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Cyclooxygenase 2 expression impairs serum-withdrawal-induced apoptosis in liver cells

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Short title: *COX-2 inhibition of liver apoptosis*

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ABSTRACT

We have investigated the mechanism of COX-2-dependent inhibition of apoptosis in liver, a key pathway underlying proliferative actions of COX-2 in liver cancers, cirrhosis, chronic hepatitis C infection, and regeneration after partial hepatectomy. Stable expression of COX-2 in Chang Liver (CHL) cells induced proliferation, with an increase in the proportion of cells in S phase but no other significant changes on cell-cycle distribution. This was associated with a marked inhibition of the apoptotic response to serum deprivation, an effect mimicked by treating empty-vector transfected controls (CHL-V) with prostaglandin E₂, and prevented in COX-2 expressing cells (CHL-C) treated with selective inhibitors of COX-2. Serum-deprived CHL-V cells displayed several indicators of activation of intrinsic apoptosis: caspases 9 and 3 activated within 6 h and caspase 8 within 18 h, Bax expression was induced, cytochrome c was released to the cytosol, and PARP-1 cleavage was evident in nuclei. COX-2 expression blocked these events, concomitant with reduced expression of p53 and promotion of Akt phosphorylation, the latter indicating activation of survival pathways. CHL were resistant to stimulation of the extrinsic pathway with anti-Fas antibody. Moreover, *in vivo* expression of GFP-labelled COX-2 in mice by hydrodynamics-based transient transfection conferred resistance to caspase 3 activation and apoptosis induced by stimulation of Fas.

INTRODUCTION

Cyclooxygenase (COX) enzymes catalyze the first step in the biosynthesis of prostaglandins (PGs) and thromboxanes [1;2]. The COX-1 isoform is constitutively expressed in many tissues [3], whereas COX-2 is induced by growth factors, tumor promoters and cytokines [2;4;5]. The role of COX-2 in the production and development of neoplastic tumors is well established, especially in colon cancer [6]. Several authors have moreover reported that increased tissue COX-2 expression and PG production may contribute to the development of other cancers, including hepatocellular carcinoma (HCC)[7]. Expression of COX-2 has been demonstrated in many contexts related to liver cell pathologies: regeneration after partial hepatectomy (PH)[8;9]; animal models of cirrhosis [10]; human hepatoma cell lines [11;12]; chronic hepatitis C infection [7;13] and HCC [14;15].

Failure of apoptosis is an important event in tumour progression and resistance to cytotoxic therapy, and a principal mechanism of selective COX-2 inhibitors (COXIBS) is their pro-apoptotic activity. Apoptosis is mediated by activation of caspases, a family of cysteine proteases [16]. Caspase activation occurs by at least two mechanisms: the extrinsic or death receptor pathway, initiated by agonists of the tumour necrosis factor superfamily such as TNF- α , Fas and Apo2 ligand/TRAIL [17], and the intrinsic or mitochondrial pathway [18]. In the mitochondrial pathway caspase activation principally occurs as a result of the release of cytochrome c from the organelle, a process closely regulated by the Bcl-2 family of proteins. Cytochrome c in the cytosol associates with Apaf-1 and ATP and pre-caspase-9 in a multi-protein complex called the apoptosome. Once activated in the apoptosome, caspase 9 in turn activates downstream executioner caspases, such as caspase-3 and caspase-7 [19]. In liver, the extrinsic and intrinsic apoptotic mechanisms are both operative. Constitutive expression of Fas is found in mouse and human liver, and this pathway appears to be very important in executing apoptosis in healthy hepatocytes and in the pathogenesis of diseases including liver injury, viral hepatitis and cirrhosis [20]. However, HCC is one of the tumours known to be resistant to Fas-mediated apoptosis, because Fas expression is down regulated and the Fas-activated signalling pathway is altered [21].

We have used two approaches to express COX-2 in liver cells in order to elucidate the mechanisms implicated in PGE₂-dependent inhibition of apoptosis. In the first, we generated liver cell lines expressing COX-2 protein by stable transfection with a vector containing the human COX-2 cDNA. In the second, we used hydrodynamics-based transient transfection to systemically administer

mice with a pcDNA3hCOX-2-GFP plasmid. Our results show that both *in vitro* and *in vivo* expression of COX-2 directly inhibits apoptosis in hepatocytes through mechanisms that involve potent inhibition of caspases 3 and 9, a decrease in p53 and Bax expression, and an increase in the survival pathway through activation of Akt.

