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• *LIVER CANCER* •

Overexpression of cyclooxygenase-2 in human HepG2, Bel-7402 and SMMC-7721 hepatoma cell lines and mechanism of cyclooxygenase-2 selective inhibitor celecoxib-induced cell growth inhibition and apoptosis

Ning-Bo Liu, Tao Peng, Chao Pan, Yu-Yu Yao, Bo Shen, Jing Leng

Ning-Bo Liu, Tao Peng, Bo Shen, Jing Leng, Laboratory of Reproductive Medicine, Department of Pathology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Chao Pan, Yu-Yu Yao, Atherosclerosis Research Center, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
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Correspondence to: Dr. Jing Leng, Laboratory of Reproductive Medicine, Department of Pathology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. lengjing@njmu.edu.cn
Telephone: +86-25-86862685 Fax: +86-25-86862685
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Abstract

AIM: To investigate the cyclooxygenase-2 (COX-2) expression level in human HepG2, Bel-7402 and SMMC-7721 hepatoma cell lines and the molecular mechanism of COX-2 selective inhibitor celecoxib-induced cell growth inhibition and cell apoptosis.

METHODS: Hepatoma cells were cultured and treated with celecoxib. Cell *in situ* hybridization (ISH) and immunocytochemistry were used to detect COX-2 mRNA and protein expression. Proliferating cell nuclear antigen and phosphorylated Akt were also detected by immunocytochemistry assay. Cell growth rates were assessed by 3-(4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium (MTT) bromide colorimetric assay. Celecoxib-induced cell apoptosis was measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and flow cytometry (FCM). The phosphorylated Akt and activated fragments of caspase-9, caspase-3 were examined by Western blotting analysis.

RESULTS: Increased COX-2 mRNA and protein expression were detected in all three hepatoma cell lines. Celecoxib could significantly inhibit cell growth and the inhibitory effect was in a dose- and time-dependent manner evidenced by MTT assays and morphological changes. The apoptotic index measured by TUNEL increased correspondingly with the increased concentration of celecoxib and the reaction time. With 50 μmol/L celecoxib treatment for 24 h, the apoptotic index of HepG2, BEL-7402 and SMMC-7721 cells was 25.01±3.08%, 26.40±3.05%, and 30.60±2.89%, respectively. Western blotting analysis showed remarkable activation of caspase-9, caspase-3 and dephosphorylation of Akt (Thr^{308}). Immunocytochemistry also showed the reduction of PCNA expression and phosphorylation Akt (Thr^{308}) after treatment with celecoxib.

CONCLUSION: COX-2 mRNA and protein overexpression in HepG2, Bel-7402 and SMMC-7721 cell lines correlate with the increased cell growth rate. Celecoxib can inhibit proliferation and induce apoptosis of hepatoma cell strains in a dose- and time-dependent manner.

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Key words: Apoptosis; Akt; Celecoxib; Caspase; Cell proliferation; COX-2; HCC; PCNA

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INTRODUCTION

Cyclooxygenase (COX), known as a PGs synthase, catalyzes the metabolism of arachidonic acid to PGs and thromboxanes, and has three isoforms: COX-1, COX-2 and COX-3[1]. COX-1 is a constitutively expressed enzyme found in most tissues and remains unaltered in cancer. COX-2 is expressed at sites of inflammation, which has led to the speculation that its inhibition could provide all the benefits of current non-steroidal anti-inflammatory drugs (NSAIDs) but without their major side effects on the gastrointestinal system[2,3] (which are due to the inhibition of COX-1). New NSAIDs, which are selective inhibitors of COX-2, can exert therapeutic efficacy without toxic effects due to the inhibition of COX-1. Celecoxib, a selective COX-2 inhibitor approved by US FDA, has been shown preclinically and clinically to have efficacy comparable to that of NSAIDs on relief of pain and inflammation in osteoarthritis.

