Ursolic acid downregulates COX-2 expression by suppressing the activation of ERK in A549 cells

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Abstract The antitumer effect and the mechanism of action of ursolic acid in A549 cells (human non-small-cell lung cancer cells) was investigated in this paper. Firstly, MTT assay was performed to test whether ursolic acid could inhibit the growth of A549 cells. Secondly, Western blot was utilized to measure the expression level of COX-2 and the activation of MAPKs. The MTT assay revealed that ursolic acid inhibited the growth of A549 cells. The result of Western blot suggested that ursolic acid inhibited the expression of COX-2 and activation of ERK and ERK specific inhibitor PD98059 suppressed the expression of COX-2 synergistically with ursolic acid. Taken together, our data suggest that ursolic acid can suppress LPS-induced COX-2 expression in A549 cells, which could be due to the inhibition of the activation of ERK.

Key words ursolic acid; lipopolysaccharide; cyclooxygenase 2; mitogen-activated protein kinases

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乌索酸通过抑制 A549 细胞中 ERK 的激活而抑制 COX-2 的表达

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摘 要 研究乌索酸对 A549 细胞的抗肿瘤作用及其机制。首先,通过 MTT 方法检测乌索酸对 A549 细胞增殖的影响;然后,用 Western blot 方法检测乌索酸对 COX-2 表达水平及 MAPKs 的作用。结果表明,乌索酸能够抑制 A549 细胞的增殖,并能抑制 COX-2 的表达及 ERK 的激活,ERK 特异性抑制剂 PD98059 能够与乌索酸协调抑制 COX-2 的表达。乌索酸能够抑制 LPS 刺激后 A549 细胞中 COX-2 的表达,其可能的作用机制是通过抑制 ERK 的激活。

关键词 乌索酸;脂多糖;环氧化酶-2;丝裂原激活蛋白激酶

1 Introduction

Ursolic acid (UA), a natural pentacyclic triterpenoid carboxylic acid, is present in a variety of medicinal plants and has attracted considerable interest in recent years for its pharmacological effects and low toxicity. UA is well-known to possess a wide range of biological functions such as antioxidative, anti-inflammation and anticancer activities. Its anti-tumor activities include inhibition of tumorigenesis[1] and tumor promotion[2], the induction of tumor cell differentiation[3] and anti-angiogenic effect[4]. However, the mechanisms by which UA induces such cellular effects are poorly understood.

Cyclooxygenase (COX) is the rate-limiting enzyme involved in the conversion of arachidonic acid to prostanoids. There are two isoforms of COX[5]. COX-1 is expressed constitutively in most cells and tissues and is very important for various physiological functions. COX-2 is expressed at a low level or is not detectable in most tissues but is highly inducible by a variety of agents including cytokines, growth factors and tumor promoters[6]. COX-2 over-expression is found in many pre-malignant and malignant conditions involving the colon, liver, pancreas, breast, lung, bladder, skin, stomach, head and neck, and esophagus[7].

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Targeted inhibition of COX-2 is now considered to be a potential therapeutic strategy to stop the occurrence or progress of cancers. UA has been reported to be able to suppress COX-2 expression in several cell types[8-10], but the mechanism has not been reported before.

The expression of COX-2 is mainly regulated by MAPKs, nuclear factor-κB (NF-κB) and activator protein-1(AP-1). MAPKs are a highly conserved family of protein serine/threonine kinases including the p38, ERK1/2, and JNK subgroups. The involvement of ERK, p38, and JNK signaling pathways in COX-2 induction has been demonstrated in many cell types. For example, asiatic acid inhibits COX-2 expression via suppression of the phosphorylation of p38, ERK1/2, and JNK in RAW 264.7 cells[11]. It has also been reported that triptolide inhibits COX-2 expression by suppressing the activity of JNK in LPS-treated microglia[12]. Recently, it was found p38 plays a distinct role in sulforaphane-induced down-regulation of COX-2 in human bladder cancer cells[13].