MATERIAL AND METHODS

Chemicals and reagents

Antibodies were from Santa Cruz Laboratories (Santa Cruz, CA), BD Biosciences (San José, CA, USA), R & D Systems Inc. (Minneapolis, MN, USA), Cayman Chemical (Ann Arbor, MI, USA), Alexis (Lausen, Switzerland) and Cell Signaling Technology (Beverly, MA). 5,5-Dimethyl-3(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU) was from Merck (Rahway, NJ). Prostaglandins were from Calbiochem EMD Biosciences Inc. (San Diego, CA). Fluorescent probes were from Molecular Probes (Invitrogen, Carlsbad, CA) and Calbiochem. Tissue culture media were from BioWhittaker (Walkersville, MD). Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA), and other reagents were from Roche Diagnostics (Mannheim, Germany) or Sigma Chemical Co. (St. Louis, MO).

Cell culture and stable transfection

The human liver cell line CCL-13 (Chang liver, CHL), an immortalized non-tumour cell line derived from normal liver, and the hepatoma cell line HepG2 were purchased from the American Type Culture Collection (Manassas, VA). The ATCC catalogue states that CCL-13 (Chang liver, CHL) cell line was derived from normal liver tissue but has HeLa markers. To evaluate the hepatocyte nature of the clones, we analysed the expression of the hepatocyte markers α -fetoprotein and albumin, which were highly expressed (data not shown) as previously described [22]. Cells were grown on Falcon tissue culture dishes (Lincoln Park, NJ) in DMEM supplemented with 10% foetal bovine serum (FBS) and antibiotics (50 μ g each of penicillin, streptomycin and gentamicin per ml) at 37°C in a humidified air/5% CO₂ atmosphere. For plasmid transfections, attached CHL and HepG2 cells at 50% confluence were exposed for 24 h to Fugene 6® reagent containing COX-2 expression vector (full-length human COX-2 cDNA cloned into pcDNA3) or control vector (pcDNA3). At the end of this period transfection media were replaced with fresh medium containing 10% FBS, and on the second day after transfection the cells were plated at 25% confluence in medium supplemented with 500 μ g/ml G418 sulphate. Single colonies of resistant cells were detectable after approximately 12 days and were selected and subcultured for 30 days. Subsequent cultures of selected cells were grown in the presence of G418. Cells stably expressing pcDNA3hCOX-2 and empty pcDNA3 vector were termed CHL-C and CHL-V, respectively. Eighteen hours before experiments, the culture medium was replaced with fresh medium containing 1% FBS.

Characterization of COX-2 vectors

pcDNA3 and pEGFP-N1 were obtained from Invitrogen and BD Biosciences (Clontech), respectively. Human full-length COX-2 cDNA [23] in pcDNA1/Amp was a kind gift of S. Prescott's lab (Huntsman Cancer Institute, Utah). The COX-2 cDNA was subsequently cloned into the pcDNA3 expression vector. COX-2-GFP was obtained by inserting 720 bp of the GFP open reading frame (excluding the initiator methionine and the termination codon) from pEGFP-N1 vector into the BspEI site of the human COX-2 cDNA. This inserts the GFP at the C-terminal end of COX-2 polypeptide, as described previously [24]. The orientation and integrity of the constructs were determined by sequencing. Large-scale preparation of plasmids was done using EndoFree plasmid mega kit (Qiagen).

Cell cycle distribution and cell counting

Cell counts were performed by initially plating 1.6×10^5 cells. At the indicated times, cells were trypsinised, stained with trypan blue and the cells that excluded trypan blue were counted under the microscope. For the analysis of cell cycle distribution, cells were resuspended in PBS, and fixed in 70% ethanol. Propidium iodide (PI) staining was performed after incubation of the cells with 0.05% PI and 0.1 mg/ml RNAase for 30 min and analysed in a CyAn MLE-R cytometer (DakoCytomation).

Immunocytochemistry

Cells (5×10^4 per well) were cultured in 24-multiwell plates on glass coverslips and maintained overnight in DMEM/1% FBS. Cells were then fixed for 15 min with 4% paraformaldehyde pH 7.0, washed with PBS and permeabilised with methanol for 10 min at -20°C . After blocking with 3% bovine serum albumin (BSA) for 1 h at room temperature (RT) the cells were incubated for 2 h at RT with the corresponding monoclonal anti-COX-2 antibodies diluted 1:150 in PBS/1% BSA. After three washes with PBS, the coverslips were incubated with a 1:500 dilution of fluorescent secondary antibody (IgG-Cy3, DakoCytomation SA, Barcelona, Spain), washed x3 with PBS, and treated with Hoechst 33258 for 30 min at RT. Fluorescence was visualized and quantified with a MRC 1024 microscope (Bio-Rad) and Lasersharp software. For tissue immunohistochemistry, liver samples were snap frozen in isopentane immersed in liquid N_2 , and serial 10 μm -thick sections were cut onto gelatinised glass with a Microm HM550 sledge microtome. After fixing and blocking, the sections were incubated overnight with GFP antiserum (diluted 1:50; Molecular Probes) in 5% horse serum at 4°C . After three washes with PBS, the sections were incubated with a 1:500 dilution of fluorescent secondary antibody (IgG-Alexa Fluor 488), washed, and analysed as above. For the detection and quantification of apoptosis, the TUNEL commercial kit for cell death detection (Roche) was used in accordance with the manufacturer's

