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Original Article

Celecoxib induces apoptosis and cell-cycle arrest in nasopharyngeal carcinoma cell lines via inhibition of STAT3 phosphorylation

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Aim: To investigate the mechanisms underlying the anticancer effect of celecoxib on nasopharyngeal carcinoma (NPC).
Methods: NPC cell lines, HNE1 and CNE1-LMP1, were treated with various concentrations of celecoxib for 48 h. The antiproliferative effect of celecoxib was assessed using MTT assay. Both cell cycle profiles and apoptosis were analyzed using flow cytometry. Western blot was used to measure the levels of signal transducer and activator of transcription 3 (STAT3), phosphorylated STAT3^{Y705} (pSTAT3^{Y705}), COX-2, Survivin, Mcl-1, Bcl-2 and Cyclin D1.
Results: Celecoxib (10–75 μmol/L) inhibited the proliferation of the NPC cell lines in a dose-dependent manner. Celecoxib (25 and 50 μmol/L) induced apoptosis and cell-cycle arrest at the G_0/G_1 checkpoint in the NPC cell lines, which was associated with significantly reduced STAT3 phosphorylation. The genes downstream of STAT3 (*ie*, Survivin, Mcl-1, Bcl-2 and Cyclin D1) were significantly down-regulated after exposure to celecoxib (25 and 50 μmol/L).
Conclusion: The anticancer effects of celecoxib on NPC cell lines results from inducing apoptosis and cell cycle arrest, which may be partly mediated through the STAT3 pathway.

Keywords: celecoxib; nasopharyngeal carcinoma; apoptosis; cell cycle; STAT3

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Introduction

Nasopharyngeal carcinoma (NPC) is the most common head and neck cancer in Southeast Asia and South China. The annual incidence rate of NPC in endemic areas is approximately 20 to 50 per 100 000 individuals[1]. NPC is characterized by its highly invasive and metastatic tendencies. Approximately 70% of NPC patients are initially diagnosed with locally advanced disease. Although aggressive concurrent chemo-radiotherapy is the standard treatment modality for NPC at the locally advanced stage, approximately 30% to 40% of NPC patients fail with local recurrence and/or distant metastasis[2]. The reported median survival time for an NPC patient with metastatic or advanced disease is only 5 to 11 months[3]. Due to the poor survival rate, more effective strategies and drugs to prevent and treat NPC are needed. The selective cyclooxygenase-2 (COX-2) inhibitor celecoxib shows great promise in this respect.

Celecoxib was the first COX-2-selective nonsteroidal anti-inflammatory drug (NSAID) approved for the clinical treatment of adult arthritis. Celecoxib exerts potent anticancer activities against various human cancers, including brain, lung, colon and liver cancers[4–7]. One mechanism through which celecoxib acts to reduce cancer development is by the inhibition of COX-2 activity. Furthermore, celecoxib can also act through COX-2-independent mechanisms involving the arrest of cell cycle progression, angiogenesis and the induction of apoptosis[8]. COX-2 is overexpressed in 43% to 75% of NPCs, contributes to nodal metastases and is associated with poorer prognosis[9, 10]. Although some studies have demonstrated certain antitumor effects of celecoxib on NPC *in vitro*, the molecular mechanisms underlying its inhibitory effects are still unclear[11–13].

Signal transducer and activator of transcription 3 (STAT3) is constitutively activated or overexpressed in a variety of human cancers. This pivotal transcription factor plays a significant role in regulating cell growth, apoptosis, angiogenesis, immune escape, invasion and metastasis. Accumulating evidence has demonstrated that STAT3 is a promising molecule target for anticancer treatment and cancer prevention[14, 15]. More recently, STAT3 has also been found to be activated in

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more than 75% of NPC cases, and this molecule plays a significant role in driving NPC progression and metastasis[16–18]. A recent study identified celecoxib as a novel inhibitor of STAT3[6]. Celecoxib was demonstrated and verified to have specificity to STAT3, and this drug has been shown to exert anticancer effects in colon cancer cells, hepatocellular carcinoma cells and rhabdomyosarcoma cells through the inhibition of STAT3 phosphorylation[6, 7, 19].

In contrast to other head and neck cancers, NPC possesses the unique feature of being strongly associated with the Epstein-Barr virus (EBV). In EBV-endemic regions, most NPC patients are EBV-infected[20]. Latent membrane protein 1 (LMP1), LMP2A, Epstein-Barr nuclear antigen 1 (EBNA1), *Bam*H I-A rightward transcripts (BARTs) and Epstein-Barr virus-encoded RNAs (EBERs) are expressed in EBV. LMP1 is the principal oncoprotein expressed by EBV, and LMP1 protein expression is detected in more than 70% of NPC patients. LMP1 plays an important role in the tumorigenesis and development of NPC and contributes to the promotion of invasion and metastasis[21]. Moreover, a recent study demonstrated that LMP1 could induce STAT3 phosphorylation through the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and Janus kinase (JAK)/STAT pathways[22].

Given the importance of STAT3 in NPC progression and the potent anticancer effect of celecoxib, we chose to investigate the potential molecular mechanisms of this drug with an emphasis on determining whether celecoxib inhibits STAT3 phosphorylation in NPC cells. We also explored whether the anticancer effects of celecoxib may be due to the induction of apoptosis and cell cycle arrest.

