Celecoxib induces apoptosis via a mitochondria-dependent pathway in the H22 mouse hepatoma cell line

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Abstract. Celecoxib is a potent nonsteroidal anti-inflammatory drug that has demonstrated promise in cancer chemoprevention and treatment. The present study was conducted to gain insight into the molecular mechanism by which celecoxib induces apoptosis in the H22 mouse hepatoma cell line. The effect of celecoxib on the viability of H22 mouse hepatoma cells was assessed with sulforhodamine B assay. Apoptosis and mitochondrial membrane potential were detected by a flow cytometric assay. The protein expression levels of Bax, Bcl-2, cytochrome *c*, caspase-3, caspase-9, apoptosis-inducing factor (AIF), peroxisome proliferator-activated receptor (PPAR)γ and nuclear factor (NF)-κB were determined by western blot analysis. The data demonstrated that celecoxib reduced the percentage of viable H22 cells in a dose- and time-dependent manner, which was associated with cell apoptosis. Furthermore, celecoxib induced apoptosis via the loss of the mitochondrial transmembrane potential (ΔΨm), the release of cytochrome *c* and AIF, and the activation of caspase-9 and caspase-3. Celecoxib also increased the abundance of the pro-apoptotic protein Bax and reduced the levels of the anti-apoptotic protein Bcl-2. The data demonstrated that celecoxib induced apoptosis in mouse liver cancer cells via the mitochondria-dependent pathway rather than the PPARγ/NF-κB signaling pathway, which indicates that celecoxib may be an effective agent in the clinical management of hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer worldwide and the third most common cause of cancer-related mortality (1). HCC usually occurs in the presence of continuous inflammation and hepatocyte regeneration during chronic hepatitis and cirrhosis; thus, mediators of inflammation are involved in the development of hepatic carcinogenesis (2). Drug treatment is the main therapy for patients in the advanced stages of disease. However, the response rate to traditional chemotherapy for HCC patients is far from satisfactory (3). Therefore, novel and effective pharmacological strategies are urgently required for the treatment of advanced HCC.

The non-steroidal anti-inflammatory drug celecoxib is a member of the cyclooxygenase-2 (COX-2) inhibitor drug family and is a potent and specific inhibitor of human COX-2 (4,5). The anticancer effects of celecoxib have been demonstrated in various different tumor types, including colorectal, lung, breast, prostate and head/neck cancer (6-8). The mechanism underlying the effects of celecoxib may be associated with COX-2-dependent or -independent pathways (9,10). A number of studies have investigated the mechanisms of the anticancer action of celecoxib. Celecoxib and derived compounds have been suggested to induce cell cycle arrest or apoptosis, inhibit tumor growth and suppress tumor neoangiogenesis (11). However, the mechanisms underlying celecoxib treatment of HCC are not yet completely understood.

In recent years, numerous advances in the understanding of HCC have been reported (12). The failure of transformed cells to undergo apoptosis markedly disrupts tissue homeostasis and allows proliferation of the resistant clone, a phenomenon frequently observed in HCC (13). Apoptosis is an evolutionarily conserved programmed mode of cell death and is critical for the sustained tissue homeostasis. Apoptosis signaling is tightly regulated by two main apoptotic pathways, termed the 'extrinsic pathway' and the 'intrinsic pathway'. These pathways involve either cell surface death receptors, or the mitochondria and the endoplasmic reticulum, respectively (14,15). We have previously focused on the effects of celecoxib on the arachidonic acid (AA) signaling pathway in H22 mouse hepatoma cells. The imbalance between AA and prostaglandin (PG)E2, characterized by increased AA at a low dosage of celecoxib and reduced PGE2 at a high dosage of celecoxib, was demonstrated to be a significant indicator of celecoxib-mediated apoptosis in H22 cells (16). The present study was designed to clarify the targeting of the apoptotic pathway by celecoxib and possible interconnections between COX-2 and the peroxisome proliferator-activated receptor (PPAR)γ/nuclear factor (NF)-κB signaling pathway.