In an effort to clarify the molecular mechanism by which ursolic acid exerts its antitumor activity, we investigated the effect of UA on COX-2 expression in LPS-stimulated A549 cells and whether MAPKs are involved in such process.

2 Materials and methods

2.1 *Reagents and cell culture*

UA (purity >98%) was provided by Prof. Wu Chen (Yichun College) and prepared as a 10 mmol/L stock solution in sterilized dimethylsulfoxide (DMSO; Sigma, USA) stored at −20 °C. LPS was purchased from Sigma-Aldrich (USA), polyclonal antibodies against human phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, COX-2 and β-actin from Santa Cruz Biotechnology (USA). The ERK inhibitor PD98059 was obtained from Promega (USA). A549 cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 units/mL penicillin and 100 mg/L streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2 *MTT assay for cell viability*

The effect of ursolic acid on cell viability and proliferation was determined by MTT assay. Briefly, cells were seeded at a density of 5×10^3 cells/well on 96-well plates and treated with various concentrations of ursolic acid and then cultured for 24, 48 and 72 h at 37 °C with 5% CO_2. At the end of culture, medium in each well was substituted with 200 μL of fresh medium containing MTT (final concentration, 250 μg/mL). After the cells had been incubated at 37 °C for 4 h, the reaction was stopped by adding 150 mL/well of DMSO, and the cells were then incubated for another 10 min. Absorbance was determined using an automatic plate reader (Bio-Rad, USA) at 570 nm with a reference filter of 630 nm.

2.3 *Western blot*

The experiments were performed as described before[14]. Briefly, cell lysates were prepared using lysis buffer. The lysate was then centrifuged and proteins in the supernatant were quantified by Bradford protein assay (Bio-Rad, Germany). Proteins (100 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, USA). Membranes were then incubated in blocking solution (10% non-fat dried milk, 0.5% Tween in TBS) for 2 h, followed by incubation with the primary antibody (1 ∶ 400) at 4 °C overnight. Blots were washed, incubated according to standard procedures and developed with ECL system.

2.4 *Statistical analysis*

Data represent the $\bar{x} \pm s$ of three independent experiments. The statistical significance of differences was determined using Student's t test. The minimal level of significance was $P < 0.05$.

3 Results

3.1 *Ursolic acid inhibited the growth of A549 cells*

The growth inhibitory effect of UA on A549 cells was initially examined. As shown in Figure 1, UA inhibited the proliferation of A549 cells in a concentration- and time- dependent manner. IC_{50} were (24.68 ± 2.05) μmol/L at 24 h, (18.32 ± 1.67) μmol/L at 48 h, and (5.05 ± 0.96) μmol/L at 72 h.

3.2 *Ursolic acid inhibited COX-2 expression*

As shown in Figure 2, COX-2 is weakly expressed in untreated A549 cells, while its expression is increased by the stimulation of LPS. Co-culture of A549 cells with LPS and 30 or 50 μmol/L of UA significantly decreased the expression of COX-2.

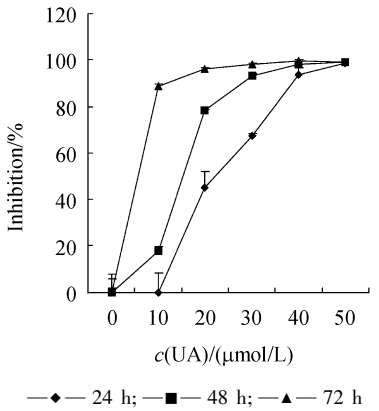


Figure 1 Effects of ursolic acid (UA) on the growth of A549 cells ($x \pm s$, $n = 3$)

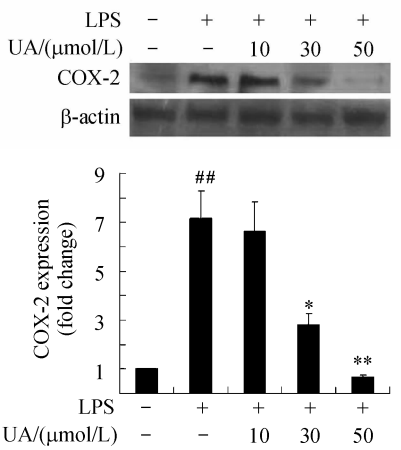


Figure 2 Effects of ursolic acid on COX-2 expression in LPS-treated A549 cells ($x \pm s$, $n = 3$)