instructions. Histological examination was performed on 4% paraformaldehyde-fixed mouse liver sections prepared by routine histological staining (hematoxylin/eosin) procedures.

Caspase assays

Cell extracts were prepared by lysing in 10 mM HEPES pH 7.9; 1 mM EGTA, 1 mM EDTA, 120 mM NaCl, 1 mM DTT, 0.5 mM PMSF, 2 µg/ml aprotinin, 10 µg/ml leupeptin, 2 µg/ml TLCK, 5 mM NaF, 1 mM NaVO₄, 10 mM Na₂MoO₄ and 0.5 % Nonidet P-40 (buffer A). After centrifugation of the cell lysate the supernatant was stored at -80°C (cytosolic extract) and protein content was assayed with the Bio-Rad protein reagent. The activities of caspases 3, 8 and 9 in cytosolic extracts were determined with the fluorogenic substrates N-acetyl-DEVD-7-amino-4-trifluoromethylcoumarin, N-acetyl-IETD-7-amino-4-trifluoromethylcoumarin, and N-acetyl-LEHD-7-amino-4-trifluoromethylcoumarin, respectively, and in accordance with the supplier's instructions (Calbiochem). The linearity of caspase assays was determined over a 30 min reaction period [25].

Cell extracts and Western blotting

Cells ($2-3 \times 10^6$) or tissue samples (100mg) were homogenized in a lysis buffer containing 10 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, 0.5% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 1 mM β-mercaptoethanol, and 0.1 mM PMSF. Extracts were vortexed for 30 min at 4°C and after centrifuging for 20 min at 13,000g the supernatants were stored at -20°C (cytosolic extracts). To obtain nuclear extracts the pellets were processed in the same volume with 20% glycerol and 0.4M KCl and mixed for 30 min at 4°C as previously described [8]. To determine the release of cytochrome c from the mitochondria to the cytosol, cell extracts were obtained by controlled lysis of the plasma membrane as previously described [25]. For Western blot analysis, whole-cell extracts were boiled for 5 minutes in Laemmli sample buffer, and equal amounts of protein (20-30 µg) were separated by 10-12% SDS-PAGE. The relative amounts of each protein were determined in total, cytosolic or nuclear cellular extracts as appropriate with the following polyclonal or monoclonal antibodies: COX-2 and COX-1 (Cayman), inhibitors of apoptosis (IAPs) (R & D Systems), Bcl-2 family proteins (Santa Cruz), cytochrome c and FAS (BD Pharmingen), p53 (Santa Cruz), PARP-1 (Alexis), and Akt/P-Akt (S473) (Cell Signaling) After incubation with the corresponding anti-rabbit or anti-mouse horseradish peroxidase conjugated secondary antibody, blots were developed by the ECL protocol (Amersham). Target protein band densities were normalized by calculating the ratio to the corresponding densities of β-actin (whole-

cell/cytosolic extracts) or Sp1 (nuclear extracts). Different exposure times were performed on each blot to ensure linearity of the band intensities. Densitometric analysis was expressed in arbitrary units.

Determination of metabolites

PGE₂ levels were determined in culture media by specific immunoassay (Amersham). Alanine aminotransferase (ALT) was assayed spectrophotometrically in plasma [26]. Protein levels were determined with the Bradford reagent.

RNA isolation and RT-PCR

Total RNA was extracted from liver with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. One µg total RNA was reverse transcribed with 50U of expand reverse transcriptase and pd(N)₆ Random Hexamer as primer (Amersham Biosciences). The resulting cDNAs were amplified with the following oligonucleotide sequences: COX-2, 5'CAGAGTTGGAAGCACTCTATGG3' (sense) and 5'CTGTTTTAATGAGCTCTGGATC3' (antisense); 18s rRNA, 5'GCAATTATCCCCATGAACGA3' (sense) and 5'CAAAGGGCAGGGACTTAATCAA3' (antisense).