Materials and methods

Chemicals and antibodies

Lyophilized celecoxib was purchased from Sigma Chemical Co, MO, USA. Stock celecoxib solutions were prepared by dissolving the powder in DMSO and then storing at -20 °C. The drugs were freshly diluted into culture medium before the commencement of each experiment, and the final DMSO concentration in the culture medium never exceeded 0.1%. Primary antibodies including rabbit anti-human phsopho-STAT3-Tyr705, Survivin, Mcl-1 and Cyclin D1 monoclonal antibodies were purchased from Epitomics Inc, Burlingame, CA, USA. Rabbit anti-human Bcl-2 and STAT3 monoclonal antibodies were obtained from Cell Signaling Technology, Danvers, USA. Goat anti-human COX-2 and rabbit anti-human β-actin polyclonal antibodies were obtained from Santa Cruz Biotechnology, USA. The horseradish peroxidase (HRP)-conjugated goat anti-rabbit and HRP-conjugated rabbit anti-goat secondary antibodies were obtained from Santa Cruz Biotechnology. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma.

Cell culture

HNE1 was derived from a poorly differentiated NPC that has lost the EB virus due to long-term *in vitro* passaging (over 100 passages)[23]. CNE1 is a poorly differentiated NPC cell line that is also EBV negative. CNE1-LMP1 is a stably transfected cell line established by introducing LMP1 cDNA into CNE1 cells[24]. The HNE1 and CNE1-LMP1 cell lines were provided by the Cancer Research Institute of Central South University (Changsha, China). All lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Grand Island, USA) supplemented with 10% newborn calf serum (Gibco, Grand Island, USA) and incubated in a 5% CO_2 atmosphere.

Growth inhibition assay

The antiproliferative effect of celecoxib on NPC cells was assessed using an MTT assay. Cells were seeded into 96-well plates and allowed to attach for 24 h. The cells were then treated with increasing concentrations of celecoxib (0, 5, 10, 25, 50 or 75 μmol/L) dissolved in DMSO (final concentration ≤0.1%) and incubated for up to 48 h. After the incubation, 20 μL of MTT dye (5 mg/mL) were added to each well and cells were incubated at 37 °C for 4 h. After removing the supernatants, the crystals were dissolved in DMSO and the absorbance was measured at 490 nm. The percentage growth inhibition was calculated as $(OD_{control} - OD_{drug})/OD_{control} \times 100\%$. The half-maximal inhibitory concentration (IC_{50}) values and the 95% confidence intervals were calculated using probit regression using SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). The experiment was performed in triplicate and repeated at least three times.

Apoptosis assay

Cells were treated with increasing doses of celecoxib (0, 10, 25, 50 or 75 μmol/L) for 48 h and then harvested and washed with phosphate buffered saline (PBS). After washing, the cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 min at 4 °C in the dark, in accordance with the manufacturer's instructions (Nanjing KeyGen Biotech Co, Nanjing, China). After incubation, the cells were immediately analyzed using flow cytometry (EPICS XL, Beckman Coulter Inc, Fullerton, CA, USA). Early apoptotic cells stained positive for Annexin V-FITC and negative for PI. Late apoptotic cells were positive for both Annexin V-FITC and PI. The experiment was performed in triplicate and repeated at least three times.

Cell cycle analysis

Cells were treated with increasing concentrations of celecoxib (0, 10, 25, 50 or 75 μmol/L) for 48 h. The adherent and the nonadherent cell fractions were then collected and fixed in 70% ice-cold ethanol overnight. Cells were washed with cold PBS, treated with RNase for 30 min at 37 °C, and then stained with PI for 30 min at 4 °C in the dark. DNA content was analyzed using flow cytometry (EPICS XL, Beckman Coulter Inc, Fullerton, CA, USA). The data were analyzed using the ModFit^{TM} for Mac version 3.0 software (Verity Software House, Topsham, ME, USA). The experiment was performed in triplicate and repeated at least three times.

Western blot analysis

Cells were treated with DMSO (control) or increasing concentrations of celecoxib (10, 25, 50 or 75 μmol/L) for 48 h. The cells were then washed with PBS and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer with phosphatase and protease inhibitors (Roche Diagnostics, Mannheim, Germany). The protein concentrations were determined using the Bradford protein assay kit (Bio-Rad Laboratories Inc, Hercules, CA, USA) according to the manufacturer's instructions. Protein samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking the membrane's non-specific binding sites using non-fat milk, the membranes were separately incubated with primary antibodies overnight and then probed with HRP-conjugated goat anti-rabbit or HRP-conjugated rabbit anti-goat secondary antibodies. The specific proteins of interest were visualized using the enhanced chemiluminescence (ECL) detection system. Densitometric quantification of the bands was performed using the AlphaEaseFC software tool (Alpha Innotech, San Leandro, CA, USA). The experiment was performed in triplicate and repeated at least three times.

Statistical analysis

All data were expressed as the mean±standard deviations (SD). Statistical analysis was performed using Student's *t* test. $P<0.05$ was considered statistically significant.

Results

COX-2 protein expression in NPC cell lines

To investigate the COX-2 protein expression patterns in HNE1 and CNE1-LMP1 cells, Western blots were performed after 48 h incubations with different doses of celecoxib (0, 10, 25, 50 or 75 μmol/L). Western blot analysis revealed that both cell lines expressed COX-2. The HNE1 cells exhibited higher protein expression levels of COX-2 than the CNE1-LMP1 cells ($P<0.01$). In the HNE1 cell line, COX-2 expression was significantly down-regulated upon celecoxib treatment at concentrations of 10, 25 and 50 μmol/L ($P<0.01$, $P<0.01$ and $P<0.01$, respectively). However, at the 50 and 75 μmol/L concentrations of celecoxib in CNE1-LMP1 cells, COX-2 expression was significantly up-regulated ($P<0.01$ and $P<0.01$, respectively) (Figure 1A and 1B).