Density value of each band was normalized to β-actin control and the fold protein expression was calculated relative to a normalized value of one given to control cells

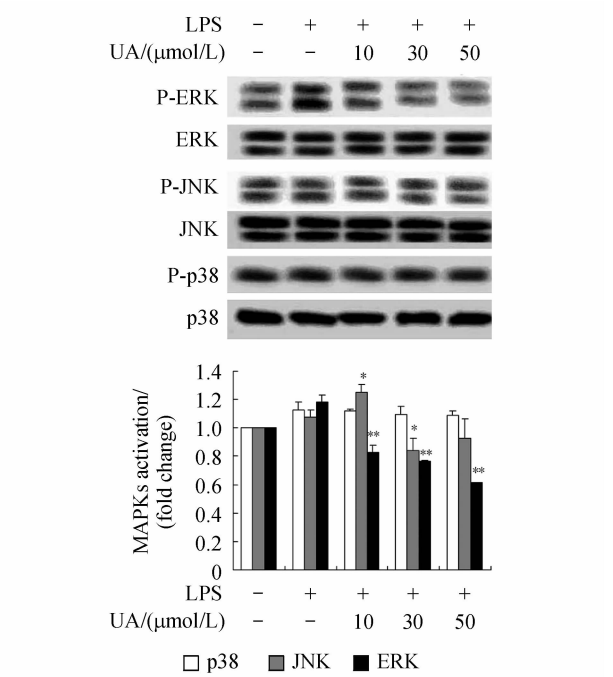
$^{\#\#}P < 0.01$ *vs* control group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* LPS group

3.3 *Ursolic acid inhibited the activation of ERK in LPS-treated A549*

As MAPKs are important for the production of many proinflammatory mediators, the effects of UA on MAPKs activation were further examined. As shown in Figure 3, Western blot analysis showed that UA inhibited the activation of the ERK, but not that of JNK and p38.

3.4 *PD98059 inhibited COX-2 expression synergistically with ursolic acid*

To further clarify whether the activation of ERK is involved in UA-induced downregulation of COX-2, we measured the change of COX-2 expression in the presence of ERK-specific inhibitor PD98059. Figure 4A showed that PD98059 suppressed the expression of COX-2 significantly. As indicated in Figure 4B, PD98059 (10 mmol/L) and UA (30 mmol/L) synergistically decreased COX-2 level. When applied alone, PD98059 and UA had only weak effects on COX-2 expression (86.62% and 73.70% of control, respectively), whereas in combination, they exerted a significant reduction on COX-2 level (only 37.39% of control).



Figurr 3 Effects of ursolic acid on MAPKs pathways in LPS-treated A549 cells ($x \pm s$, $n = 3$)

The intensity of the bands was quantified by densitometry and normalized to that of total MAPK proteins. The fold protein expression was calculated relative to a normalized value of one given to control cell

$^{*}P < 0.05$, $^{**}P < 0.01$ *vs* LPS group

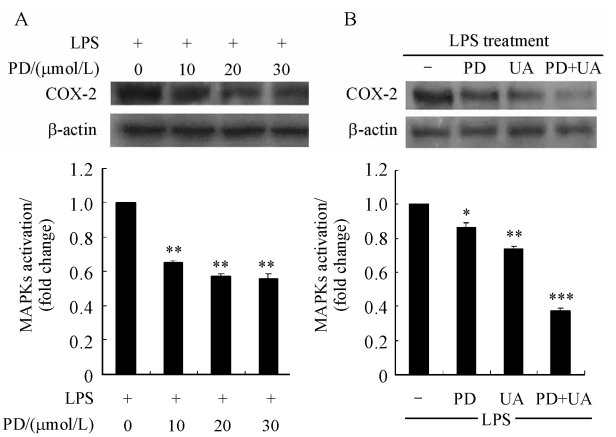


Figure 4 Effects of PD98059 (PD) on COX-2 expression in LPS-treated A549 cells ($x \pm s$, $n = 3$)