Recently, COX-2 has been suggested to be associated with carcinogenesis and high expression of COX-2 has been

found in many cancers[4-6] such as colorectal cancer, prostatic cancer, breast cancer and gastric cancer. Although selective COX-2 inhibitors could reduce the growth of various cancer cell lines[7-9] and display anti-tumor effect by sensitizing cancer cells to apoptosis[10-12], it is unknown whether COX-2 contributes to the malignant growth and whether inhibition of COX-2 function modifies the malignant potential of tumors.

In hepatocarcinoma (HCC), the expression pattern of COX-2 protein is well correlated with the differentiation grade, suggesting that abnormal COX-2 expression plays an important role in hepatocarcinogenesis[13]. However, the exact anti-tumor mechanisms are not well known. Leng *et al*.[8] have proved that COX-2 could promote HCC cell growth and selective COX-2 inhibitor celecoxib could mediate cell apoptosis in HepG2 and Hep3B cell lines. In this article, we investigate the expression pattern of COX-2 on HepG2 and two other human HCC cell lines, BEL-7402 and SMMC-7721 and evaluate the efficacy of selective COX-2 inhibitor celecoxib in order to understand the potential role of COX-2 in promoting HCC cell growth and to provide theoretical evidence for searching a new agent for the treatment of HCC.

MATERIALS AND METHODS

Materials

Human HepG2, BEL-7402 and SMMC-7721 hepatoma cell lines were obtained from The Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). RMPI1640, DMEM and MEM culture medium were purchased from Gibco BRL. Fetal bovine serum (FBS) was provided by Sijiqing Biological Engineering Material (Hangzhou, China). Rabbit anti-COX-2 was from Cayman. Mouse anti-caspase3 was from Boster Biological Technology (Wuhan, China). Rabbit anti-caspase9, anti-actin, rabbit anti-phospho-Akt/PKB (Thr^{308}), sheep anti-rabbit, sheep anti-mouse secondary antibody, Western blotting luminol reagent were purchased from Santa Cruz. *In situ* cell death detection kit (TUNEL) and sensitive *in situ* hybridization detection kit were purchased from Roche. Rainbow molecular weight markers were from Amersham. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was provided by Amersco. Celecoxib was provided by Scenry Chemical (Hefei, China).

Cell lines and culture conditions

SMMC-7721 cell line was cultured in DMEM supplemented with 100 mL/L heat-inactivated FBS and 100 U/mL penicillin/streptomycin. Bel-7402 cell line was cultured in RPMI 1640 supplemented with 100 mL/L heat-inactivated FBS and 100 U/mL penicillin/streptomycin. HepG2 cell line was cultured in MEM supplemented with 100 mL/L heat-inactivated FBS, 100 U/mL penicillin/streptomycin and 1% nonessential amino acid. All cells were grown in 50 mL/ L CO_2 humidified atmosphere at 37 ℃.

Morphological changes

Celecoxib-treated cells and control were digested by trypsin, washed with phosphate-buffered saline (PBS) and centrifuged at 1 500 r/min for 5 min. After the cells were mixed with glycerine and egg white, the mixtures were fixed in alcohol for 30 min. Then the mass was embedded and fabricated to paraffin section with H&E staining. Morphological changes were observed under light and inverted microscopes.

In situ hybridization (ISH) assays

The digoxigenin-labeled COX-2 oligonucleotide probe for ISH was prepared with the sequence 5'-TGATATCA-TCTAGTCCGGAGCGGGAAG-3'(TaKaRa Bio). ISH was performed according to the instructions of the enhanced sensitive ISH detection kit. Briefly, the sections were deparaffinized with xylene, dehydrated with graded ethanol and embryos were incubated in 1 mL of 20 μg/mL proteinase K for 20 min. Re-fix embryos were incubated for 10 min in 4% formaldehyde. Then all were removed and 20 μL pre-hybridization solution was added for 2 h. Solution was removed and replaced with 20 μL hybridization solution to hybridize overnight at 37 ℃. After these, embryos were washed twice in 2× SSC and 0.01× at 37 ℃ SSC for 5 min each, replaced with anti-digoxygenin-alkaline phosphatase antibody and 40 μL AP-coupled antibodies at 37 ℃ for 1 h. The last wash was replaced with AP reaction buffer and the reaction was terminated when it became dark.

