# Involvement of cell cycle regulatory proteins and MAP kinase signaling pathway in growth inhibition and cell cycle arrest by a selective cyclooxygenase 2 inhibitor, etodolac, in human hepatocellular carcinoma cell lines

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**Recent studies have shown that selective cyclooxygenase-2 (COX-2) inhibitors induce growth inhibition and cell cycle arrest in hepatocellular carcinoma (HCC) cell lines. However, the mechanism by which COX-2 inhibitors regulate the cell cycle and whether or not growth signal pathways are involved in the growth inhibition remain unclear. In this study, we investigated the mechanisms of growth inhibition and cell cycle arrest by etodolac, a selective COX-2 inhibitor, in HCC cell lines, HepG2 and PLC/PRF/5, by studying cell cycle regulatory proteins, and the MAP kinase and PDK1-PKB/AKT signaling pathways. Etodolac inhibited growth and PCNA expression and induced cell cycle arrest in both HCC cell lines. Etodolac induced $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ expression and inhibited CDK2, CDK4, CDC2, cyclin A and cyclin B1 expression, but did not affect cyclin D1 or cyclin E. HGF and 10% FBS induced ERK phosphorylation, but phosphorylation of p38, JNK and AKT was down-regulated by etodolac. PD98059, a selective inhibitor of ERK phosphorylation, induced growth inhibition, the expression of $p27^{Kip1}$ and cell cycle arrest. In conclusion, $p21^{WAF1/Cip1}$, $p27^{Kip1}$, CDK2, CDK4, CDC2, cyclin A, cyclin B1 and the MAP kinase signaling pathway are involved in growth inhibition and cell cycle arrest by a selective COX-2 inhibitor in HCC cell lines. (Cancer Sci 2004; 95: 666–673)**

Cyclooxygenase 2 (COX-2) is a highly inducible, rate-limiting enzyme involved in the production of prostaglandins (PGs), prostacyclins and thromboxanes. COX-2 is expressed in response to a variety of proinflammatory agents and cytokines.[1,2] Overexpression of COX-2 has been demonstrated in various tumors such as colon cancer, pancreatic cancer and hepatocellular carcinoma (HCC).[3–5] Nonsteroidal anti-inflammatory drugs (NSAIDs) are currently being assessed for use in the prevention and treatment of colorectal cancer, based on epidemiologic, preclinical and experimental evidence.[6,7] Although several studies have shown the up-regulation of COX-2 in cirrhotic tissues adjacent to HCC and well-differentiated HCC, the precise role of COX-2 in hepato-carcinogenesis remains unclear.[5,8] A recent report of ours has also shown that NS-398, a selective COX-2 inhibitor, inhibited growth and induced cell cycle arrest in HCC cell lines.[9] However, the mechanism by which COX-2 inhibitors cause growth inhibition and cell cycle arrest in HCC cells is not known.

There is increasing evidence that perturbation of cell cycle regulation is an important contributing factor to cancer, including HCC.[10,11] There are two key checkpoints in the cell cycle, the G1-S and G2-M checkpoints. Cyclins, cyclin-dependent kinases (CDKs) and cyclin/CDK complexes have been found to control the progression of cells through the G0-G1, S and G2-M phases of the cell cycle and to regulate the two checkpoints.[12,13] CDK inhibitors bind to the cyclin/CDK complexes and inhibit their activity, resulting in the inhibition of cell cycle progression. Two structurally defined classes of CDK inhibitors have been identified: the p21 family, including $p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and the INK4 family, including $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$.[14] At present, the relationship between these cell cycle regulatory proteins and cell cycle arrest by COX inhibitors in HCC cells remains largely unknown.

When cells are stimulated by growth factors, an important signaling pathway is activated by phosphorylation, i.e., the mitogen-activated protein (MAP) kinase pathway, which includes extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. ERK, JNK and p38 are key molecules at the convergence of several signal transduction pathways, and they transduce the converged signals into the nucleus, resulting in various cellular responses, including proliferation, differentiation and apoptosis.[15,16] The PDK1-PKB/AKT pathway is also important in growth factor-dependent survival of cell lines and primary cultures of diverse cell types, including liver cancer.[17,18] The involvement of the MAP kinase signaling pathway and the PDK1-PKB/AKT pathway in NSAID-induced growth inhibition in prostate cancer cell was recently reported.[19] However, whether these signaling pathways are also involved in growth inhibition and cell cycle regulation by COX-2 inhibitors in HCC remains unclear.

In this study, we investigated the mechanism of cell cycle arrest by etodolac and its effects on signaling pathways by examining changes in cell cycle regulatory proteins and by monitoring the phosphorylation of several key signal proteins in HCC cell lines. Our findings showed that $p21^{WAF1/Cip1}$, $p27^{Kip1}$, CDK2, CDK4, CDC2, cyclin A and cyclin B1 are involved in cell cycle arrest by etodolac. Etodolac may also inhibit cell growth and regulate the cell cycle by blocking the MAP kinase signaling pathway in HCC cells.