Anti-proliferative effect of celecoxib on NPC cell lines

To investigate whether celecoxib was capable of inhibiting NPC cell growth, HNE1 and CNE1-LMP1 cells were treated with increasing concentrations of celecoxib (0, 5, 10, 25, 50 or 75 μmol/L) for 48 h, followed by an MTT assay. As shown in Figure 1C and 1D, we observed that the inhibitory effect of celecoxib occurred in a dose-dependent manner. The average IC_{50} values of celecoxib in HNE1 and CNE1-LMP1 were 32.86±1.13 μmol/L and 61.31±4.30 μmol/L, respectively.

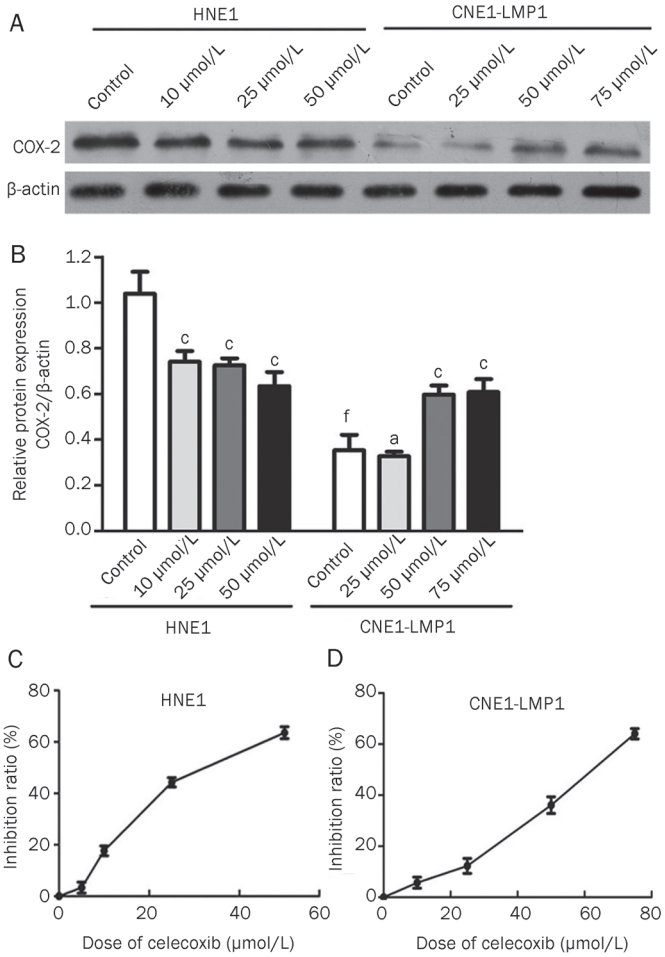

Figure 1. Celecoxib regulated COX-2 expression levels and inhibited cell proliferation in human nasopharyngeal carcinoma cells. (A and B) COX-2 was consistently expressed in both HNE1 and CNE1-LMP1 cell lines. Western blot analysis of the control cells and cells treated with various concentrations celecoxib (0, 10, 25, 50 or 75 μmol/L). HNE1 cells expressed higher levels of COX-2 than CNE1-LMP1 cells. COX-2 expression levels were significantly reduced in HNE1 cells but were significantly increased in the CNE1-LMP1 cells after drug treatment. (C and D) Celecoxib induced dose-dependent growth inhibition in both HNE1 and CNE1-LMP1 cells. Values displayed are the mean±SD ($n=3$). $^{a}P>0.05$, $^{c}P<0.01$ *vs* control; $^{f}P<0.01$ CNE1-LMP1 control *vs* HNE1 control.

Celecoxib induces apoptosis in NPC cell lines

Because celecoxib has been reported to exert an anticancer effect by mediating apoptosis in a COX-2-independent manner in many tumors[25], we examined whether the inhibitory effect of celecoxib was associated with the induction of apoptosis. Flow cytometric analysis using Annexin V/PI double staining in both celecoxib-treated and control cells was used to detect apoptosis. After treatment with increasing doses of celecoxib (0, 10, 25, 50 or 75 μmol/L) for 48 h, induction of apoptosis was observed in both HNE1 and CNE1-LMP1 cells in a concentration-dependent manner. Celecoxib induced significant increases in the percentage apoptotic cells in HNE1 cells

at concentrations of 25 and 50 μmol/L (P<0.01 and P<0.01, respectively) and in CNE1-LMP1 cells at concentrations of 50 and 75 μmol/L (P<0.05 and P<0.01, respectively) (Figure 2).