Westen blotting

Celecoxib-treated three cell lines and control were lysed by 4 g/L trypsin containing 0.2 g/L EDTA, then collected after being washed twice with cold PBS. Total protein extract from cells was prepared using cell lysis buffer [50 mmol/L Tris-HCL (pH 8.0), 150 mmol/L NaCl, 1%Triton X-100, 100 μg/mL PMSF, 1 μg/mL aprotinin]. The extract (40 ug) was electrotransferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in TBST (10 mmol/L Tris-HCL, pH 8.0, 100 mmol/L NaCl, and 0.05% Tween-20) for 1 h at room temperature and then incubated with appropriate primary antibody overnight at 4 ℃, followed by incubation with horseradish peroxidase-conjugated secondary antibody at 1∶2 000 dilution for 1 h at room temperature. The polyclonal anti-COX-2 and anti-actin were used at 1∶1 000 dilution. The polyclonal anti-caspase-3, anti-caspase-9, anti-phospho-Akt/PKB were used at 1∶400 dilution. The immunoblots were visualized by enhanced chemiluminescence.

Immunocytochemical assays

Briefly, the sections were subjected to immunostaining using an ultrasensitive streptavidin–peroxidase technique (DAKO). Endogenous peroxidases were blocked by incubating with 5 mL/L H_2O_2 for 30 min at room temperature. The sections were subsequently treated for 10 min with Triton X-100, and incubated for 30 min at 37 ℃ with normal non-immune serum before overnight incubation at 4 ℃ with specific antibody at a dilution of 1∶200. The sections were then treated with biotin-conjugated second antibody before streptavidin-peroxidase was added. For color reaction, diaminobenzidine (DAB) was used. If the positive signals were present, the cytoplasm or the nuclei were stained brown. For negative control, the antibody was replaced by PBS.

Cell growth assays (MTT assays)

The number of viable cells was determined by MTT assays. Each cell sample was plated at a density of 10 000 cells/well in 96-well cluster dishes before treatment. On the same day, the medium was changed and celecoxib was added in concentrations and analyzed at indicated time points. After addition of MTT solution (10/100 μL medium) in each well, the cells were incubated at 37 ℃ for 4 h, and then 100 μL DMSO was added to dissolve the dark blue crystals. The absorbance was measured in an ELISA plate reader with a test wavelength of 570 nm.

Cell cycle analysis by FCM

Celecoxib-treated cells were fixed with 700 mL/L alcohol for 15 min at 4 ℃, and then stained with propidium iodide (PI, Sigma, USA). The red fluorescence of DNA-bound PI in individual cells was measured at 488 nm with a FACSCalibur (Becton Dickinson, USA) and the results were analyzed using CELL QuestV1.22 software.

Apoptosis detection (TUNEL assay)

The apoptotic cells were identified by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer's instructions. The cells were counterstained with royal blue. In each experiment, 400 cells were examined using four high-power fields each (magnification×400). The experiments were performed at least twice independently.

Image analysis

The results of TUNEL and immunocytochemistry were analyzed in a blinded fashion by image analysis system (RXA_2, Leica) using *Qwin* software. The apoptosis index (AI) was determined by observing more than 400 nuclei for each sample.

Statistical analysis

Statistical significance was assessed by the nonparametric ANOVA test with SPSS 10.0 software and $P<0.05$ was considered statistically significant.

RESULTS

COX-2 expression in hepatoma cell lines

ISH, Western blotting and immunocytochemical staining were performed on HepG2, Bel-7402 and SMMC-7721 cell lines. Significant mRNA and protein of COX-2 were detected in all test cell lines. A representative Western blotting showed that the COX-2 expression level of the HCC cell lines in sequence was SMC-7721>BEL-7402>HepG2 (Figure 1). COX-2 protein was located in the cytoplasm of the cells shown by immunocytochemical staining. No staining was observed in the negative control slide.

Growth inhibitory effect of celecoxib

The effects of celecoxib on the cell viability were determined by MTT assay. Cells became sparse, round and detached from the dishes when treated with 6.25, 12.50, 25.00, 50.00, and 100.00 μmol/L concentrations of celecoxib for 24 h (Figure 2), and resulted in 11.9%, 23.9%, 43.8%, 62.4% and 72.7% cell growth reduction, respectively, in HepG2 cells (Figure 3).