## Materials and Methods

**Reagents.** Etodolac was a generous gift from Nihon Shinyaku Co. (Kyoto, Japan). Rabbit polyclonal antibodies against phos-

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Abbreviations: COX-2, cyclooxygenase-2; HCC, hepatocellular carcinoma; NSAIDs, nonsteroidal anti-inflammatory drugs; CDK, cyclin-dependent kinase; MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; PCNA, proliferating cell nuclear antigen; PDK1, phosphoinositide-dependent kinase; PKB, protein kinase B; $PGE_2$, prostaglandin $E_2$.

pho-Ser$^{308}$-AKT, phospho-Ser$^{473}$-AKT, phospho-p38 and phospho-JNK were from BioSource International, Inc. (Camarillo, CA). Mouse monoclonal anti-p21$^{WAF1/Cip1}$, anti-p27$^{Kip1}$, anti-PCNA and anti-cyclin B1, goat polyclonal anti-phospho-ERK and anti-COX-2 and rabbit polyclonal anti-ERK, anti-CDK2 and anti-cyclin E were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-cyclin D1 was from Dako Cytomation Co., Ltd. (Kyoto, Japan). PD98059 was from Calbiochem (La Jolla, CA). Human recombinant HGF was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Cell culture.** Two human hepatocellular carcinoma cell lines HepG2 and PLC/PRF/5, were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C. The MTT method was used to monitor cell growth. Etodolac was dissolved in ethanol. The concentration of ethanol in the medium remained constant for all groups (<0.5%).

As a control experiment, MRC-5, a human embryonic lung-derived fibroblast cell line, which exhibits a low level of COX-2 expression, was also obtained from the American Type Culture Collection. MRC-5 cells were treated in the same way as HCC cells.

**Western blotting.** Cells ($5\times10^5$) were sonicated and homogenized in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 45 μg/ml aprotinin, 100 m*M* sodium orthovanadate). The supernatant of the homogenate was used for protein determination with a BCA Protein Assay Kit (Pierce, IL) and electrophoresis. Samples with equal amounts of total protein were applied to SDS-PAGE gel in order to compare the levels of protein expression among samples. Actin was also used to confirm equal loading. Electrophoretic transfer to polyvinylidene difluoride membranes was followed by immunoblotting with different primary antibodies. Signals were developed with an ABC kit (Vector, Burlingame, USA) and diaminobenzidine.

**Measurement of $PGE_2$ in medium.** After treatment of the cells with etodolac in medium containing 10 m*M* arachidonic acid to provide sufficient substrate for 24 h, the medium was collected. The level of $PGE_2$ in each sample was measured by using a $PGE_2$ High Sensitivity ELISA Kit (R&D Systems, Minneapolis, USA).

**Flow cytometric analysis for cell cycle distribution.** Cell cycle distribution was determined by flow cytometric analysis of DNA content (Becton Dickinson, San Jose, USA) after 72 h of etodolac treatment. Cell suspensions were fixed overnight in 10 ml of 70% ethanol and incubated with RNase at a concentration of 0.25 mg/ml at 37°C for 1 h. Cells were treated with propidium iodide (50 μg/ml) for 30 min. DNA histograms were analyzed using Lysis-II software (Becton Dickinson) to evaluate the cell cycle components.

**Statistical analysis.** Statistical significance was determined using the Wilcoxon two-sample test and Kruskal-Wallis test. The criterion of significance was set at $P<0.05$.

## Results

**Dose-dependent inhibitory effect on growth and PCNA expression following treatment with etodolac.** Two of the cell lines used in this study expressed COX-2, an important target of etodolac treatment in HCC cell lines, at the mRNA and protein levels, as reported previously.[9] Growth-inhibition experiments were performed by treatment of cells with various doses of etodolac for 72 h. Etodolac exhibited a dose-dependent growth-inhibitory effect on HepG2 and PLC/PRF/5 (Fig. 1A). As a control experiment, MRC-5 cells were treated in the same way as HCC cells; no growth inhibition was observed.

The expression of PCNA, an important growth marker and DNA replication regulator, was also detected. Ten micrograms of total protein from etodolac- treated cells was separated by SDS-PAGE and western blotting was carried out using an anti-PCNA monoclonal antibody. A dose-dependent down-regulation of the expression of PCNA in two cell lines was observed after 72-h treatment with various concentrations of etodolac (Fig. 1B). The down-regulation of PCNA expression is consistent with the results of growth inhibition described above.

**No correlation between edotolac-induced growth inhibition and generation of $PGE_2$; growth-inhibitory effect unaffected by adding $PGE_2$.** To confirm the inhibitory effect of etodolac on COX-2 enzymatic activity and to determine the correlation between growth inhibition and COX-2 expression/PG production, we measured $PGE_2$ levels in the medium by ELISA and the expression of COX-2 in cell lysates by western blotting. The $PGE_2$ levels in the medium were reduced in a dose-independent manner by etodolac treatment for 24 h, and no significant correlation was observed between growth inhibition and the $PGE_2$