Hydrodynamic transient transfection experiments

100 µg of plasmid dissolved in 2 ml isotonic NaCl were injected into the tail veins of 18-22 g adult male Swiss CD1 mice (Charles River) over 8 s (hydrodynamic injection) [27]. Five animals were used for each condition. 24 h after injection, animals were intraperitoneally injected with a single dose of purified hamster anti-mouse FAS monoclonal antibody Jo2 (0.3 µg/g body wt) freshly dissolved in 0.9% NaCl. This dose was chosen on the basis of previous studies [28]. Animals were killed five hours later, and livers were rapidly removed and freeze-clamped in liquid N₂. Animals were treated in accordance with Institutional Care Instructions.

Data analysis

Data are expressed as means ± standard deviation (SD). Statistical significance of differences between the CHL-C and CHL-V groups was evaluated by the U of Mann-Whitney test. All tests have been calculated two-tail, and the significance has been considered at $P < 0.05$.

RESULTS

Characterization of CHL liver cells expressing COX-2

The human Chang liver cell line (CHL) was transfected with the COX-2 expression vector pcDNA3hCOX-2 or the control vector pcDNA3, and selected with 500 µg/ml G418. Among 20 clones analysed, we selected two clones: a control clone CHL-V, transfected with the empty vector; and a COX-2 clone, CHL-C, transfected with pcDNA3hCOX-2 and selected on the basis of its high COX-2 expression. These clones were used for all subsequent experiments. As shown in Fig. 1A, COX-2 protein was significantly expressed in CHL-C cells, as determined by Western blot, but was not expressed in WT or CHL-V cells. COX-1 protein was expressed at low levels and without significant differences in CHL and in the transfected cells. Immunocytochemistry revealed clear staining for COX-2 in the endoplasmic reticulum and perinuclear membranes (Fig. 1B). RT-PCR of COX-2 mRNA confirmed increased COX-2 mRNA expression in CHL-C cells (Fig. 1C). COX-2 expression in liver cells was functional; Fig. 1D shows that CHL-C cells released significantly greater quantities of PGE₂ into the culture medium than did CHL-V (3.7-fold increase). This PGE₂ synthesis was markedly increased after incubation of the cells for 30 min with 30 µM arachidonic acid (AA). The contribution of COX-2 to this process was confirmed by inhibition (>90%) of AA-inducible PGE₂ synthesis after treatment with the COX-2 selective inhibitor DFU (5 µM). When the cells were incubated in the presence of indomethacin (50 µM), PGE₂ levels returned to the control (or even lower) values. These data indicate that COX-2 is the major contributor to PGE₂ synthesis, whereas the contribution of COX-1 could be evaluated in less than 5% of the PGE₂ levels in CHL-C cells. Fig. 1E shows the clear morphological differences between CHL-V and CHL-C cells, with CHL-C being larger and exhibiting a less rounded shape than the CHL-V control cells.

COX-2 expression in liver cells promotes an increase in S-phase cells and inhibits apoptosis

The cell number of CHL-C after 72 h of culture in 10% FBS was greater (129%) than that of CHL-V (Fig. 2A). To assess the role of COX-2 in cell proliferation we analysed the dependence of CHL-V and CHL-C cell number on FBS concentration. As Fig. 2B shows, COX-2-dependent proliferation was evident in the absence and presence of serum (229%, 129%). Analysis of cell cycle distribution by propidium iodide (PI) staining and FACS revealed that COX-2-expression is associated with an increase in the number of cells in S-phase both at 0 and 10% FBS (133%, Fig. 2C), with no

significant changes in the G₀/G₁ and G₂-M populations observed, when considering nonapoptotic cells (Fig. 2D). Figure 2E shows the distribution of apoptotic and nonapoptotic cells in the different steps of the cell cycle.

Flow cytometry of PI-stained cells revealed an inhibition of apoptosis in CHL-C (COX-2-expressing) cells cultured in the absence of FBS (Fig. 3A-C). After 72 h of culture only 4% of CHL-C cells were apoptotic, compared with 11% of CHL-V cells (Fig. 3B). In order to correlate the number of PI positive cells induced after FBS removal with COX-2 expression, the levels of PGE₂ were measured at various times, and as shown in Fig. 3B (inset), a correlation exists between an increase in PGE₂ synthesis and a decrease in the number of PI positive cells. The inhibition of apoptosis could be mimicked in CHL-V control cells by treating with exogenous PGE₂ (Fig. 3D), and the action of COX-2 metabolites in the reduced apoptosis in CHL-C cells was confirmed by treatment with increasing concentrations of DFU, which progressively increased the numbers of apoptotic cells (Fig. 3E). COX-2 expressing CHL were also resistant to the induction of apoptosis by 10 nM staurosporine, camptothecin or a combination of TNF- α and actinomycin D, with 30% fewer cells in apoptosis compared to CHL-V controls (data not shown). We also overexpressed COX-2 in HepG2, as a different cell line with a basal constitutive COX-2 expression (Fig. 3F) obtaining the same results than in CHL cells. COX-2 overexpression, but not the constitutive level, caused a decrease in the number of apoptotic cells after serum deprivation (Fig. 3G).