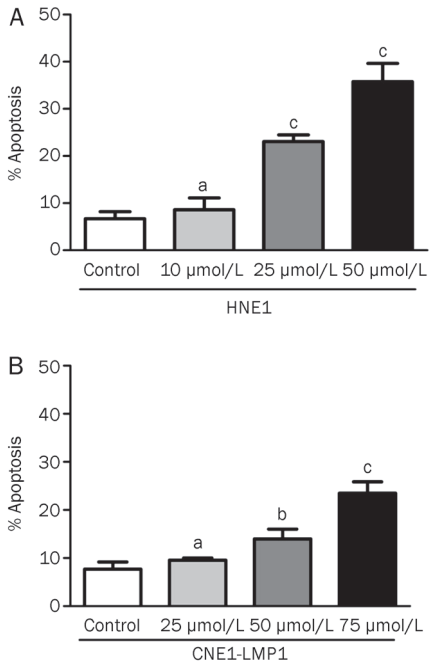

Figure 2. Celecoxib induced apoptosis in HNE1 and CNE1-LMP1 cells. After 48 h of incubation with drugs, flow cytometry analysis using Annexin V and propidium iodide (PI) double staining was performed on both control and celecoxib-treated cells. The population in the figure is the total number of apoptotic cells, which includes both early and late apoptotic populations. Early apoptotic cells were positive for Annexin V-FITC and negative for PI. Late apoptotic cells were positive for both Annexin V-FITC and PI. Significant apoptosis was observed in HNE1 cells (A) with 25 and 50 μmol/L of celecoxib, and in CNE1-LMP1 cells (B) with 50 and 75 μmol/L of celecoxib. The data are presented as the mean±SD (n=3). [a]P>0.05, [b]P<0.05, [c]P<0.01 *vs* control.

Celecoxib induces cell-cycle arrest in NPC cell lines

To investigate whether cell-cycle arrest contributed to cell growth inhibition by celecoxib, cell-cycle progression was assayed. Flow cytometric analysis was performed on NPC cells treated with increasing concentrations of celecoxib (0, 10, 25, 50 or 75 μmol/L) for 48 h. As displayed in Figure 3, celecoxib induced a marked G_0/G_1 cell-cycle arrest in NPC cell lines in a concentration-dependent manner. Compared with the DMSO control treatment group, a significant increase in the G_0/G_1 population was observed in both NPC cell lines. At 25 and 50 μmol/L concentrations of celecoxib, significant increases (P<0.01 and P<0.01, respectively) in the proportion of cells arrested in the G_0/G_1 cell-cycle phase were observed in HNE1 cells. Subsequently, significant inhibition of S phase transition (P<0.01 and P<0.01 at 25 and 50 μmol/L, respectively) and G_2/M phase (P<0.05 and P<0.01 at 25 and 50 μmol/L, respectively) were observed. In CNE1-LMP1 cells there were significant increases in the proportion of cells that were arrested in the G_0/G_1 phase of the cell cycle after celecoxib treatment at concentrations of 25, 50 and 75 μmol/L (P<0.01, P<0.01 and P<0.01, respectively). Subsequently, significant inhibition of S phase transition (P<0.05 and P<0.01 at 50 and 75 μmol/L, respectively) and G_2/M phase (P<0.01 and P<0.01 at 50 and 75 μmol/L, respectively) were observed (Figure 3B and 3C).

Celecoxib inhibits STAT3 phosphorylation

To investigate whether celecoxib could inhibit STAT3 phosphorylation, Western blotting was used to analyze the levels of phosphorylated STAT3 after celecoxib treatment. The HNE1 and CNE1-LMP1 cell lines were treated with increasing concentrations of celecoxib (0, 10, 25, 50 or 75 μmol/L). As displayed in Figure 4, we observed that the amount of phosphorylated $STAT3^{Y705}$ (pSTAT3) was significantly reduced after the treatment of celecoxib at concentrations of 10, 25 or 50 μmol/L (P<0.01, P<0.01 and P<0.01, respectively) in HNE1 cells. Additionally, phosphorylated $STAT3^{Y705}$ was significantly down-regulated in CNE1-LMP1 cells after celecoxib treatment at concentrations of 25, 50 and 75 μmol/L (P<0.01, P<0.01 and P<0.01, respectively). The pSTAT3 expression levels in the CNE1-LMP1 cell line were higher than those in the HNE1 cell line (P<0.01) (Figures 4A and 4B), and the amount of total STAT3 expressed was consistent after treatment with increasing celecoxib doses.

To provide further evidence of the inhibition of STAT3 phosphorylation, we analyzed the important downstream proteins of STAT3 by Western blot. Survivin, Mcl-1, Bcl-2 and Cyclin D1 proteins displayed reduced expression levels after treatment with escalating doses of celecoxib (0, 10, 25, 50 and 75 μmol/L) in HNE1 and CNE1-LMP1 cells (Figure 4A). In HNE1 cells, celecoxib significantly down-regulated Survivin (P<0.01 and P<0.01 at 25 and 50 μmol/L, respectively), Mcl-1 (P<0.01 and P<0.01 at 25 and 50 μmol/L, respectively), Bcl-2 (P<0.01, P<0.01 and P<0.05 at 10, 25 and 50 μmol/L, respectively) and Cyclin D1 (P<0.01, P<0.01 and P<0.01 at 10, 25 and 50 μmol/L, respectively). In CNE1-LMP1 cells, Survivin (P<0.01, P<0.01 and P<0.01 at 25, 50 and 75 μmol/L, respectively), Mcl-1 (P<0.01, P<0.01 and P<0.01 at 25, 50 and 75 μmol/L, respectively), Bcl-2 (P<0.01 and P<0.01 at 50 and 75 μmol/L, respectively) and Cyclin D1 (P<0.01, P<0.01 and P<0.01 at 25, 50 and 75 μmol/L, respectively) were significantly down-regulated after celecoxib treatment (Figure 4C and 4D). This provides additional evidence that celecoxib inhibits STAT3 phosphorylation.