Density value of each band was normalized to β-actin control and the fold protein expression was calculated relative to a normalized value of one given to control cells

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control group

4 Discussion

As one of the most common cancers in the world, lung cancer is the leading cause of cancer deaths throughout the world. Two main types of lung cancer are small cell lung cancer and non-small-cell lung cancer (NSCLC), the latter making up about 75% of all lung cancers. Elevated level of COX-2 is found in almost all NSCLC pre-invasive precursor lesions and invasive lung carcinomas[15]. In the present study, we found that UA inhibited the growth of A549 cells, a kind of human NSCLC cells, in a dose- and time-dependent manner.

Our studies also demonstrated that UA significantly decreased LPS-stimulated COX-2 expression in A549 cells. Previously it has been reported that UA could inhibit the expression of COX-2 in PMA-treated human mammary epithelial cells[8] and TNF-induced leukemic cell[16] yet the relationship between UA and COX-2 expression is rarely studied in cancer cells. Multiple lines of evidence suggest that COX-2 is involved in tumor proliferation, invasion, angiogenesis and resistance to apoptosis. COX-2 expression is elevated in various malignancies including colon, gastric, esophageal, prostate, pancreatic, breast and lung carcinomata[17-22]. Several investigators have observed that COX-2 over-expression is sufficient to transform normal cells to malignant neoplasms in animal models of carcinogenesis[23-25]. Thus, COX-2 has become an important molecular target and COX-2 inhibition appears to be an exciting anti-tumor strategy. The traditional non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin exert their effect through the inhibition of both COX-1 and COX-2 with a property to reduce the risk of cancers and inhibit tumor development. However, their regular use is associated with various side-effects including gastric ulceration, coronary thrombosis and renal disorder, which is thought to be due to the inhibition of COX-1. Recently developed selective COX-2 inhibitors could reduce these adverse effects. However, certain COX-2 inhibitors, like rofecoxib and valdecoxib, increased cardiovascular risk (myocardial infarction or stroke) and thereafter, these drugs were withdrawn from the market[26-27]. Therefore, UA may be a potential COX-2 inhibitor without aforementioned unwanted effects to be used in combination with other cancer therapies.

MAPKs control a number of cellular events, including differentiation, proliferation, and death[28]. To further explore the mechanism by which ursolic acid modulates LPS-stimulated COX-2 expression, we examined whether UA affects the activation of MAPKs and whether the downregulation of COX-2 expression by UA is through the MAPK pathway. Our data obtained from Western blot strongly suggest that UA inhibits COX-2 expression via the ERK1/2 pathway but not that of p38 and JNK1/2 and ERK specific inhibitor PD98059 not only downregulated COX-2 expression alone, but also significantly enhanced the reduction in COX-2 expression caused by UA. Previous studies have shown the mechanism of other agents on COX-2 expression. For example, asiatic acid inhibits iNOS, COX-2, IL-6, IL-1β and TNF-α expression through the down-regulation of NF-κB activation via suppression of IKK and MAP kinase (p38, ERK1/2, and JNK) phosphorylation in RAW 264.7 cells[11]. Triptolide inhibits PGE_2 production and COX-2 protein expression in LPS-treated microglia cells through suppressing the activation of JNK and the transcription factor NF-κB[12]. The function mechanism of regulating COX-2 expression by these agents appears to be varied and complicated. Our data indicated that UA downregulated COX-2 expression via MAPK signaling pathway. However, whether the action can be realized by other signaling pathways, like NF-κB or PI3K/Akt pathways, still needs further research.

In conclusion, this study show that UA inhibited the growth of A549 cells and suppressed COX-2 expression induced by LPS. The suppression of COX-2 expression by UA is via the ERK1/2 pathway. Although UA is a promising anti-tumor agent, further extensive evaluations are necessary to determine the risks and benefits of this triterpenoid and much work needs to be done to improve its solubility.

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