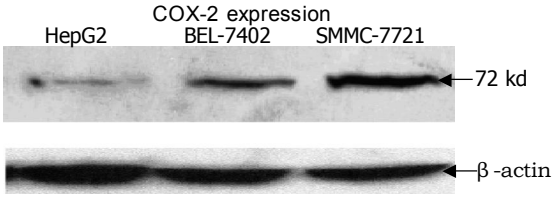

Figure 1 Expression of COX-2 in HepG2, BEL-7402 and SMMC-7721 HCC cell lines shown by Western blotting.

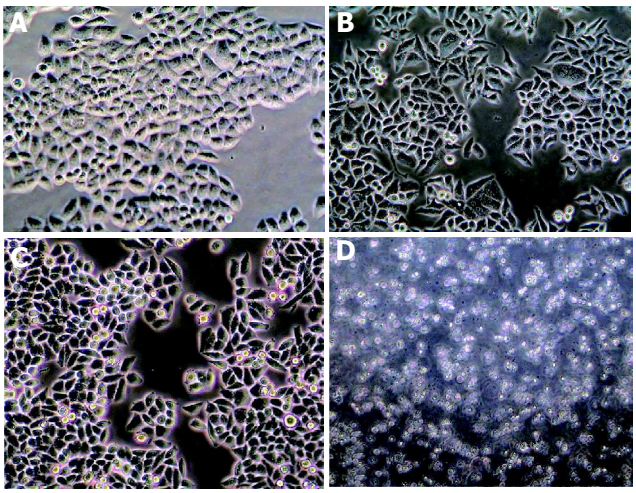

Figure 2 Growth inhibitory effect of celecoxib on SMMC-7721 cell lines. **A**: Normal control; **B**: 25.00 μmol/L celecoxib for 6 h; **C**: 25.00 μmol/L celecoxib for 24 h; **D**: 50.00 μmol/L celecoxib for 48 h. (Original magnification, ×200).

Down-regulation of PCNA expression in celecoxib-treated hepatoma cells

The effects of celecoxib on PCNA expression in cultured HCC cells were assessed by immunocytochemical staining. PCNA-positive cells were more abundant in control cells than in celecoxib-treated cells (Figure 4).

Human HCC cell apoptosis induced by celecoxib

Morphologic changes were evident in the three cell lines after treatment with celecoxib for 24 h. Progressive cytoplasmic shrinkage and nuclear condensation (the typical morphologic signs of apoptosis under light microscope) could be observed. To further make sure of these apoptotic morphologic changes, TUNEL staining was performed. As shown in Figure 5, celecoxib induced HepG2 cell apoptosis in a dose-dependent manner, the percentage of TUNEL-positive cells increased from 2.80±0.84% to 25.01±3.08% after treatment with 6.25 or 50 μmol/L celecoxib for 24 h, respectively. Celecoxib-treated BEL-7402 and SMMC-7721 cell lines showed the same results.

Results of FCM also showed apoptosis induced by celecoxib in SMMC-7721 cell lines. A subdiploid (subG1) peak was observed after being treated with 6.25, 12.