Celecoxib inhibits interleukin-6 (IL-6)-induced STAT3 phosphorylation

To investigate whether celecoxib may inhibit interleukin-6 (IL-6)-induced STAT3 phosphorylation, Western blotting was performed. We chose the HNE1 cell line due to its low levels of endogenous $pSTAT3^{Y705}$. Cells were cultured in serum-free medium for 24 h and then pretreated with 25 μmol/L or 50 μmol/L of celecoxib for 2 h. The cells were then treated with 25 ng/mL of human IL-6 (Peprotech Inc, Rocky Hill, NJ,

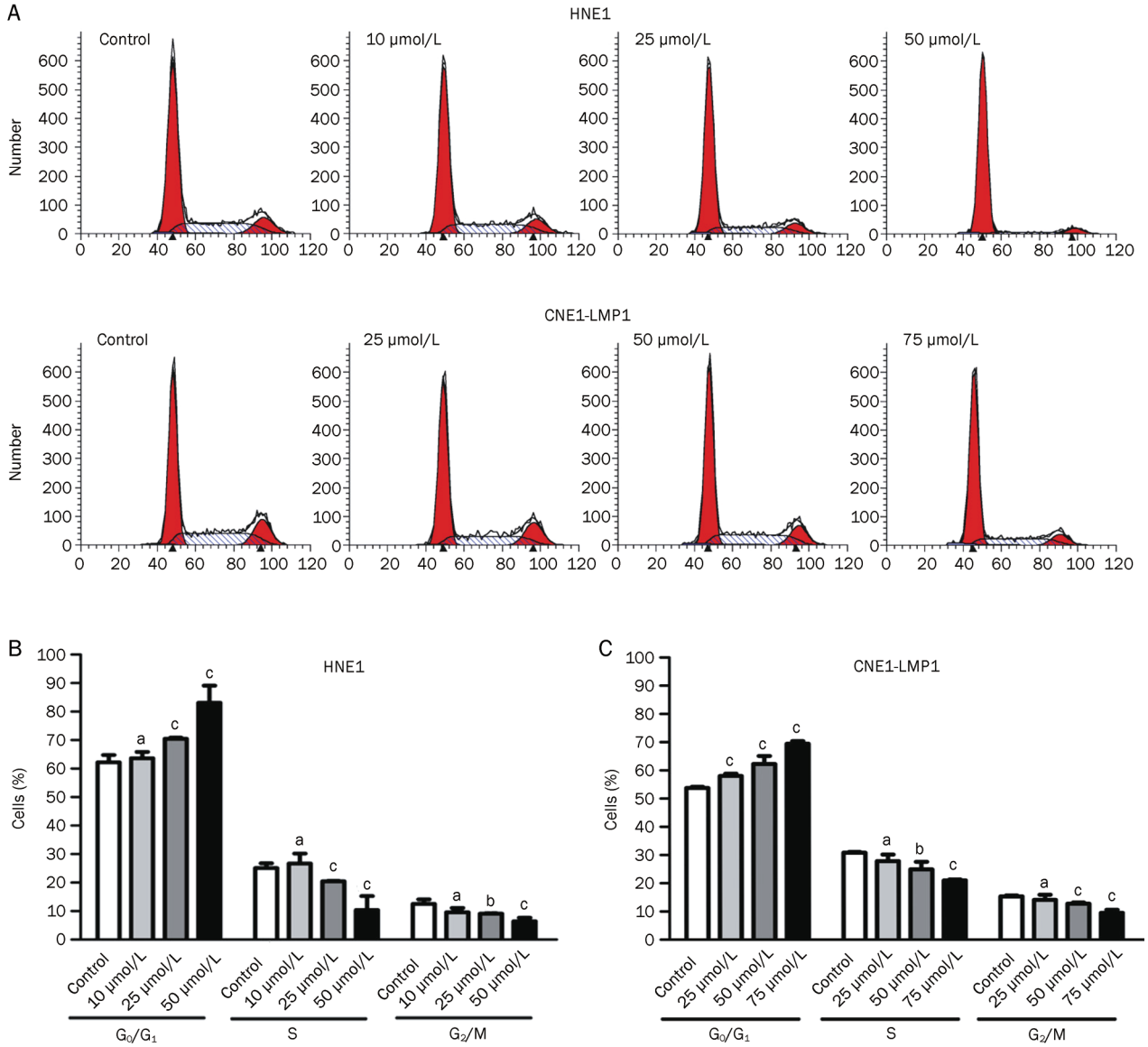

Figure 3. Celecoxib caused cell-cycle arrest in HNE1 and CNE1-LMP1 cells. Flow cytometry analysis of cells treated with DMSO (control) or celecoxib (10, 25, 50 or 75 μmol/L) for 48 h. Cells were fixed with 70% ethanol, stained with PI, and analyzed by flow cytometry. (A) Representative DNA histogram of cells after treatment with control or various concentrations of celecoxib for 48 h. Significant G_0/G_1 checkpoint arrest was observed in HNE1 (B) treated with 25 and 50 μmol/L of celecoxib, and this arrest was also observed in CNE1-LMP1 cells (C) treated with 25, 50 or 75 μmol/L of celecoxib. The data shown are the mean± SD from triplicate replicates and three independent experiments. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

USA) for 30 min. After IL-6 treatment, $pSTAT3^{Y705}$ and STAT3 protein levels were analyzed by Western blot. We observed that IL-6 significantly promoted STAT3 phosphorylation ($P<0.01$) and that celecoxib significantly inhibited IL-6-induced STAT3 phosphorylation at concentrations of 25 and 50 μmol/L ($P<0.01$ and $P<0.01$, respectively) (Figure 5A and 5B).