COX-2 expression leads to altered expression and activity of apoptosis markers

The data presented so far provide convincing evidence that PGE₂ synthesized by COX-2 suppresses apoptotic signals in CHL-C liver cells. To investigate the mechanisms through which this occurs we first analysed the activities of caspases 3, 8 and 9 by fluorimetry (measured as DEVDase, IETDase and LEHDase activities, respectively). As shown in Fig. 4, the activities of caspases 3 and 9 were markedly lower in CHL-C cells than in control CHL-V cells. Caspase 8 activity was also inhibited in CHL-C cells, but to a lesser extent and with a delay with respect to caspases 3 and 9. These results are in agreement with data obtained in gastric mucosal cells [29], where COX-2 expression also suppresses the activation of caspases 8 and 9 in addition to caspase 3.

Figure 5 shows the effect of COX-2 expression on the levels of several proteins governing apoptosis fate. As Fig. 5A,B C shows, p53 expression declined notably in CHL-C cells at 0 time and within 6 h of culture in medium without FBS, whereas it was maintained in CHL-V controls. Bax expression, scarcely detected in both cell lines at 10% serum, was increased in CHL-V cells from 6 h

without FBS, but was not observed even after 72 h in CHL-C cells (Fig. 5D). The protein levels of Bcl-2, XIAP and Mcl-1 did not vary between CHL-V or CHL-C cells; however, Bcl-xL decreased in CHL-V cells from 6 to 48 h of culture in medium without FBS, whereas it was maintained in CHL-C cells (Fig. 5). Release of cytochrome c to the cytosol with the corresponding decrease in the mitochondria and cleavage of poly (ADP-ribose) polymerase (PARP-1) were also observed only in CHL-V cells (Fig. 6A,B).

The PI3K/Akt pathway plays a central role in integrating diverse survival signals. The levels of phospho-Akt (Ser⁴⁷³) increased in cells expressing COX-2. Moreover, when the CHL-C cells were incubated with DFU to inhibit COX-2 the increase in P-Akt was not observed (Fig. 6E), suggesting a role for COX-2 in liver cell survival. The same mechanism occurs in HepG2-COX-2 expressing cells (not shown).

These results indicate that the anti-apoptotic effect induced by COX-2/PGE₂ is mediated by the inhibition of the mitochondrial intrinsic pathway. But the inhibition of caspase 8 (Fig. 4C) suggested the possible involvement of death receptor signalling, known to be important in hepatocyte apoptosis and in the pathogenesis of liver diseases. As shown in Fig. 5 A the levels of Fas protein were scarcely detected in either cell line. The above mentioned failure of FAS antibody to induce apoptosis (data not shown) indicates that CHL-V and CHL-C cells are resistant to FAS-mediated apoptosis, as occurs in many liver cell lines and in HCC [21].

Transient expression of COX-2 inhibits liver apoptosis in vivo.

To examine whether COX-2 expression inhibits liver-cell apoptosis in living animals, we developed an *in vivo* model to deliver the COX-2 gene to the liver via hydrodynamics-based transfection. COX-2 was expressed in approximately 35% of the liver cells of animals transfected with a construct encoding GFP-COX-2, as indicated by the associated GFP fluorescence (Fig. 7A). Under these conditions, the levels of ALT determined 24 h after transfection were similar between animals transfected with GFP or with GFP-COX-2, and were in the range of the animals receiving saline (Fig. 7B). The transfected COX-2 was active, since intrahepatic levels of PGE₂ increased in the GFP-COX-2 mice but not in the GFP animals (Fig. 7C). As Fig. 7D-F show, transfection of liver with GFP-COX-2 impaired Fas-dependent apoptosis as indicated by the inhibition of caspase-3 activity and reduced staining in TUNEL analysis. Moreover, the analysis of apoptosis markers in the liver of GFP-COX-2 mice demonstrates an increase in the levels of P-Akt and in the Bcl-2/Bax ratio (Fig. 7G).