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Materials and methods

Reagents and antibodies. Celecoxib (purity, >98%) was purchased from Sunheat Chemicals (Shanghai, China). High-glucose Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco-BRL (Carlsbad, CA, USA). Dimethyl sulfoxide, methanol, ethanol, chloroform, phosphoric acid and acetic acid were obtained from Beijing Chemistry Company (Beijing, China). Sulforhodamine B and proteinase inhibitors were bought from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), SDS, tetramethylethylenediamine, glycine, ammonium, persulfate, acrylamide, Tris, Trizol, agarose and Tween-20 were purchased from Beijing Dingguo Changsheng Biological Technology Co., Ltd (Beijing, China). The protein assay kit, radioimmunoprecipitation assay (RIPA) lysis buffer, Rhodamine 123 and Cell Apoptosis Assay kit were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Antibodies against β-actin (mouse, monoclonal, sc-47778), Bax (mouse, monoclonal, sc-7480), B-cell CLL/lymphoma 2 (Bcl-2; mouse, monoclonal, sc-7382), cytochrome *c* (mouse, monoclonal, sc-13561), caspase-3 (rabbit, polyclonal, sc-98785), caspase-9 (mouse, monoclonal, sc-133109), apoptosis-inducing factor (AIF; mouse, monoclonal, sc-55519), PPARγ (rabbit, polyclonal, sc-7196) and NF-κB (rabbit, polyclonal, sc-298) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture. The H22 mouse hepatoma cell line was originally obtained from the American Type Culture Collection (Rockville, MD, USA) and stored at -80˚C. The H22 cells were cultured in DMEM supplemented with 10% FBS (v/v), 100 μM penicillin G (Sigma Aldrich, St. Louis, MO, USA) and 0.1 mg/ml streptomycin (Sigma Aldrich). The cultures were maintained in a humidified incubator at 37˚C in 5% CO_2.

Determination of cell viability by a sulforhodamine B (SRB) assay. The cells were seeded in 96-well plates in medium with 10% FBS. Following incubation with celecoxib at concentrations ranging between 25 and 400 μM for 48 h, the cells were fixed with 10% trichloroacetic acid (Sigma Aldrich), and 0.4% (w/v) SRB in 1% acetic acid was added to stain the cells. Unbound SRB was washed away with 1% acetic acid and SRB-bound cells were rendered soluble with 10 mM Trizma base (pH 10.5; Sigma Aldrich). The absorbance was read at a wavelength of 570 nm with a 680 microplate enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad Laboratories, Hemel Hempstead, UK). Using the following absorbance measurements: Time zero (T_0), control growth (C) and cell growth in the presence of the compound (T_x), the percentage growth was calculated at each compound concentration level. Percentage growth inhibition was calculated as: $100-[(T_x-T_0)/(C-T_0)] \times 100$. The 50% growth inhibition (IC_{50}) value was determined to be the compound concentration that resulted in a 50% reduction of the total protein increase in the control cells during the compound incubation.

Determination of apoptosis by Annexin V/propidium iodide (PI) staining. Apoptosis was measured using a fluorescein isothiocyanate-Annexin V apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). Following treatment, the cells were harvested by trypsinization, washed with ice-cold PBS and suspended in binding buffer at a density of $1x10^6$ cells/ml. The cell suspension was stained with 5 μl Annexin V and PI, and analyzed by a FACSort flow cytometer (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA).

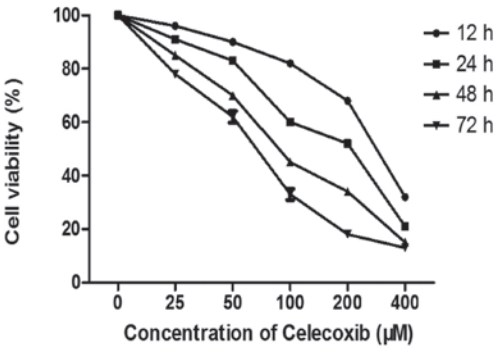

Figure 1. Time- and dose-dependent reduction in cell viability induced by celecoxib in H22 mouse hepatoma cell lines. The results are presented as the mean ± standard error of the mean from three independent experiments.