Discussion

STAT3 is a novel anticancer drug target. Constitutive STAT3 activation has a critical role in tumor development and tumorigenesis in multiple tumors and cell lines. Critical gene expression changes induced by aberrant activation of STAT3 may promote cancer cell growth and survival via the upregulation of Cyclin D1, c-Myc, Pim-1[26, 27]. Additionally, STAT3 may inhibit apoptosis via the upregulation of Bcl-2, Survivin and Mcl-1[28–30]. STAT3 may also promote cancer cell invasion and metastasis through the induction of matrix metalloprateinase-2 (MMP-2)[31] and increase angiogenesis via the induction of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1-alpha (HIF-1α)[32]. Increasing evidence has shown that the inhibition of constitutively activated STAT3 could lead to cancer cell death and tumor regression[33–35].

The activation or overexpression of STAT3 is very common in NPC. Indeed, it is detected in more than 75% of NPC cases.

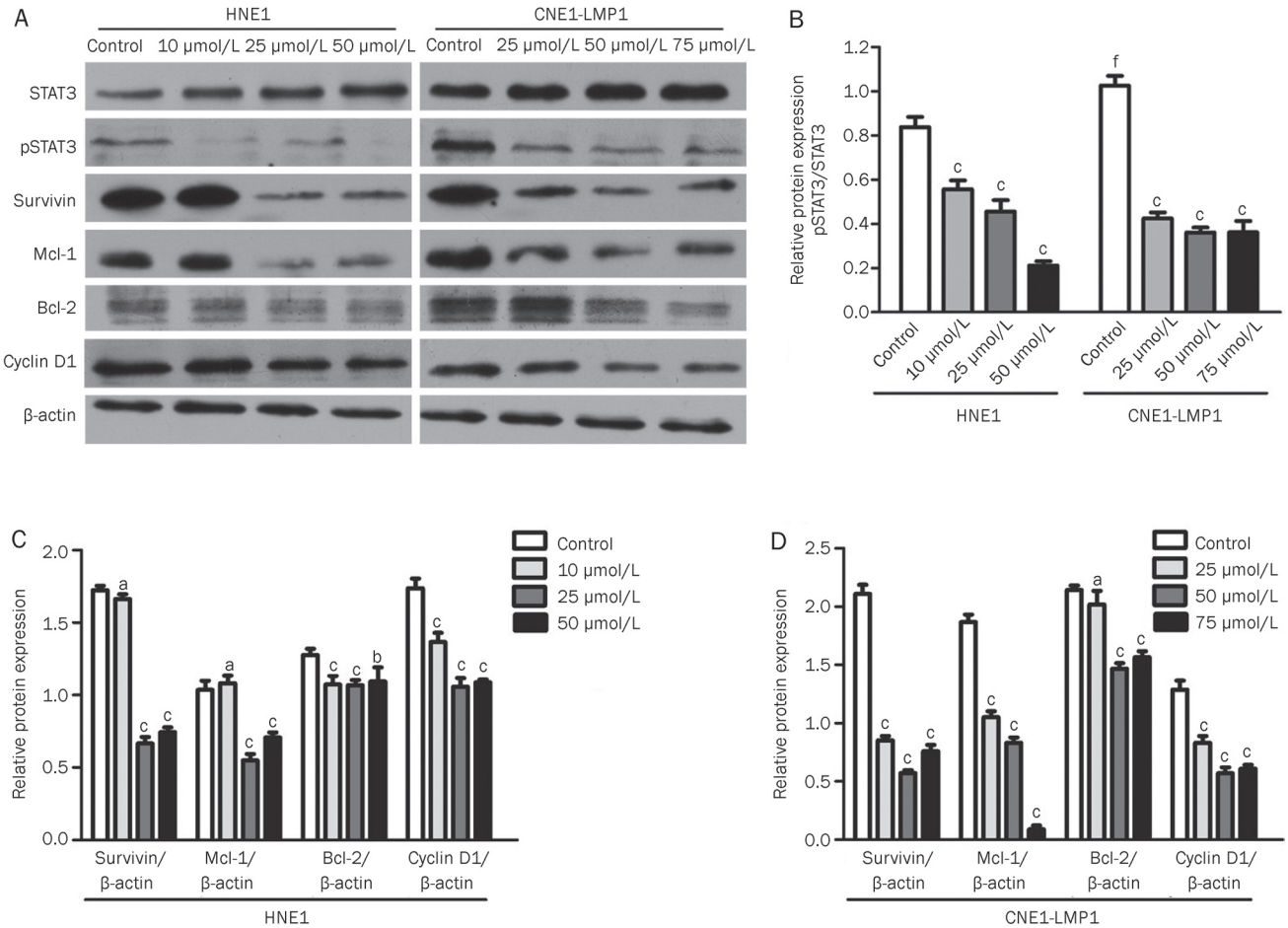

Figure 4. Celecoxib inhibited STAT3 phosphorylation and down-regulated the levels of STAT3 downstream targets. HNE1 and CNE1-LMP1 cells were treated with DMSO (control) or celecoxib (10, 25, 50 or 75 μmol/L) for 48 h. Celecoxib significantly inhibited STAT3 phosphorylation in both cell lines. The pSTAT3 expression level was higher in the CNE1-LMP1 cell line than that in the HNE1 cell line (A and B). Celecoxib also down-regulated the downstream proteins of STAT3 (Survivin, Mcl-1, Bcl-2 and Cyclin D1) (C and D). The blots displayed are from a representative experiment repeated three times with similar results. $^{a}P>0.05$, $^{b}P<0.05$, $^{c}P<0.01$ *vs* control; $^{f}P<0.01$ CNE1-LMP1 control *vs* HNE1 control.