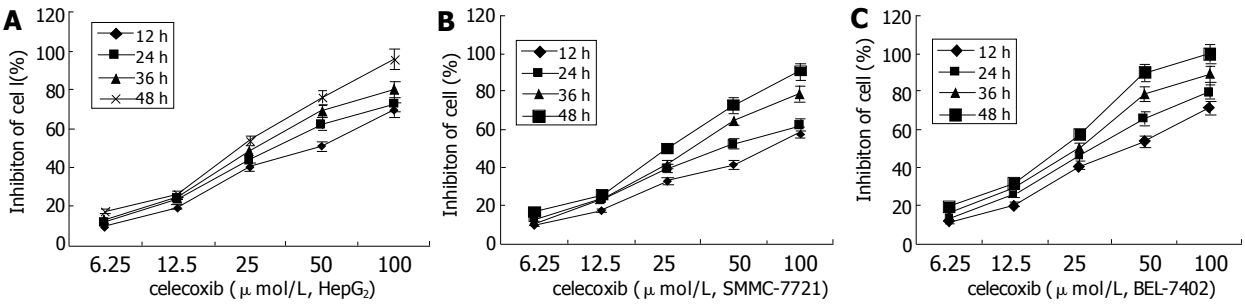

Figure 3 Growth inhibitory effects of celecoxib on HepG2 (**A**), SMMC-7721 (**B**) and BEL-7402 (**C**) HCC cells.

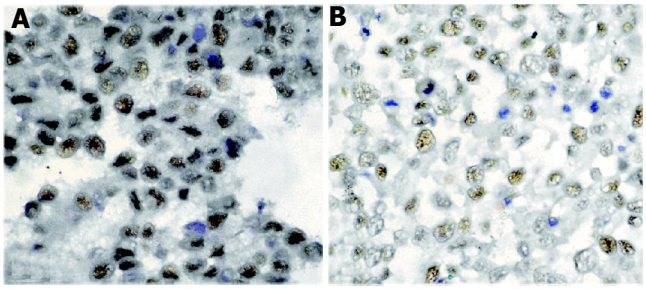

Figure 4 Immunocytochemical staining of PCNA in HepG2 cells in the presence or absence of celecoxib. **A**: normal control; **B**: 25.00 μmol/L celecoxib for 24 h.

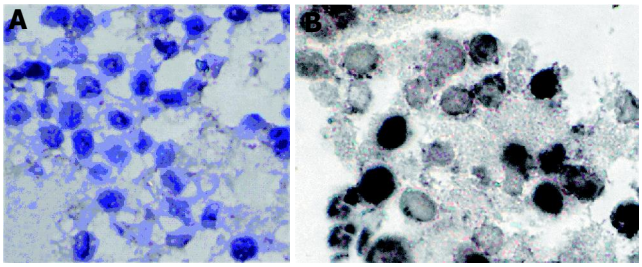

Figure 5 Cell apoptosis induced by celecoxib. **A**: Normal control; **B**: Apoptosis of BEL-7402 cell assessed by TUNEL.

50, 25.00 and 50.00 μmol/L celecoxib for 24 h, and the apoptosis rate was 4.84%, 16.10%, 22.80% and 33.80%, respectively, compared to 0.81% in normal control (Table 1).

Caspase-9 and caspase-3 activated by celecoxib

Western blotting analysis revealed that celecoxib induced activation of caspase-9 and caspase-3 (Figure 9). Activation of caspase-3 was reflected by a decrease in the 32-kd precursor and a concomitant increase in the 20-kd cleaved band. Activation of caspase-9 was reflected by conversion of the 47-kd precursor to the active 35- and 37-ku proteolytic cleavage products. The activation level of caspase-9 and caspase-3 was in a dose- and time-dependent manner to the celecoxib treatment (Figure 6).