DISCUSSION

The important role of COX-2 as inhibitor of apoptotic pathways has been demonstrated *in vivo* in transgenic mouse models of COX-2 expression in several tissues. Transgenic mice expressing COX-2 in mammary gland developed tumours, and these animals had reduced levels of the proapoptotic proteins Bax and Bcl-X_L and elevated levels of Bcl-2 [30]. In basal keratinocytes, the results are conflicting, since both epidermal hyperplasia and suppression of tumour development have been described [31;32]. Transgenic mice expressing COX-2 in neurons develop age-dependent cognitive deficiencies due to an increase in neuronal apoptosis [33]. A recent study demonstrated hyperplastic gastric tumours in transgenic mice expressing COX-2 and microsomal prostaglandin E synthase [34].

Although a number of experimental studies have demonstrated a clear positive correlation *in vitro* between COX-2 expression and the inhibition of apoptosis, the underlying molecular mechanisms are still not fully understood. Most of this work has been done in colon or gastric carcinoma cell lines, where DuBois et al. [35] demonstrated that rat intestinal epithelial cells (RIE) stably transfected with COX-2 were resistant to butyrate-induced apoptosis. The same authors reported that these cells exhibit a delayed G₁ transit, and this may be related to the resistance to apoptosis. Our results demonstrate that COX-2 expression in liver cells is not associated to significant changes in cell-cycle distribution but is related to the inhibition of cellular apoptosis. Cell cycle arrest in G₀/G₁ has been described in COX-2-expressing endothelial, NIH 3T3, COS-7 and embryonic kidney 293 cells [24], but this effect did not require the synthesis of prostaglandins. We did not find significant changes in G₀/G₁ or G₂/M cell populations, but the inhibition of apoptosis in COX-2 expressing liver cells was dependent on PGE₂ synthesis.

COX-2 inhibitors provide an indirect means of establishing a relationship between COX-2-dependent PGs and apoptosis. However, the doses which induce apoptosis are much higher than those needed to inhibit COX-2 activity, indicating that these inhibitors may be acting via COX-2-independent effects, depending on the compound used and the cell type. Some NSAIDs, such as sulindac, potently induce apoptosis in human gastric cancer cells [36], while others such as SC-58125 induce both apoptosis and cell cycle arrest in HCA-7 cells [37]. A prostate apoptosis response gene (Par-4) was found to be up-regulated following exposure of HCA-7 cells to NS-398 [38]. Sulindac, salicylate and aspirin have been shown to inhibit NF-κB signalling by directly blocking the activity of IκB kinase, leading to reconstituted sensitivity of cancer cells to apoptosis [39]. Additionally, indomethacin, a non-

selective COX inhibitor, can act as a direct ligand of PPAR γ , a suppressor of tumor cell proliferation [40].

In liver, increased COX-2 expression has recently been demonstrated in patients with HCC, especially in tissue with cirrhosis as well as in well-differentiated tumour tissue [41]. In HCC, COX-2 expression seems to be up regulated at early stages and down regulated in advanced stages. Silencing of the COX-2 gene by promoter hypermethylation has been reported to regulate the growth of some HCC cell lines [42]. However, *in vitro* studies revealed that NS-398, celecoxib and sulindac effectively inhibited growth of human hepatoma cell lines [11;12]. In mice implanted with hepatoma, nimesulide inhibits tumour growth by inducing apoptosis and overexpression of Bax over Bcl-2 [43]. Moreover, combinations of COX-2 and MEK inhibitors synergistically increase apoptosis in human HCC [44].

Several signalling pathways have been proposed as mediators of COX-2-dependent inhibition of apoptosis. PGE $_2$ inhibits apoptosis in gastric mucosa cells via the mitochondrial pathway and PKA activation [29]. In cholangiocarcinoma cells, celecoxib suppressed Akt phosphorylation, released cytochrome c to the cytosol and activated caspases -9 and -3 [45]. There are a few reports, which describe the direct effect of COX-2 expression on apoptosis in liver or HCC cells. Leng et al. [46] reported an enhanced phosphorylation of Akt in Hep 3B cells transiently and permanently transfected with a COX-2 expression vector but Bcl-2 is not induced. Celecoxib reduced Akt activation and induced caspase-9 and -3 activation with a concomitant release of cytochrome c. Our results clearly demonstrate that caspase-3 and -9 activities are inhibited in COX-2 expressing cells and the activity of caspase -8 is also decreased in CHL-C cells. The absence of cytochrome c in the cytosol of CHL-C cells after exposure to apoptotic stimulus clearly implies that COX-2 PG products act on the mitochondrial pathway to prevent apoptosis. We did not find significant changes in the expression of IAP proteins. This is in agreement with results obtained in cholangiocarcinoma cells, although cAMP-dependent induction of c-IAP2 has been reported to mediate the anti-apoptotic action of PGE $_2$ synthesized by COX-2 in intestinal epithelial cells [47]. The levels of Bcl-2 remained unaffected by COX-2 expression in our experiments, in agreement with previous findings in liver cells [12].