Growing evidence indicates that STAT3 activation contributes to the progression and invasiveness of NPC[16–18]. Therefore, the activation of STAT3 provides a promising and valid target for NPC therapeutic modalities.

Even though a great number of studies have demonstrated that celecoxib could exert its anticancer effect in a wide variety of tumor types through COX-2-dependent and/or COX-2-independent pathways, studies on the anticancer effect and molecular mechanisms of celecoxib in NPC are scarce[11–13]. The majority of NPC patients are EBV infected, and LMP1 is the main EBV oncoprotein expressed in these tumors. Therefore, the HNE1 (EBV-negative) and CNE1-LMP1 cell lines were chosen for this study. The results presented in our study clearly illustrate that celecoxib significantly inhibits cell proliferation in both of the NPC cell lines in a dose-dependent manner. However, the mechanism of the anticancer effect of celecoxib is not fully dependent on COX-2 expression. COX-2 protein expression levels in HNE1 cells are higher than in CNE1-LMP1 cells. Additionally, the COX-2 expression pattern was different between the two cell lines after treatment with celecoxib, with down-regulated COX-2 expression observed in HNE1 cells, but up-regulated COX-2 expression in CNE1-LMP1 cells. The mechanism for increased COX-2 expression in CNE1-LMP1 cells is still unclear.

The observation that celecoxib can increase COX-2 expression was reported in breast cancer MDA-MB-231 cells, 184 hTERT breast cancer cells and human osteosarcoma MG-63 cells[36–38]. One or more products produced by COX may be capable of inhibiting COX expression via a negative feedback loop. Celecoxib treatment could remove the negative feedback and lead to COX-2 induction. In addition, Murono *et al* determined that LMP1 could induce COX-2 expression in NPC cell lines, increase prostaglandin E2 production through COX-2 induction and that celecoxib could inhibit prostaglandin E2 production[39]. Therefore, LMP1 may induce COX-2 expression to up-regulate prostaglandin E2 expression after the inhibition of prostaglandin E2 expression by celecoxib. Furthermore, celecoxib is known to inhibit the phosphatidylinositol 3-kinase

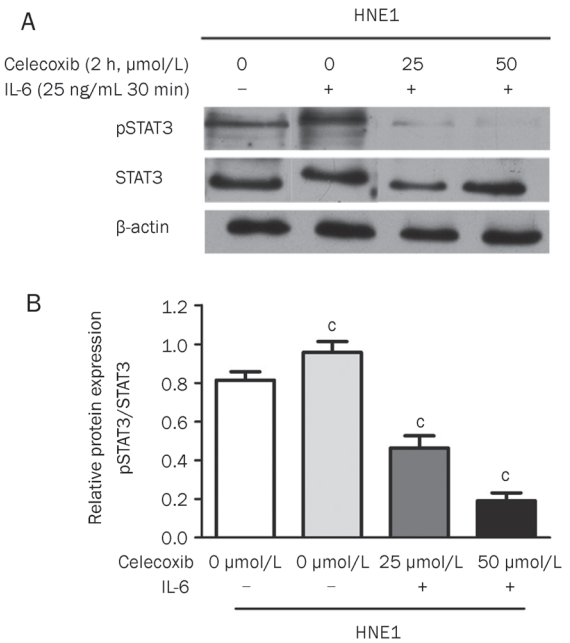

Figure 5. Celecoxib inhibited STAT3 phosphorylation induced by IL-6. HNE1 cells were pretreated with celecoxib for 2 h followed by 25 ng/mL of IL-6 for 30 min. Western blot analysis was performed for STAT3 phosphorylation. IL-6 promoted pSTAT3 expression, which was significantly suppressed by celecoxib at concentrations of 25 and 50 μmol/L (A and B). The blots displayed are from a representative experiment repeated three times with similar results. $^{c}P<0.01$ *vs* control.

(PI3K)/Protein Kinase B (Akt) pathway[40]. Monick *et al* reported that P13K inhibition resulted in an increase in COX-2 protein[41]. PI3K negatively regulates the activation of the p38 pathway, and active p38 is necessary for COX-2 production. Therefore, celecoxib could up-regulate COX-2 expression via the downregulation of PI3K.

Regardless of the COX-2 expression patterns in HNE1 and CNE1-LMP1 cells, at the molecular level, in accordance with the recent similarly reported findings in colon cancer cells, hepatocellular carcinoma cells and rhabdomyosarcoma cells[6, 7, 19], celecoxib strongly inhibited STAT3 phosphorylation in both NPC cell lines, which is a critical step in the activation of downstream signaling. pSTAT3 expression levels in CNE1-LMP1 cells were higher than in the HNE1 cells. One potential mechanism for this discrepancy may be that LMP1 could activate STAT3 phosphorylation through the MAPK/ERK and JAK/STAT pathways[22]. Subsequently, the critical downstream proteins Survivin, Mcl-1, Bcl-2 and Cyclin D1, were down-regulated, ultimately leading to cell growth inhibition, cell cycle arrest and apoptosis. To the best of our knowledge, the anticancer effect of celecoxib on the STAT3 signaling cascade in NPC cells has not yet been investigated. Our results revealed that the STAT3 pathway may be a COX-2-independent anticancer mechanism for celecoxib.