Measurement of mitochondrial membrane potential ($\Delta\Psi m$). The cells were treated with celecoxib. At 30 min prior to incubation termination, Rhodamine 123 solution (final concentration of 10 μg/ml) was added to the cells and incubated for the final 30 min at 37˚C. The cells were harvested and the accumulation of Rhodamine 123 was determined using FACScan flow cytometric analysis.

Preparation of cell extracts and western blot analysis. Subsequent to treatment, the cells were harvested with trypsinization, centrifuged and lysed in 0.1 ml lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1mM phenylmethanesulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 50 mM NaF and 100 mM sodium orthovanadate. Total protein was quantified, mixed with sample buffer and boiled at 90˚C for 5 min. Equal quantities of protein (30 mg) were separated by gel electrophoresis in 8 or 12% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The blots were blocked in Tris-buffered saline containing 0.05% Tween-20 (TBST) and 5% non-fat dry milk for 1 h at room temperature. The membrane was incubated overnight at 4˚C with the primary antibodies. Following repeated washings with TBST, the membranes were incubated with the HRP-conjugated mouse anti-rabbit secondary antibody or HRP-conjugated rabbit anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature prior to additional washes with TBST. Detection of antibody binding was performed using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Amersham, UK). Equal loading was verified using the antibodies against β-actin. All western blot analyses were repeated three times.

Statistical analysis. The data are reported as the mean ± standard error of the mean (n=3 per group). Statistical analysis was performed using the unpaired Student's t-test and $P<0.05$ was considered to indicate a statistically significant difference.

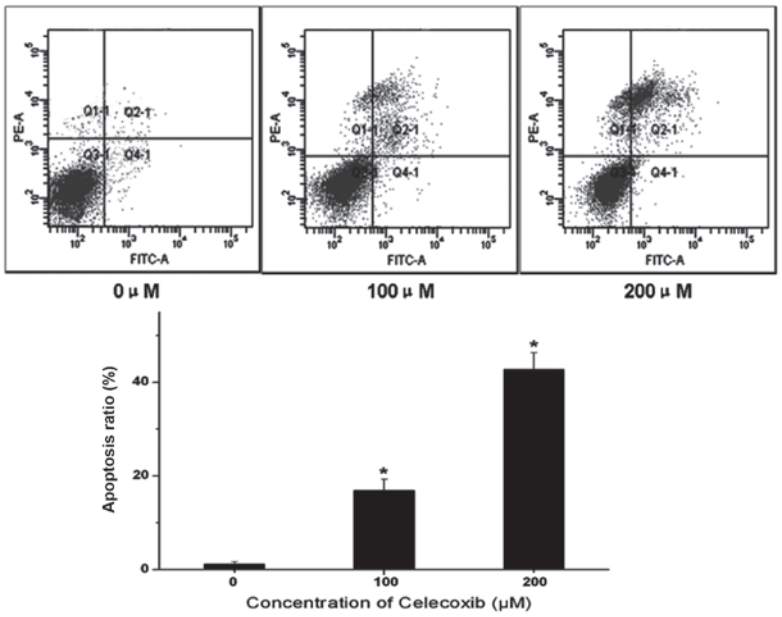

Figure 2. Celecoxib-induced apoptosis in H22 mouse hepatoma cells was assessed by flow cytometric analysis with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining. Cells were incubated either without celecoxib, or in the presence of 100 or 200 μM celecoxib for 24 h. Undamaged cells were stained negative for Annexin V-FITC and PI (bottom left quadrant). Following incubation with celecoxib for 24 h, a notable number of apoptotic cells were stained positive Annexin V-FITC and negative PI (bottom right quadrant). Data are presented as the mean ± standard error of the mean from triplicates and three independent experiments. Two other independent experiments produced similar results. *P<0.05, compared with control.