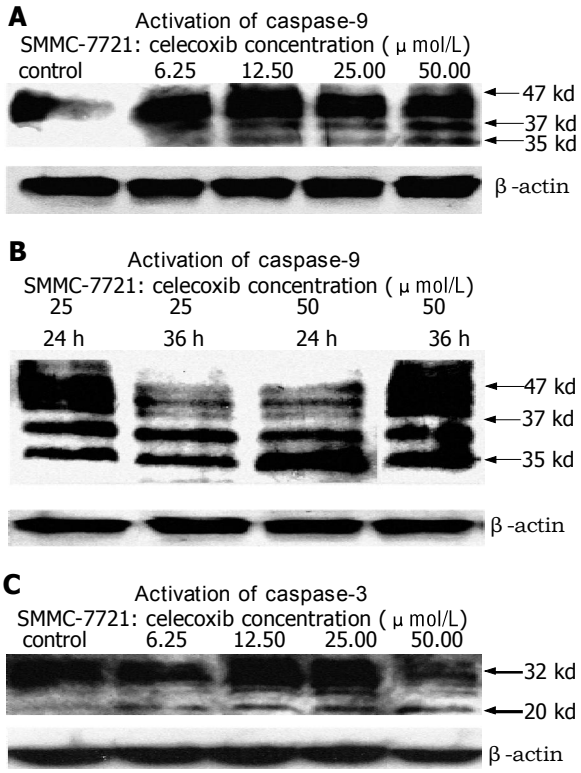

Figure 6 Activation of caspase-9 and caspase-3 after treatment with celecoxib. **A:** Activation of caspase-9 after being treated with celecoxib for 12 h; **B:** Activation of caspase-9 after being treated with different concentrations of celecoxib for different time; **C:** Activation of caspase-3 after being treated with celecoxib for 12 h.

Phosphorylation of Akt reduced by celecoxib

Table 1 AI induced by celecoxib (mean±SD)

Cell line	Control	6.25	12.5	25	50
BEL- 7402	0.60±0.89	3.40±1.14	7.60±2.41	15.20±3.49	26.40±3.05
SMMC-7721	1.20±0.84	4.20±1.48	9.80±2.59	19.60±3.36	30.60±2.89
HepG2	1.00±1.00	2.80±0.84[a]	5.40±1.52	15.00±1.58	25.01±3.08

[a]$P<0.05$ *vs* control

To evaluate the effect of celecoxib on Akt/PKB activity, HepG2, BEL-7402 and SMMC-7721 cells were treated with celecoxib for 4, 12 or 24 h, and phosphorylation at Thr^{308} was examined by specific phospho-Akt antibody. Western blotting analysis showed that celecoxib significantly reduced Akt/PKB phosphorylation (Figure 7). Immunocy-tochemistry assay also displayed dephosphorylation of Akt Thr^{308} (Figure 8) in a dose- and time-dependent manner when analyzed with Leica-*Qwin* computer image analysis system. The results are shown in Figure 9.

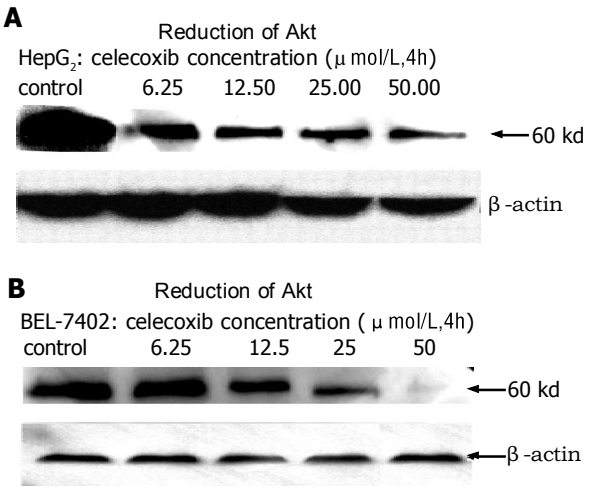

Figure 7 Dephosphorylation of Akt/PKB after treatment with celecoxib for 4 h. **A**: HepG2 cell line; **B**: BEL-7402 cell line.

DISCUSSION

COX-2, an isoform of cyclooxygenase, is inducible by a variety of factors including cytokines, growth factors, and tumor promoters[2,3]. It was reported that COX-2 expression increases in many human cancers including HCC[13]. The mechanism leading to COX-2 overexpression in HCC is still unknown. It was demonstrated that overexpression of COX-2 increases cell survival by inhibiting cell apoptosis and stimulating angiogenesis, and enhancing cellular adhesion to the matrix proteins[14,15]. In this article, we have observed a high expression of COX-2 mRNA and protein in human HepG2, Bel-7402 and SMMC-7721 hepatoma cell lines. These results suggest that studies are warranted to determine the role of COX-2 in hepatocarcinogenesis. Furthermore, we have shown that celecoxib, a selective COX-2 inhibitor, could strongly suppress cell proliferation.

Morphologic changes and results obtained by MTT assay support the suggestion that celecoxib down-regulates cell growth activity. It was reported that COX-2 inhibitors may have antineoplastic effects during the early stages of hepatocarcinogenesis[16].