COX-2 has been reported to promote survival of human lung adenocarcinoma cells by up-regulating the level of the anti-apoptotic protein Mcl-1 and activating the PI3K/Akt-dependent pathway [43;48]. Our results demonstrate that PGE $_2$ produced by COX-2 does not change the levels of Mcl-1 but induces a significant increase in Akt phosphorylation, indicating a role for Akt in COX-2-dependent survival of HCC cells. p53 activation plays an important role in the induction of apoptosis by various

agents. Once activated, p53 negatively regulates cell cycle progression and induces apoptosis by regulating the proapoptotic gene Bax and several death promoting molecules in many cell lines [49]. The regulation of p53 is complex and implicated different mechanisms such as stability, nuclear accumulation and activity. COX-2 expression negatively affect p53 activity via mechanism that could involve physical interaction and COX-2 positive prostate cancer cells exhibited a decrease p53 stability, nuclear accumulation and activity after hypoxia, both via direct effects on p53 activation and via activation of Mdm2, the primary negative regulator of p53 [50]. In this sense, our results showed an important decrease in p53 levels in CHL-C-COX-2 expressing cells.

The results obtained *in vivo* from mice hydrodynamically transfected with COX-2 clearly demonstrated that PGs produced by COX-2 expression protected the liver against Fas-mediated apoptosis in agreement with the results obtained from the *in vitro* liver cell lines. Interestingly, the levels of Bcl-2 that are very low in liver, were up-regulated significantly after hydrodynamic transfection of COX-2.

In summary, the data presented in this study indicate that PGs produced by COX-2 in liver, both *in vitro* and *in vivo*, inhibit apoptosis dependent on the intrinsic mitochondrial pathway. Given the frequent expression of COX-2 in HCC, COX-2 would be expected to provide these cancers with a survival advantage. This is an important finding and may explain both the anti-apoptotic-hepatoprotective effect of PGE₂ after acute liver injury and the important contribution of COX-2 to the development of tumorigenesis through increased resistance to apoptosis in HCC.

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FIGURE LEGENDS

Fig. 1. COX-2 expression in CHL liver cells increases PGE₂ production.

Chang liver cells (CHL) were stably transfected with empty pcDNA3 vector (CHL-V) or pcDNA3hCOX-2, encoding human COX-2 (CHL-C). (A) COX-2 and COX-1 protein expression in whole-cell extracts detected by Western blot. COX-2 and COX-1 expression were normalized by calculating the ratios of COX-2 and β -actin band densities, expressed in arbitrary units (a.u.). A representative experiment is shown beneath the chart. (B) Immunocytochemical analysis of COX-2 by confocal microscopy. COX-2 staining is in red and nuclear staining with Hoechst 33258 in blue. The chart shows the relative fluorescence intensities. (C) RT-PCR analysis of COX-2 mRNA expression. COX-2 mRNA amounts were calculated as arbitrary units normalized to the expression of 18S rRNA, to control for the quantity and integrity of total RNA. (D) PGE₂ production by COX-2 expressing CHL. PGE₂ concentrations were determined by ELISA in the culture media of untreated cells or cells treated at 37°C for 30 min with 30 μ M AA \pm 5 μ M of the COX-2 inhibitor DFU and \pm 50 μ M of the non-selective COX inhibitor indomethacin (E) Light microscopy showing morphological changes in CHL cells expressing COX-2. All data presented are the means \pm SD of five independent experiments. # P < 0.05 vs. the CHL control cells. * P < 0.05 vs. the CHL-V cells. \blacklozenge P < 0.05 vs. paired AA-treated cells without DFU or indomethacin treatment.

Fig. 2. Effect of COX-2 expression on cellular growth and cell cycle distribution.

(A) Time-dependent proliferation of CHL-V and CHL-C. Cells were seeded at 1.6×10^5 on 6-cm plates in medium containing 10% FBS, and after the indicated times in culture cells were trypsinized, stained with trypan blue and counted (trypan blue negative cells). (B) Effect of FBS concentration on cell growth at 72 h. Cells were seeded as before, but in media containing differing concentrations of FBS as indicated. (C) Flow cytometry analysis of the proportion of CHL-V and CHL-C populations in S phase at 0% and 10% FBS (72 h). (D) Cell cycle analysis by FACS of CHL-V and CHL-C cells at 72 h. (E) The same experiment as in D but considering also apoptotic cells. Cells were resuspended in PBS, fixed with 70% ethanol, stained with PI and analyzed in a CyAn MLE-R cytometer. All data presented are the means \pm SD of four independent experiments. * P < 0.05 vs. the CHL-V control cells.