Results

Celecoxib inhibits cell proliferation. The effect of celecoxib on cell viability was determined by an SRB assay. The H22 cells were treated with celecoxib at concentrations ranging between 25 and 400 μM for 12, 24, 48 and 72 h. As shown in Fig. 1, celecoxib exposure significantly reduced the viability of the H22 cells. The celecoxib IC_{50} values were 256.6±1.5 μM, 155.4±0.6 μM, 99.2±0.4 μM and 70.6±0.6 μM at 12, 24, 48 and 72 h, respectively. These results suggest that celecoxib induces cell death and inhibits cell proliferation in a dose- and time-dependent manner.

Celecoxib induces apoptosis in H22 cells. To further understand whether celecoxib-induced cell death is mediated by apoptosis or necrosis, apoptotic cell death was evaluated using Annexin V/PI double staining, which specifically labels apoptotic cells. Fig. 2 shows that H22 cells without celecoxib treatment were mostly detected in the Q3 quadrant, indicating that these cells were viable. Following celecoxib treatment, however, an increased number of late apoptotic cells were detected in the Q2 quadrant. Therefore, celecoxib markedly increased the proportion of apoptotic cells in a dose-dependent manner. These data suggest that apoptotic cell death events contribute to the growth inhibitory effect of celecoxib.

Celecoxib alters mitochondrial function and ΔΨm. Mitochondrial function is critical to cell viability. Disruption of the mitochondrial membrane potential has been reported to irreversibly result in cell apoptosis, the release of cytochrome *c* and a reduction in adenosine triphosphate (ATP) generation. In order to gain an improved understand of the mechanism of celecoxib-induced H22 cell apoptosis, Rhodamine 123 was used to ascertain the mitochondrial membrane potential through examining the fluorescent intensity. As shown in Fig. 3, the fluorescence intensity was reduced with increases in celecoxib concentration. A concentration-dependent reduction in Rhodamine 123 fluorescence was detected following celecoxib treatment, compared with the control group. This indicates that celecoxib was able to induce mitochondrial membrane potential disruption in H22 cells.

Celecoxib induces apoptosis via the mitochondria-dependent pathway. In chemically-induced apoptosis, mitochondria are central in the cellular commitment to apoptosis through cytochrome *c*-dependent or -independent pathways. To elucidate the molecular mechanism of celecoxib-induced apoptosis in H22 cells, the expression levels of proteins associated with apoptosis were examined. H22 cells were exposed to the indicated celecoxib concentrations for 24 h (Fig. 4). The data revealed that celecoxib induced the release of cytochrome *c* from the mitochondria to the cytosol and AIF from the cytosol to the nucleus in a concentration-dependent manner. Caspase-3 and caspase-9, well-known to be activated downstream of cytochrome *c*, were also cleaved in a concentration-dependent manner. By contrast, the expression levels of Bcl-2 protein, an anti-apoptotic molecule, were reduced in a concentration-dependent manner. However, celecoxib induced an increase in Bax expression levels; the complementary pro-apoptotic Bax proteins of the Bcl-2-family are essential for the activation of the intrinsic death pathway. These data suggest that celecoxib induced mitochondria-dependent apoptosis through the mitochondria-dependent pathway.