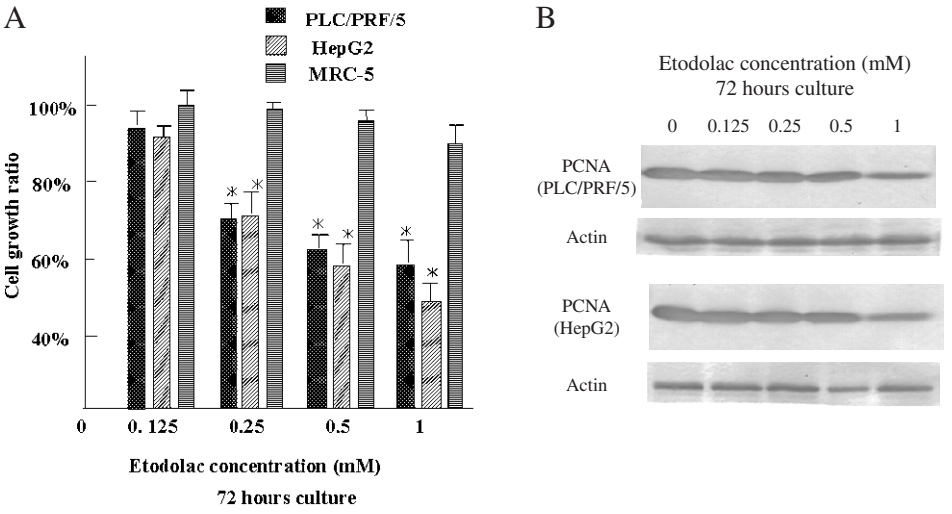


**Fig. 1.** (A) Dose-dependent growth-inhibitory effect of etodolac in HepG2 and PLC/PRF/5 cells. Etodolac was added at 0.125 m*M*, 0.25 m*M*, 0.5 m*M* or 1 m*M*, and the cells were cultured for 72 h in 96-well plates. The MTT method was used to monitor cell growth. The cell growth ratio (%) was calculated by dividing the number of etodolac-treated cells by the number of untreated cells. Data from three independent experiments are shown as the mean±SD. * $P<0.05$. No significant growth inhibition was observed in MRC-5 cells. (B) Effect of etodolac on the expression of PCNA. The expression of PCNA was down-regulated in HepG2 and PLC/PRF/5 cells by etodolac treatment. The cells were treated for 72 h with etodolac at 0.125 m*M*, 0.25 m*M*, 0.5 m*M* or 1 m*M*. Total protein was extracted from $5\times10^5$ cells and samples containing 10 μg of total protein were used for western blotting. PCNA expression was inhibited in a dose-dependent manner. Representative results of western blotting are shown from three independent experiments.

level (Fig. 2A) ($P<0.05$). The growth-inhibitory effect was not influenced by adding $PGE_2$ up to 1000 pg/ml in either of the cell lines during 72-h culture (Fig. 2B). No significant change in COX-2 expression was found by western blotting after treatment for 24 to 72 h with various concentrations of etodolac (Fig. 2C). These results suggest that a COX-2-independent mechanism may be involved in the anti-tumor effect of etodolac.

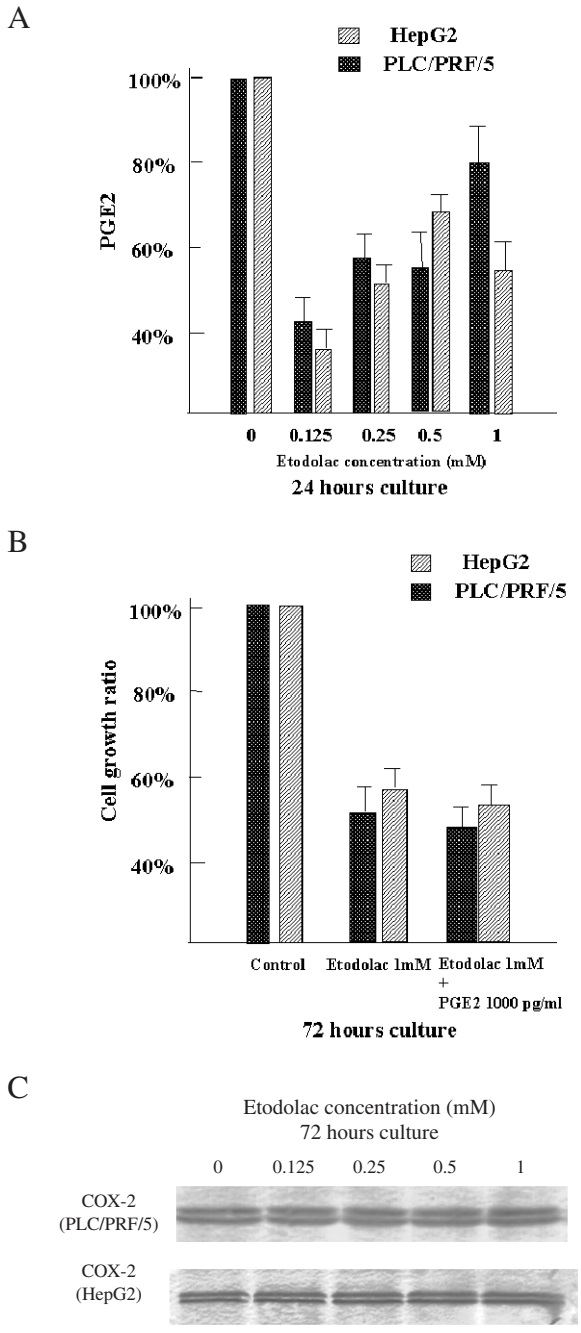


**Fig. 2.** Lack of correlation between the generation of $PGE_2$ in the medium and growth inhibition. (A) The $PGE_2$ levels in the medium were reduced in a dose-independent manner by etodolac treatment for 24 h. No significant correlation was observed between growth inhibition and the inhibitory effect on $PGE_2$ generation. (B) The growth-inhibitory effect was not influenced by adding $PGE_2$ at 1000 pg/ml to the culture for 72 h in either of the cell lines. (C) No significant change in COX-2 expression were found after etodolac treatment for 72 h in either of the cell lines.

**Induction of cell cycle arrest in two HCC cell lines by etodolac.** DNA histograms from flow cytometric analysis showed a reduced number of cells in the S phase, and accumulation in G0-G1 in both HepG2 and PLC/PRF/5 after a 72-h treatment with various concentrations of etodolac (Fig. 3, A and B). Significant accumulation in G2-M was observed for HepG2 cells (Fig. 3A), but no significant change was observed in G2-M for PLC/PRF/5 cells (Fig. 3B). A significant correlation was observed between growth inhibition and the accumulation of HepG2 and PLC/PRF/5 cells in G0-G1 ($P<0.05$).

