complete the adjustment function [18]. PPAR-γ is located at the intersection of various signal transduction routes in the body. It can exhibit multiple and complex biological effects, with characteristics of endogenous prostaglandins [19-21]. It also acts as an

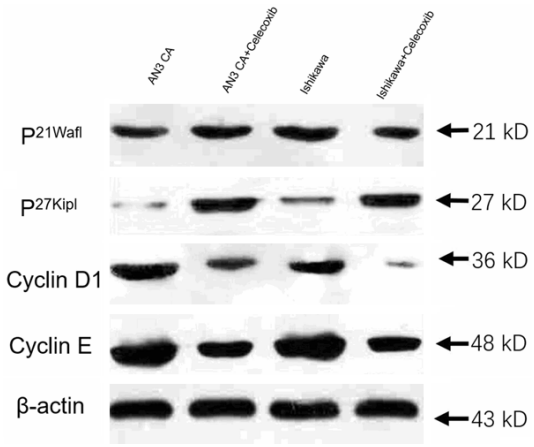

Figure 8. The expressions of cell cycle-associated proteins of Ishikawa and AN3 CA cells treated with IC_{50} celecoxib for 48 h.

important target for the anti-tumor effect of NSAIDs through the COX-2 non-dependent route [22-24]. Thus, PPAR-γ could be an important downstream control gene for COX-2.

In this study, we first screened for high COX-2-expressing Ishikawa and low COX-2-expressing AN3 CA endometrial cancer cell lines. An ELISA revealed that Ishikawa cells showed a relatively high PGE_2 content, while the AN3 CA cells had a relatively low PGE_2 content. Celecoxib showed good cytotoxicity in both Ishikawa and AN3 CA tumor cells, wherein celecoxib inhibited cell proliferation, induced tumor apoptosis, and arrested the cell cycle, suggesting that it has a tumor inhibiting effect on endometrial cancer cells through a COX-2-dependent route. Furthermore, our results showed that a low concentration of celecoxib, namely 12.5 μM, did not show any significant inhibition or apoptosis induction on AN3 CA cells ($P>0.05$), but a high concentration of celecoxib (50 μM, 100 μM) treatment significantly inhibited AN3 CA tumor cells ($P>0.05$). After exogenous addition of high concentration PGE_2, neither the Ishikawa nor the AN3 CA tumor cells showed a complete reversal of the anti-tumor effect of celecoxib. These results show that celecoxib can inhibit endometrial cancer through a COX-2-independent route. The results showed that after IC_{50} celecoxib treatment, the relative content of the PPAR-γ protein in the Ishikawa and AN3 CA tumor cells was significantly increased ($P<0.05$), indicating that the COX-2-independent route of tumor inhibition in endometrial cancer may be via the PPAR-γ protein activation. This is consistent with previous results where Wick *et al.* [25] reported that for small cell lung cancer, which does not express the COX-1 or COX-2 gene, the NSAID sulindac sulfide effectively inhibited tumor proliferation and growth in mice, thus indicating that the expression of PPAR-γ-reactive elements was induced, stabilizing the tumor, and antagonizing growth after PPAR-γ treatment.

Conclusion

Our study is the first to report that the tumor inhibiting effect of celecoxib has been demonstrated through both COX-2-dependent and -independent pathways in endometrial cancer. This may provide new biological targets and therapeutic strategies for the treatment of endometrial cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

COX-2, Cyclooxygenase-2; PGE_2, prostaglandin-E_2; PPAR-γ, peroxisome proliferator-activated receptor γ; EEC, Endometrioid endometrial carcinoma; NSAIDs, non-steroidal anti-inflammatory drugs; RT-PCR, Real-time polymerase chain reaction; IC_{50}, half-maximal inhibitory concentration; SCC, squamous cell carcinoma.

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Supplementary Data 1. Inhibition effect of celecoxib on Ishikawa cell proliferation (%)

Time (h) \ Celecoxib concentration (μM)	0	12.5	25	50	75	100
24	100	82.70	72.64	61.08	48.43	40.25
48	100	78.13	67.19	40.82	36.52	25.19
72	100	81.25	65.55	37.50	23.93	18.45

Supplementary Data 2. Inhibition effect of celecoxib on AN3 CA cell proliferation (%)

Time (h) \ Celecoxib concentration (μM)	0	12.5	25	50	75	100
24	100	94.87	91.58	70.70	66.30	50.92
48	100	92.74	88.86	64.65	51.09	43.58
72	100	90.22	84.96	58.51	46.92	37.68