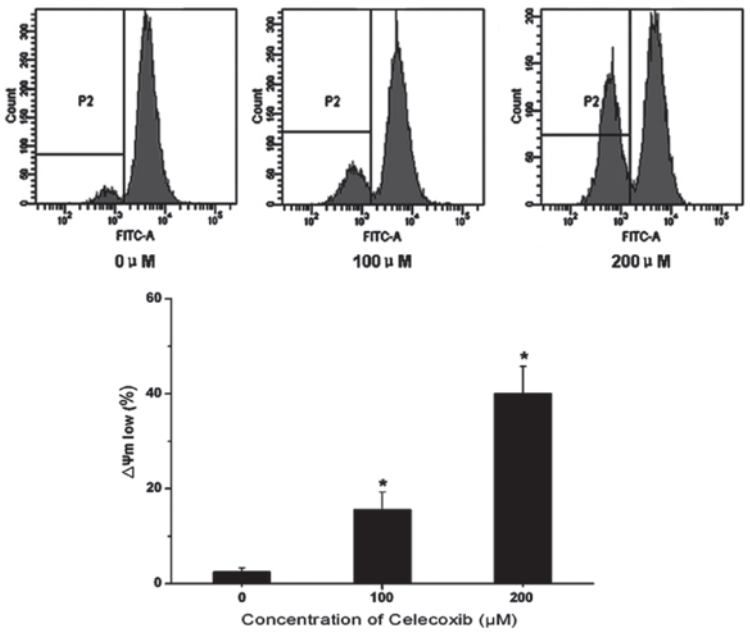

Figure 3. Flow cytometric assay of mitochondrial membrane potential changes detected with Rhodamine 123 dye in H22 cells following exposure to 100 or 200 μM celecoxib for 24 h. The change in the fluorescence intensity of Rhodamine 123 indicated the loss of mitochondrial membrane potential. Data are presented as the mean ± standard error of the mean from triplicates and three independent experiments. Two other independent experiments produced similar results. *P<0.05, compared with control.