**Etodolac treatment induces expression of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$.** To determine the role of two important CDK inhibitors, $p21^{WAF1/Cip1}$ and $p27^{Kip1}$, in cell cycle arrest induced by etodolac, their expression was examined by western blotting after etodolac treatment. Our results showed that $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ were induced by 24-h etodolac treatment (Fig. 4) and the effect was dose-dependent in both HepG2 and PLC/PRF/5 cells. The

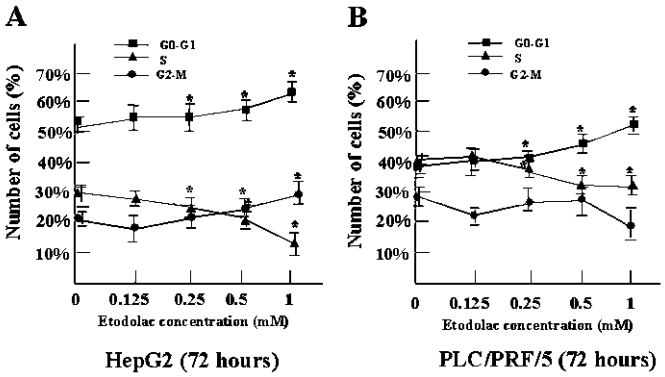


**Fig. 3.** Effect of etodolac on cell cycle arrest in HepG2 and PLC/PRF/5 cells. Etodolac at a concentration of 0.125 m*M*, 0.25 m*M*, 0.5 m*M* or 1 m*M* was added, and cells were cultured for 72 h. A reduction in cell entry into the S phase and accumulation of cells in the G0-G1 phase in both HepG2 and PLC/PRF/5 were observed. No significant change in the number of cells at the G2-M phase was noted in PLC/PRF/5 cells, and accumulation of cells in G2-M was observed in HepG2 cells. Data from three independent experiments are shown as the mean±SD. * $P<0.05$.

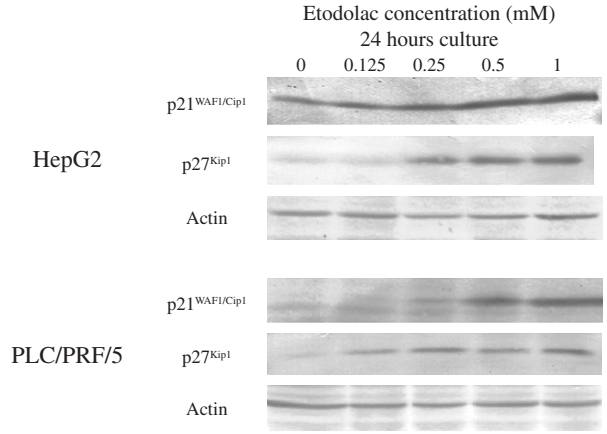


**Fig. 4.** Effect of etodolac on the expression of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ in HepG2 and PLC/PRF/5 cells. Etodolac were added at a concentration of 0.125 m*M*, 0.25 m*M*, 0.5 m*M* or 1 m*M* and the cells were cultured for 24 h. Samples containing 50 μg of total protein were used for western blotting. A dose-dependent induction of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ was observed. Representative bands from western blotting are shown for five independent experiments.

two cell lines showed different degrees of sensitivity to etodolac treatment, as shown in Fig. 4. $P21^{WAF1/CIP1}$ was induced at 0.125 m*M* in HepG2 cells, but a clear inducing effect was only seen at more than 0.25 m*M* in PLC/PRF/5. However, $p27^{Kip1}$ is induced at lower concentrations in PLC/PRF/5 than in HepG2 cells. $P27^{KIP1}$ was induced at 0.125 m*M* in PLC/PRF/5, but a clear inducing effect was only seen at more than 0.25 m*M* in HepG2 cells.

**Etodolac inhibited expression of CDK2, CDK4, CDC2, cyclin A and cyclin B1, but not cyclin D1 or cyclin E.** We next investigated whether etodolac regulates the expression of CDK2, CDK4, CDC2, cyclin A, cyclin D1 and cyclin E, which are important regulatory proteins of the cell cycle. As shown in Fig. 5, CDK2, CDK4, CDC2, cyclin A and cyclin B1 expression were inhibited in a dose-dependent manner after 24-h treatment with etodolac. A time-dependent inhibitory effect was also observed from 24 to 72 h (data not shown). No inhibitory effect was observed on the expression of cyclin D1 and cyclin E from 24 to 72 h.

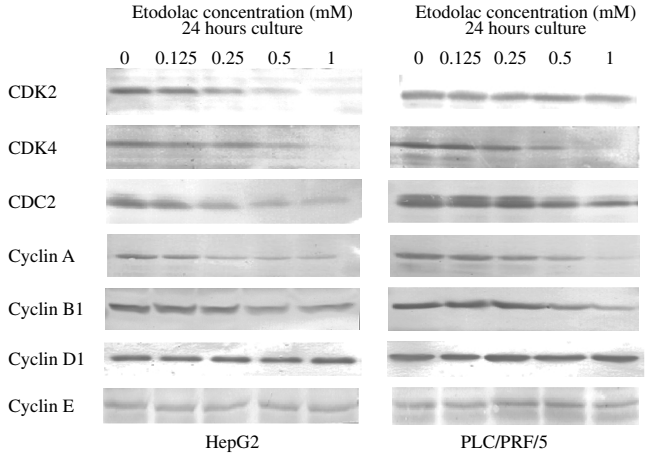


**Fig. 5.** The expression levels of CDK2, CDK4, CDC2, cyclin A, cyclin B1, cyclin D1 and cyclin E following etodolac treatment. The cells were cultured for 24 h after addition of etodolac at 0.125 m*M*, 0.25 m*M*, 0.5 m*M* or 1 m*M*. Total protein was extracted from $5\times10^5$ cells and samples containing 50 μg of total protein were used for western blotting. The expression of CDK2, CDK4, CDC2, cyclin A and cyclin B1 was inhibited in HepG2 and PLC/PRF/5 cells. No significant change was observed in the expression of cyclin E or cyclin D1. Representative results of western blotting are shown from three independent experiments.