IL-6 induces STAT3 phosphorylation in various human cancers[42–44]. We hypothesized that celecoxib could exert anticancer effect by blocking inflammatory pathways, such as the IL-6/STAT3 pathway. In agreement with previously published studies[6, 7, 19], our results indicated that celecoxib could inhibit IL-6-induced STAT3 phosphorylation in HNE1 cells, demonstrating that celecoxib has great promise as a molecular targeting therapy for human cancers with aberrant IL-6 expression.

Studies in various cancers have indicated that celecoxib possesses a unique capacity to induce apoptosis independent of COX-2 inhibition, making celecoxib a potent anticancer drug[8, 25]. At the cellular level, our results showed that celecoxib induced apoptosis in both the HNE1 and CNE1-LMP1 cell lines and that apoptosis was associated with the down-regulation of the anti-apoptotic proteins Survivin, Mcl-1 and Bcl-2. Survivin, Mcl-1 and Bcl-2 are important downstream apoptotic genes in the IL-6/STAT3 pathway. Constitutively active STAT3 up-regulates Survivin, Mcl-1 and Bcl-2, leading to the dysregulation of apoptosis and thereby promoting cancer cell survival and proliferation. The apoptosis induction and the down-regulation of critical downstream anti-apoptotic proteins (Survivin, Mcl-1 and Bcl-2) further indicated that celecoxib could work via the inhibition of STAT3 phosphorylation. Several studies demonstrated that inhibition of 3-phosphoinositide-dependent kinase 1 (PDK-1) and its downstream substrate, PKB/AKT, plays a central role in the induction of apoptosis and cell cycle arrest[8]. However, whether this is the case in NPC still requires further investigation.

It is well described in the literature that celecoxib inhibits cell growth and survival in several different tumor types through the induction of G_0/G_1 cell cycle arrest. However, no consensus exists concerning the underlying molecular mechanisms of these effects. Consistent with previous studies[36, 45, 46], our results demonstrated that celecoxib induced G_0/G_1 cell cycle arrest in HNE1 and CNE1-LMP1 cell lines and that this arrest was accompanied by the down-regulation of Cyclin D1[12, 45–47], which is one of the major cyclins known to be up-regulated in cancers. Cyclin D1 activates Cyclin Dependent Kinase 2 (CDK2) and CDK4, leading to the phosphorylation of the retinoblastoma protein (pRb), which forces its release from the E2F transcription factor. Consequently, the cell cycle progresses from G_1 to S phase[48]. In our studies, the celecoxib-induced cell-cycle arrest in G_0/G_1 phase occurred, at least partially, via the downregulation of Cyclin D1 expression. It has been reported in other cancer types that celecoxib induces G_0/G_1 cell-cycle arrest by down-regulating c-Myc, Cyclins A and B, up-regulating cell-cycle inhibitors $p21^{wafl}$ and $p27^{kipl}$ and losing CDK activity[8]. It is unclear whether these mechanisms are involved in NPC cells after celecoxib treatment.

In addition to the above work, preclinical findings and a Phase I clinical trial suggest celecoxib could synergistically enhance radiation therapy treatment in prostate, lung and recurrent head and neck cancers[49–51]. Radiotherapy (RT) is the primary treatment modality for non-metastatic NPC. A recent Phase I clinical trial in NPC determined that celecoxib can be safely administered concurrently with radiotherapy at doses of up to 800 mg/d with good rates of local control and overall

survival[52]. However, whether celecoxib improved treatment efficacy is still unclear due to the lack of a control arm. Therefore, further studies are required to investigate whether synergistic effects exist with combination of celecoxib and radiation therapy in NPC *in vitro* and *in vivo* and to elucidate the underlying molecular mechanisms of this treatment.

There are certain limitations in the present study. First, different cell lines may respond differently to celecoxib; therefore, additional cell lines must be investigated to fully elucidate the underlying mechanism of the proliferation inhibitory effect with celecoxib treatment. Additionally, further studies are needed to differentiate the anticancer effect of celecoxib in NPC on different pathways. Celecoxib could act through COX-2-dependent and/or COX-2-independent mechanisms, including the COX-2 pathway, the PI3K/AKT pathway, the ERK pathway, the IL-6/STAT3 pathway, and other pathways. Elucidating the precise molecular mechanism would provide a precise molecular target for future NPC therapy. *In vivo* studies also need to be evaluated.

In summary, at the cellular level, the present study demonstrated that celecoxib inhibited growth and induced apoptosis and cell-cycle arrest at the G_0/G_1 phase in NPC cells. At the molecular level, pSTAT3 was inactivated in parallel with reduced activation of the downstream anti-apoptotic proteins Survivin, Mcl-1, Bcl-2 and the cell-cycle regulatory protein Cyclin D1. Taken together, the anticancer effects of celecoxib in NPC may be partly achieved through the STAT3 pathway.

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Author contribution

Dong-bo LIU and Guang-yuan HU performed the research and wrote the manuscript; Guo-xian LONG, Hong QIU and Qi MEI performed some of the experiments and analyzed the data; and Guo-qing HU designed the experiments.

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