PCNA is an essential protein found in proliferating eukaryotic cells, and plays a crucial role in DNA replication, repair, and control of cell proliferation[17,18]. This protein is involved in the synthesis of both leading and lagging DNA strands, providing an anchorage site and increasing the processivity of DNA polδ and DNA polε, which is the basis of PCNA serving as a DNA synthesis marker for evaluating cell proliferation[19,20]. Therefore, overexpression of COX-2 may be mitogenic in HCC cells through up-regulation of PCNA expression. Selective COX-2 inhibitor celecoxib can block the activity of COX-2 and down-regulate PCNA expression, thus causing cell growth inhibition.

Apoptosis plays a central role in tumor development and lack or failure of apoptosis leads to the development of many tumors including hepatocarcinoma[21,22]. Thus, induction of apoptosis of tumor cells is an effective approach to delay the tumor progress. In this study, we found that celecoxib could induce the characteristic features of apoptosis including morphological changes, TUNEL-positivity, FCM and caspase-9/3 activation of the three tested HCC cell lines.

There are at least two extrinsic and intrinsic broad pathways that lead to apoptosis[23-25]. The "extrinsic" pathway begins with the binding of Fas ligand (FasL or CD95L) to the Fas receptor (CD95) and results in recruiting FADD and pro-caspase-8 to the complex. Concentration of pro-caspase-8 results in its autocatalysis and activation. Activated caspase-8 cleaves pro-caspase-3, which then undergoes autocatalysis to form active caspase-3, a principle effector caspase of apoptosis. The "intrinsic" apoptosis pathway always begins with mitochondrial damage and results in release of cytochrome C from damaged mitochondria. In cytosol or on the surface of mitochondria, cytochrome C is bound to the protein Apaf-1 (apoptotic protease activating factor) which then activates an initiator caspase, caspase-9, then activates caspase-3[26,27]. Caspase families play an important role in apoptosis signaling pathway. They are present in the cytoplasm in normal condition as inactive pro-enzymes, most of which are activated by proteolytic cleavage when undergoing apoptosis[28,29]. Both caspase-8 and caspase-9 can activate the effector caspase, caspase-3, by proteolytic cleavage and subsequent processes result in the nuclear DNA fragmentation and formation of apoptotic bodies, indicating that activation of caspase-3 is a central event in the process of apoptosis.

In this article, Western blotting analysis revealed that celecoxib induced the activation of caspase-9 and caspase-3. Activation of caspase-9 is reflected by conversion of the 47-kd inactive precursor to the active 35-kd and 37-kd proteolytic cleavage products. Activation of caspase-3 is indicated by a decrease of the 32-kd precursor accompanied with a concomitant increase in the 20-kd cleaved band. Leng *et al*.[8], reported that celecoxib-treated HepG2 cells significantly release cytochrome C in the cytosol. Thus we propose the hypothesis that celecoxib allows the release of cytochrome C from mitochondria and activates caspase-9 and caspase-3 in sequence and finally induces apoptosis of HCC cells.

Our works have also shown that celecoxib down-regulates the phosphorylation of Akt/PKB at Thr^{308} in a dose- and time-dependent manner. Akt/PKB[30,31], the major downstream effector of PI3-kinase, is a Ser/Thr