**Etodolac down-regulated the inducing effect of HGF on cell growth and on ERK activation in both HepG2 and PLC/PRF/5 cells.** To investigate whether etodolac regulates the effect of growth factor on HCC cell lines, 10 ng/ml recombinant human HGF with or without etodolac (0.5 m*M*) was added to HepG2 and PLC/PRF/5 in culture medium containing 10% FBS for 48 h. The HGF-induced cell growth was inhibited by etodolac treatment in both HepG2 and PLC/PRF/5 cells (Fig. 6A).

We further studied whether etodolac regulates HGF-induced ERK activation in HCC cell lines. PLC/PRF/5 and HepG2 cells, which were cultured for 48 h in medium without serum, were pretreated with etodolac for 1 h and stimulated with HGF (10 ng/ml) for 1 h. HGF-induced phosphorylation of ERK was down-regulated in both HepG2 and PLC/PRF/5 cells by pretreatment with etodolac. This finding suggests that etodolac down-regulated HGF-induced cell growth by blocking the HGF growth signaling pathway (Fig. 6B).

**Etodolac inhibited phosphorylation of ERK-1/2 in PLC/PRF5 in the presence of 10% FBS.** Furthermore, to determine the effect of etodolac on the MAP kinase signaling pathway in the presence of 10% FBS, the activities of phosphorylated ERK, p38, and JNK were also assessed. Etodolac inhibited phosphorylated ERK-1/2 dose-dependently in PLC/PRF/5 cells in the presence of 10% FBS (Fig. 7A), but this kind of effect was not seen in HepG2 with 10% FBS (data not shown). The inhibitory effect of etodolac was only apparent at 18 to 24 h after treatment (Fig. 7B). Total ERK-1/2 (phosphorylated and nonphosphorylated) levels were not affected by treatment with etodolac. The phosphorylation status of p38 and JNK was not affected by treatment by etodolac in PLC/PRF/5 (Fig. 7C) or HepG2 cells (data not shown) with 10% FBS at 24 to 72 h.

**A selective inhibitor, PD98059, of ERK activation significantly inhibited cell growth of PLC/PRF/5 and induced the expression of $p27^{Kip1}$.** To confirm the role of the MAP kinase pathway in cell growth and cell cycle, ERK activation was inhibited by PD98059, a selective inhibitor of ERK activation, at 2.5–20 μg/ml in PLC/PRF/5 cells. Cell growth, $p21^{WAF1/Cip1}$, $p27^{Kip1}$

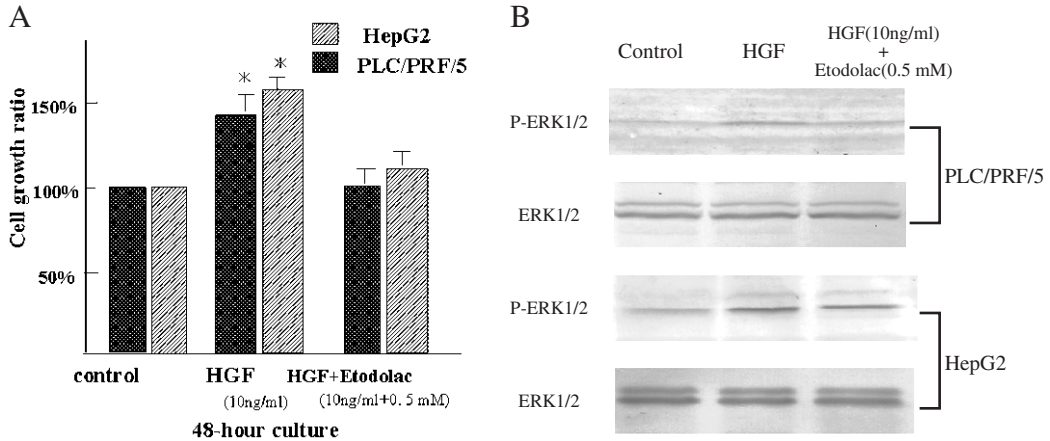


**Fig. 6.** The effect of etodolac on HGF-induced cell growth and phosphorylated ERK. (A) The cells were treated with HGF (10 ng/ml) or HGF (10 ng/ml)+etodolac (0.5 m*M*) in the medium with 10% FBS for 48 h in 96-well plates. Cell growth was monitored by the MTT method. HGF-induced cell growth in both HepG2 and PLC/PRF/5 cells was inhibited effectively by etodolac. * $P<0.05$. (B) Pretreatment of the cells with or without etodolac (0.5 m*M*) for 1 h after the cells had been cultured in serum-free medium for 48 h. HGF (10 ng/ml) was then added for 1 h and the cells were collected to extract total protein for western blotting. The level of HGF-induced phosphorylated ERK was inhibited by etodolac pretreatment in both HepG2 and PLC/PRF/5 cells. Samples containing 50 μg of total protein were used for western blotting. Representative results of western blotting are shown from three independent experiments.