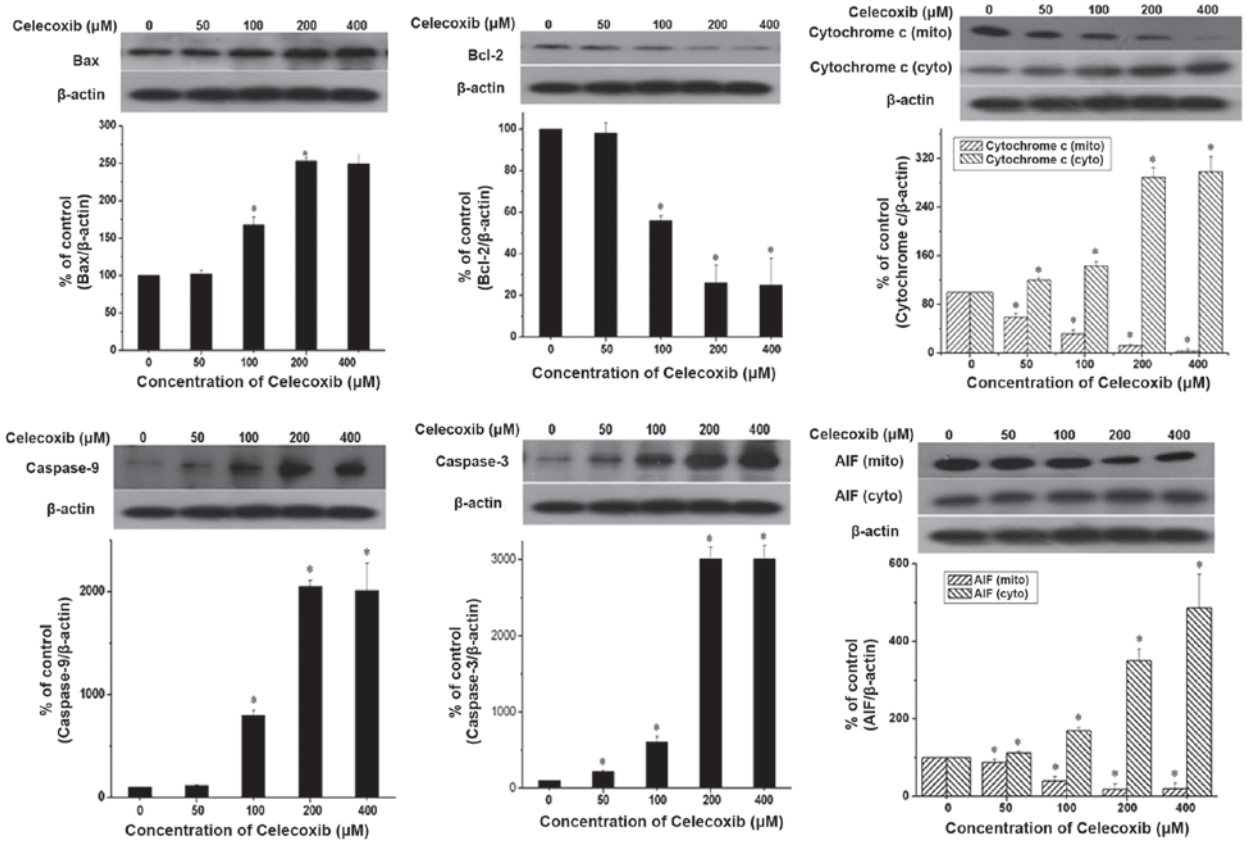

Figure 4. Protein expression levels of Bax, Bcl-2, cytochrome *c*, caspase-3, caspase-9 and apoptosis-inducing factor (AIF) in H22 mouse hepatoma cells treated with different doses of celecoxib. Expression and relative quantification of protein levels are expressed relative to the control. Values are presented as the mean ± standard error of the mean (n=3). *P<0.05.

Celecoxib does not induce apoptosis through the PPARγ/NF-κB signaling pathway. Since PPAR-γ agonists are known to exhibit growth-inhibitory effects in tumor cells, including HCC cell lines, the effect of celecoxib on PPAR-γ

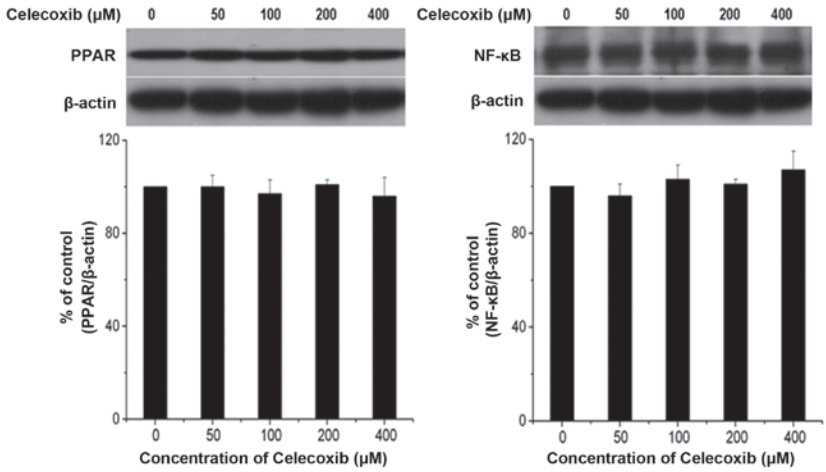

Figure 5. Protein expression levels of peroxisome proliferator-activated receptor (PPAR)γ and nuclear factor (NF)-κB in H22 mouse hepatoma cells treated with different doses of celecoxib. Expression and relative quantification of protein levels are expressed relative to the control. Values are presented as the mean ± standard error of the mean (n=3).

expression levels in H22 cells was investigated. In Fig. 5, western blot analysis of PPARγ and NF-κB did not reveal a differential effect on PPARγ and NF-κB expression levels during celecoxib-induced apoptosis, indicating that celecoxib induced apoptosis via the mitochondria-dependent pathway rather than the PPARγ/NF-κB signaling pathway.

Discussion

Celecoxib may reduce the risk of cancer formation by altering AA metabolism, which has been implicated in the development of cancer. Although the antitumor mechanism of celecoxib is not completely understood, a large number of studies have demonstrated that celecoxib prevents carcinogenesis through inhibition of COX-2 activity and the resulting reduction in PGE2 expression levels (17). In previous studies, the inhibitory effects of low celecoxib doses on hepatoma H22 cells were indicated to be induced by AA released through cPLA2 activity (16). Although celecoxib at low doses induced different changes in the AA metabolism pathway compared with high doses, celecoxib was, to the best of our knowledge, found for the first time to increase the ratio of AA to PGE2 in a dose-dependent manner, correlating with the repressing of cell viability on H22 cells. Therefore, the increased ratio of AA to PGE2 may be an important indicator of celecoxib cytotoxicity and may be used to evaluate antitumor activity (16).