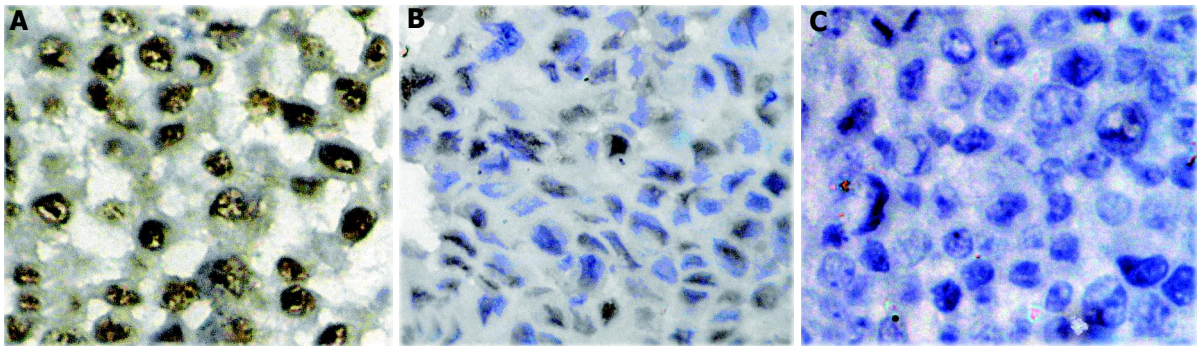

Figure 8 Down-regulation of phosphorylation of Akt Thr308 after treated with celecoxib. **A:** Normal control; **B:** SMMC-7721 cell line; (**C**): Negative control.

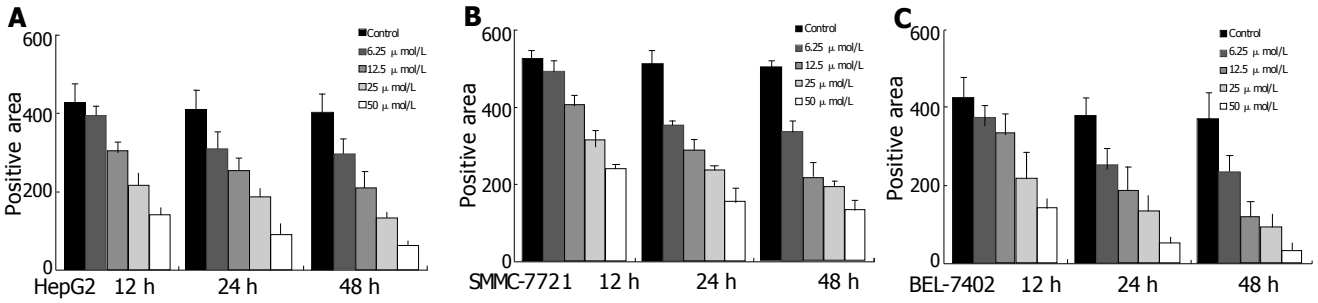

Figure 9 Dose- and time-dependent effects of reduced phosphorylation of Akt (Thr^{308}) on HepG2 cell (**A**), SMMC-7721 cell (**B**), BEL-7402 cell (**C**).

protein kinase that exerts a crucial role in the regulation of several cellular signaling pathways. Akt/PKB is a regulator of cell survival and apoptosis, and its activation can protect a variety of cells against apoptosis. Akt/PKB is phosphorylated at two regulatory sites, Thr^{308} and Ser^{473}, essential for its activation. Activated Akt/PKB can phosphorylate BAD, IκB kinase, glycogen synthase kinase-3β, and forkhead transcription factors[32,33], leading to their inactivation and cell survival. Interestingly, it was reported that caspase-9 activity is regulated by phosphorylation[34]. Akt phosphorylates pro-caspase-9 at Ser^{196} and inhibits proteolytic processing of pro-caspase-9.

Celecoxib blocks the activation of Akt, thereby attenuating the activity of a major anti-apoptotic pathway and inducing cell apoptosis. It remains unclear how celecoxib affects Akt phosphorylation because it does not affect PI3-kinase activity directly[35]. Other potential consequences of COX-2 inhibition, such as modulation of the RAS-signaling pathway, p53 expression, and other members of the BCL-2 family, such as MCL-1[36,37], activation of the sphingomyelin-ceramide pathway, and interference with the NF-κB[38] need to be investigated in future.

In conclusion, COX-2 is overexpressed in human HepG2, BEL-7402 and SMMC-7721 hepatoma cells and may play a key role in tumor cell proliferation and carcinogenesis. Selective COX-2 inhibitor celecoxib can suppress the growth of HCC cells by inhibiting cell growth and inducing cell apoptosis. The mechanism of apoptosis induced by celecoxib is due to activation of the caspase cascade, which is correlated with phosphorylation of Akt/PKB. These results suggest that selective COX-2 inhibitor offers potential as an effective chemotherapeutic and chemopreventive strategy against human liver cancer.

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