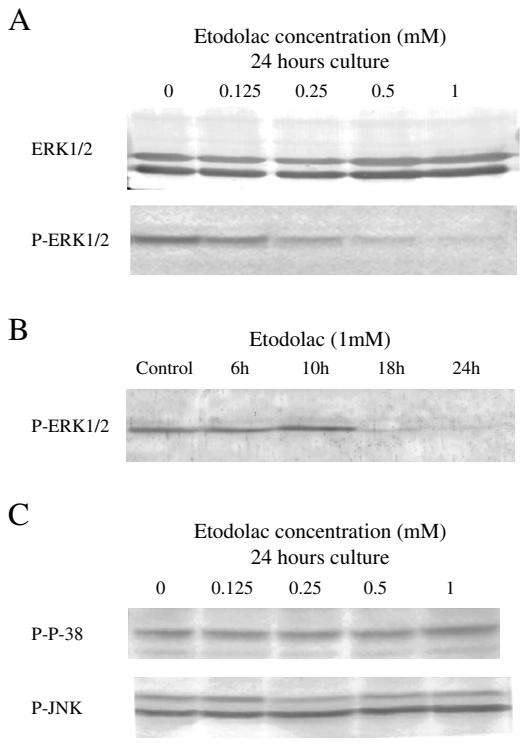


**Fig. 7.** The effect of etodolac on the MAP kinase signaling pathway. PLC/PRF/5 cells were cultured for 24 h after addition of etodolac at 0.125 m*M*, 0.25 m*M*, 0.5 m*M* or 1 m*M* in the medium with 10% FBS, and western blotting was performed. The phosphorylation status of ERK, p38 and JNK was detected by western blotting with anti-phospho-ERK, phospho-p38 and phospho-JNK antibodies, respectively. (A) etodolac inhibited the phosphorylation of ERK-1/2 at concentrations from 0.125 m*M*. A dose-dependent effect was observed. (B) Time-course studies were performed at 6, 10, 18 and 24 h following treatment with 1 m*M* etodolac. The phosphorylation of ERK-1/2 was obviously inhibited from 10 to 18 h. (C) No significant change was observed in phosphorylated p38 or JNK after etodolac treatment. Samples containing 50 μg of total protein were used for western blotting. Representative results of western blotting are shown from three independent experiments.

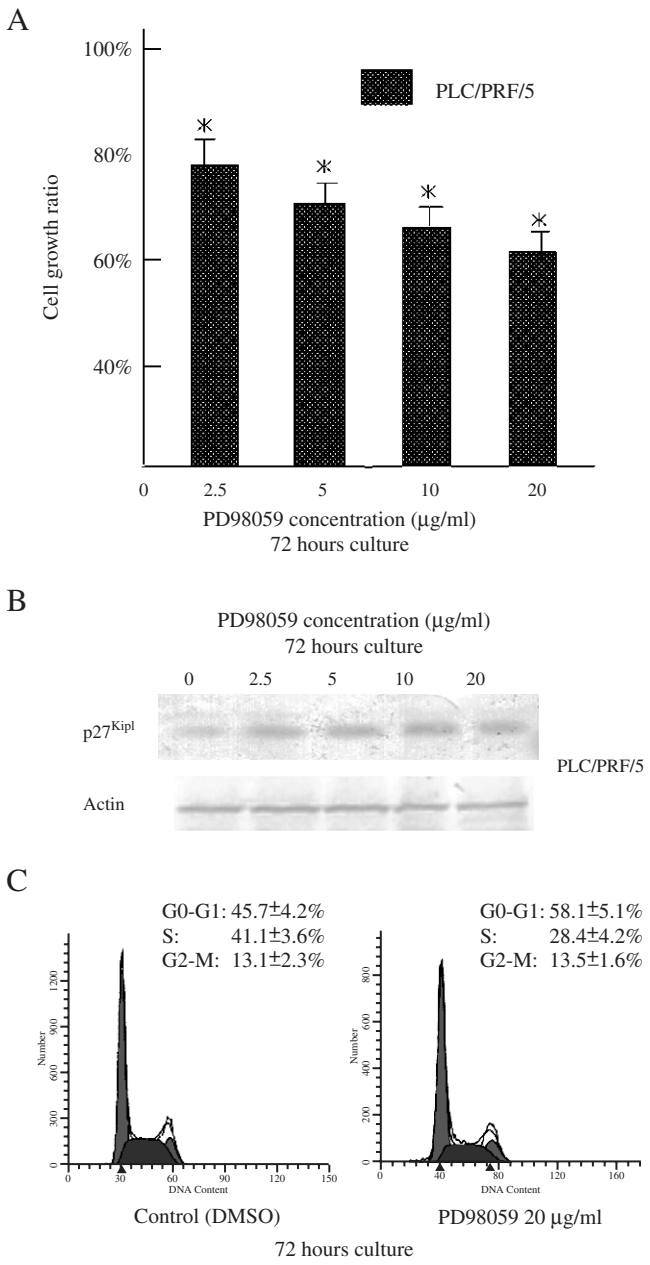


**Fig. 8.** The effect of PD98059 on cell growth, the expression of $p27^{Kip1}$ and the cell cycle in PLC/PRF/5. (A) PLC/PRF/5 cells were treated in medium with 10% FBS for 72 h with PD98059 at 2.5 μg/ml, 5 μg/ml, 10 μg/ml or 20 μg/ml. The MTT method was used to monitor cell growth. Cell growth was inhibited significantly after PD98059 treatment. * $P<0.05$. (B) Western blotting was performed to detect the expression of $p27^{Kip1}$. The expression of $p27^{Kip1}$ was induced in PLC/PRF/5 cells. Samples containing 50 μg of total protein were used for western blotting. (C) G1-S arrest was observed by means of flow cytometric analysis after etodolac treatment ( 20 μg/ml) for 72 h in PLC/PRF/5 cells. DNA histograms are representive of three independent experiments.

expression and the cell cycle were analyzed. Cell growth was inhibited in a dose-dependent manner after treatment for 72 h (Fig. 8A). The expression of $p27^{Kip1}$ was induced (Fig. 8B), while that of $p21^{WAF1/Cip1}$ was not affected, and G1-S arrest was induced after PD98059 treatment for 72 h (Fig. 8C). These results suggest that inhibiting ERK activation may induce $p27^{Kip1}$ expression, cell cycle arrest and growth inhibition in PLC/PRF/5 cells. This finding implies that etodolac may regulate cell cycle arrest by inhibiting the MAP kinase signaling pathway and inducing CDK inhibitor expression in HCC cells.