Recently, data have indicated that the effects of celecoxib, a selective COX-2 inhibitor, are COX-2-dependent and -independent in HCC (18,19). The pro-apoptotic effects of celecoxib were first attributed to the inhibitory action of the drug on COX-2. By inhibiting COX-2, celecoxib has been suggested to interfere with prostaglandin-mediated upregulation of anti-apoptotic proteins (20,21). However, a study has demonstrated that celecoxib is able to suppress tumor growth without an apparent involvement of the target protein, COX-2 (22). Important molecular targets of the COX-2-independent celecoxib activity include protein kinase B and its upstream kinase 3-phosphoinositide-dependent kinase-1 (23), cyclin-dependent kinase inhibitors and cyclins, the anti-apoptotic proteins survivin, Bcl-2 and Mcl-1, as well as sarcoplasmic/endoplasmic reticulum calcium ATPase (24,25). Thus, celecoxib acts as a multifunctional drug. Although celecoxib constitutes a prototype of drugs that induce cell death independently from COX-2, inhibition of COX-2 in COX-2-expressing cells may also contribute to the cytotoxic effects of the drug. COX-2 inhibition may be particularly important for the *in vivo* effects of celecoxib in COX-2-expressing tumors, as COX-inhibition affects prostaglandin-mediated angiogenesis in xenografts and newly formed tumors (26).

Intrinsic apoptotic signaling occurs in response to stimuli such as DNA damage, growth-factor withdrawal and exposure to certain chemotherapeutic agents, which result in the release of cytochrome *c* and other pro-death factors from the intermembrane spaces of the mitochondria, and subsequent downstream signaling through the initiator caspase-9. The mitochondria are important for apoptosis. With external or internal apoptotic signal stimulation in tumor cells, the mitochondrial membrane permeability changes, and cytochrome *c*, AIF and other apoptotic factors are released into the cytoplasm. Cytochrome *c* may result in cell apoptosis following the formation of the apoptosome with Apaf-1 and caspase-9, activating caspase-3, caspase-6 or caspase-7 downstream. The Bcl-2 family proteins, including the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, are important factors that regulate the changes of the mitochondrial membrane permeability, and are important in the mitochondrial apoptotic pathway (27).

PPARγ is a ligand-activated transcription factor with a DNA-binding domain that recognizes response elements in the promoter regions of specific target genes associated with inflammation, cell proliferation, apoptosis and differentiation (28,29). NF-κB is aberrantly activated in tumor cells, contributing an advantage in cellular survival and proliferation (30). The NF-κB activation mechanism in tumor cells is not well-elucidated, but it appears complex and varies in different tumor types. In a previous study, celecoxib upregulated expression of PPARγ and inhibited growth of Lewis cancer cells in a dose-dependent manner (31). Whether increased PPARγ expression levels occurred directly or indirectly due to elevated AA levels remains unclear. However, our recent

data has not demonstrated a differential effect on PPARγ and NF-κB expression levels during celecoxib-induced H22 cell apoptosis. The mechanism of celecoxib action appears to vary according to the capacity of the cell type being treated. Celecoxib effects depend not only on the conditions under which it is administered to the cells and the cell type, but also on key pathways, such as COX-2, PPARγ and NF-κB. This highlights the requirement to closely analyze the mechanisms underlying celecoxib action within and among different tumor types.

In conclusion, the results of the present study demonstrate that celecoxib reduced the percentage of viable H22 cells in a dose- and time-dependent manner, which was associated with cell apoptosis. Celecoxib induced apoptosis via the mitochondria-dependent pathway, including through mitochondrial dysfunction, release of AIF and cytochrome *c* from the mitochondria, and the activation of caspase-9 and caspase-3. Celecoxib also increased the abundance of the pro-apoptotic protein Bax and reduced the levels of the anti-apoptotic protein Bcl-2. Therefore, the data indicate that celecoxib may be an effective therapy in HCC.

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