**The PDK1-PKB/AKT pathway was not inhibited by edotolac at 24 to 72 h.** We also studied the PDK1-PKB/AKT signaling pathway, which is a target for COX-2 inhibitors in human prostate cancer cells, according to a recent report.[19] Two phosphorylated sites, $AKT^{308}$ and $AKT^{473}$ were detected by western blotting. No significant change was observed in either HepG2 or PLC/PRF/5 cells (Fig. 9). Our results do not suggest that the PDK1-PKB/AKT pathway is a target for etodolac in HCC cells.

## Discussion

HCC is one of the leading causes of cancer death in the world. However, the exact molecular mechanisms of hepatocarcinogensis and means for effective prevention and treatment are still unclear. COX-2 is associated with liver pathogenesis, including fibrosis and carcinogenesis, and COX-2 inhibitors have exhibited significant anti-proliferative effects in several human HCC cell lines.[20–23] Here we observed dose-dependent growth inhibition and cell cycle arrest by a selective COX-2 inhibitor, etodolac, on two HCC cell lines. Our findings suggest that a selective COX-2 inhibitor might be effective for the chemopre-

vention and treatment of HCC.

Several mechanisms have been proposed for the regulation by NSAIDs of the molecular pathways of cellular proliferation in colon carcinoma, including modulation of Ras and MAP kinase signal transduction, nuclear factor κB protein activation and cyclin expression.[24–29] However, the key mechanism by which COX-2 inhibitor affects HCC cell growth remains unclear. Our data do not provide evidence that the growth-inhibitory effect is dependent on COX-2 expression or the level of its important product, $PGE_2$. Similar results have been reported for pancreatic and colorectal cancer and HCC cell lines.[4, 9, 30] Some mechanism different from COX-2 expression and PG production might be involved in growth inhibition by etodolac in HCC cells. To understand the mechanism of growth inhibition of HCC by COX-2 inhibition, we studied how etodolac regulates the expression of several key cell cycle regulatory proteins and growth factor signaling pathways. Possible mechanisms are summarized in Fig. 10. Etodolac regulated the cell cycle by inducing the expression of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ and inhibiting the expression of CDK2, CDK4, CDC2, cyclin A and cyclin B1, but had no effect on cyclin E or cyclin D1. Etodolac also inhibited growth by blocking the MAP kinase signaling pathway.

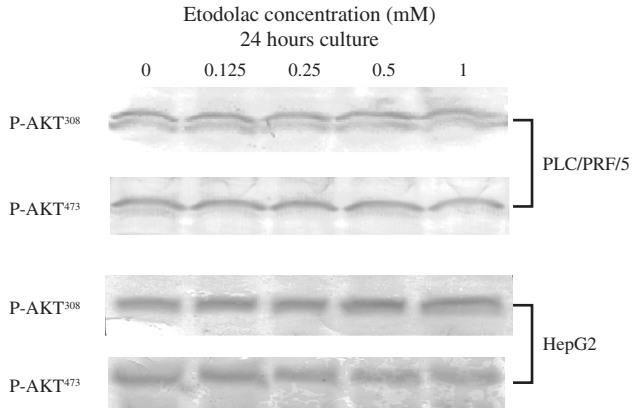


**Fig. 9.** The effect of etodolac on the PDK1-PKB/AKT signaling pathway. HepG2 and PLC/PRF/5 cells were cultured for 24 h after addition of etodolac at 0.125 m*M*, 0.25 m*M*, 0.5 m*M* or 1 m*M* with 10% FBS, and western blotting was performed. The phosphorylation status of representative proteins of the PDK1-PKB/AKT signaling pathway was examined. AKT were detected by western blotting with anti-phospho-$AKT^{308}$ and phospho-$AKT^{473}$ antibodies. No significant change was observed in phosphorylated $AKT^{308}$ and $AKT^{473}$. Samples containing 50 μg of total protein were used for western blotting. Representative results of western blotting are shown from three independent experiments.

As shown in Fig. 10, specific cyclin/CDK complexes are activated at different periods during the cell cycle. Cyclin D1/CDK4 and cyclin D1/CDK6 are activated in mid-G1, whereas cyclin E/CDK2 complexes are required for the G1/S transition, cyclin A/CDK2 for the progression of DNA synthesis, and cyclin A-B/CDC2 for the G2/M transition.[11] $P21^{WAF1/Cip1}$ and $p27^{Kip1}$ inhibited the activity of cyclin D1/CDK4, cyclin E/CDK2, cyclin A/CDK2 or CDC2 and cyclin B1/CDC2 complexes and regulated the G1-S and G2-M checkpoints.[31, 32] In addition, $p21^{WAF1/Cip1}$ blocks PCNA-dependent DNA replication, resulting in G1-S and G2-M arrest.[33] The observed up-regulation of $p21^{WAF1/Cip1}$and $p27^{Kip1}$ expression and down-regulation of CDK2, CDK4, CDC2, cyclin A and cyclin B1 expression are consistent with the findings on cell cycle arrest. A similar effect of induction on the expression of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ by another COX-2 inhibitor, celecoxib, in cholangiocarcinoma has been reported recently.[34]

The ERK pathway is generally thought to be a central signaling cascade in growth stimulation by growth factors.[35, 36] In this study, we showed that etodolac inhibits HGF- and 10% FBS-in-

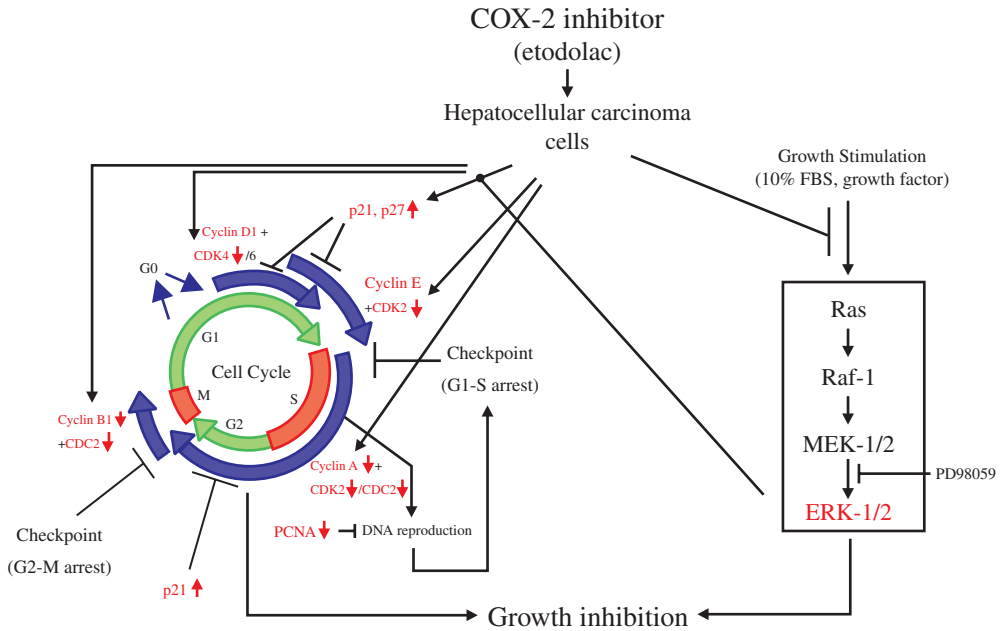


**Fig. 10.** Possible mechanisms of action of COX-2 inhibitor (etodolac) in HCC cell lines. Etodolac affected cell growth by inducing cell cycle arrest and by blocking growth signaling pathways in HCC cells. Expression of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ was induced and expression of CDK2, CDK4, CDC2, cyclin A and cyclin B1 was inhibited by etodolac treatment. $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ protein inhibited the activity of CDKs and participated in G1-S arrest. Down-regulation of CDK4 expression may induce G1-S arrest. CDK2 and $p21^{WAF1/Cip1}$ not only regulate the progression from G1 to S phase, but also play important roles in S and G2-M phases. Down-regulation of CDK2, CDC2, cyclin A, cyclin B1 and elevation of $p21^{WAF1/Cip1}$ protein might play roles in causing G2-M arrest. Expression of cyclin D1 and cyclin E was not affected by etodolac. Reduction of PCNA expression affected DNA replication. Etodolac blocked the growth signaling pathway by down-regulation of ERK activation. Inactivation of ERK also induced $p27^{Kip1}$ expression and participated in cell cycle arrest.

duced ERK activity and proliferation of HCC cells. This finding showed that a COX-2 inhibitor might inhibit HCC cell proliferation by interfering with growth signals mediated by growth factors. An MEK inhibitor, PD98059, suppressed cell proliferation, up-regulated $p27^{Kip1}$ expression and induced cell cycle arrest in HCC cells. This finding not only showed that external stimulation may modulate the cell cycle via the ERK pathway in HCC cells, but also suggested that a COX-2 inhibitor may cause cell growth inhibition and cell cycle arrest by inhibiting the MAP kinase pathway. Expression of both $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ was induced by etodolac, but only $p27^{Kip1}$ was induced by PD98059. Thus, inhibiting the ERK pathway is not the sole mechanism of cell cycle arrest by etodolac. Further study is needed to explore whether some other growth signaling pathway is involved in cell cycle arrest by etodolac in HCC. ERK pathway has been reported to transmit the signals of growth and apoptosis in other experimental models of colorectal carcinoma. Inhibition of phosphorylation of ERK by a COX-2 inhibitor, sulindac sulfide, has an anti-proliferative effect, ERK activation was also shown to result in COX-2 expression and apoptosis by another COX-2 inhibitor, NS-398.[16, 37] Further study is also needed to explore whether ERK transmits the apoptosis signal blocked by the COX-2 inhibitor in HCC cells, as well as colorectal carcinoma.

The majority of responses to etodolac were common in HepG2 and PLC/PRF/5 cells, such as induction of cell cycle regulatory proteins and inhibition of HGF-induced growth and ERK activation. However, several responses differed between the two cell lines. For example, etodolac induced cell cycle arrest in G1-S and G2-M in HepG2 cells, but only in G1-S in PLC/PRF/5 cells. Although $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ were induced in both cell lines, they showed different concentration dependences in response to etodolac. In general, $p27^{Kip1}$ is responsible for regulating the G1-S checkpoint, while $p21^{WAF1/Cip1}$ regulates the G1-S and G2-M checkpoints.[31, 33, 38, 39] The molecular mechanisms involved and the exact role of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ in HCC cells need to be clarified. Our data showed that etodolac inhibited HGF-induced ERK-phosphorylation, but not 10% FBS-induced ERK phosphorylation in HepG2 cells. Other growth factors besides HGF might exist in 10% FBS and interfere with the response of HepG2 to etodolac. On the other hand, a recent report showed that NS-398 inhibited HGF-induced phosphorylation of ELK1 in HepG2 cells.[40] Future studies should address whether differences between HGF- and 10% FBS-induced ERK phosphorylation are present, and whether or not other signal proteins of the MAP kinase signaling pathway are involved in the growth inhibition.

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