Fig. 3. COX-2 expression inhibits propidium iodine staining in liver cells.

Cells resuspended in PBS were PI stained, and cell cycle analysis was determined by flow cytometry. (A) Effect of FBS concentration on the percentage of apoptotic CHL-V and CHL-C cells after 72 h of culture. (B) Time-dependent apoptosis and PGE₂ concentrations in CHL-V and CHL-C cells cultured at 0% FBS. (C) Representative DNA histograms and G1 profiles showing the PI staining from CHL-V and CHL-C cells cultured at 0 and 10% FBS for 72 h. (D) Effect of exogenous PGE₂ on the percentage of apoptotic cells. (E) Effect of DFU on the percentage of apoptotic CHL-C cells (0% FBS, 72 h of culture). (F) Western blot analysis of COX-2 expression in HepG2-V and -C. (G) Apoptosis of HepG2-V and HepG2-C cells at 0% FBS after 72 h of culture. All data presented are the mean \pm SD of four independent experiments. * $P < 0.05$ vs. paired CHL-V control cells. $\blacklozenge P < 0.05$ vs. cells at time = 0 (B) or untreated cells (D and E).

Fig. 4. Expression of COX-2 suppresses caspase-like activities.

Activities of caspase 3 (A), 9 (B) and 8 (C), measured as DEVDase, IETDase and LEHDase activities, respectively, in CHL-V control and CHL-C COX-2-expressing cells cultured without FBS. Caspase activities were measured by fluorimetric assay with specific fluorogenic substrates (see Materials and Methods). One unit of protease activity was defined as the amount of enzyme required to release 1 pmol of AMC/min. All data presented are the means \pm SD of five independent experiments. * $P < 0.05$ vs. the corresponding CHL-V control cells.

Fig. 5. Altered expression of apoptosis indicators in COX-2-expressing liver cells.

Representative Western blots showing the expression of p53, Bax, Bcl.2, xIAP, cIAP-1 and Fas in CHL-V control cells and COX-2-expressing CHL-C cultured without FBS (A,B). Densitometric analysis of the expression of p53 and Bax (C,D). The expression of target proteins was normalized to that of β -actin (whole-cell and cytosolic extracts), and the ratios are presented in arbitrary units. The data presented are the means \pm SD of five independent experiments. * $P < 0.05$ vs. the corresponding CHL-V control cells.

Fig. 6. Cytochrome c, PARP-1 and P-Akt changes in CHL-V and CHL-C cells.

The presence of cytochrome c in the cytosolic and mitochondrial fractions after controlled lysis of the cells, and the levels of PARP-1 were analyzed by Western blot (A-D). Blots were normalized with β -actin (whole-cell and cytosolic extracts) or Sp-1 (nuclear extracts). Total and P-Akt levels were determined in the same total cell extract in the presence or absence of 5 μ M DFU (E). Results show representative Westerns (n=3) and data are the means \pm SD of five independent experiments. * P < 0.05 vs. the corresponding CHL-V control cells.

Fig. 7. Hydrodynamic transfection of mice with GFP-COX-2 inhibits Fas-induced liver apoptosis.

Animals were transfected with 100 μ g of plasmid encoding GFP or GFP-COX-2. Control animals were treated with saline. (A) GFP fluorescence in liver sections taken 24 h after transfection to verify the expression of GFP or GFP-COX-2 fusion protein. (B) (ALT) serum activity determined as an index of transfection-induced liver injury after 24 h. (C) Intrahepatic PGE₂ concentrations were determined 24 h after transfection. (D) Caspase-3 activity (DEVDase) in liver extracts taken 5 h after intraperitoneal injection with the Fas-activating antibody Jo2 (0.3 μ g/g body weight). (E) Apoptosis in liver sections taken 5 h after injection with Jo2. The fluorograms show representative results of TUNEL analysis (red fluorescence) and nuclear staining with Hoechst 33258 (blue). The bar chart shows the percentage of TUNEL positive cells (F). Protein levels of P-Akt and Bcl-2/Bax ratio in liver extracts from saline, GFP and GFP-COX-2 mice (G). All data presented are the means + SD of eight animals per condition. * P < 0.05 vs. GFP-transfected animals.

Figure 1

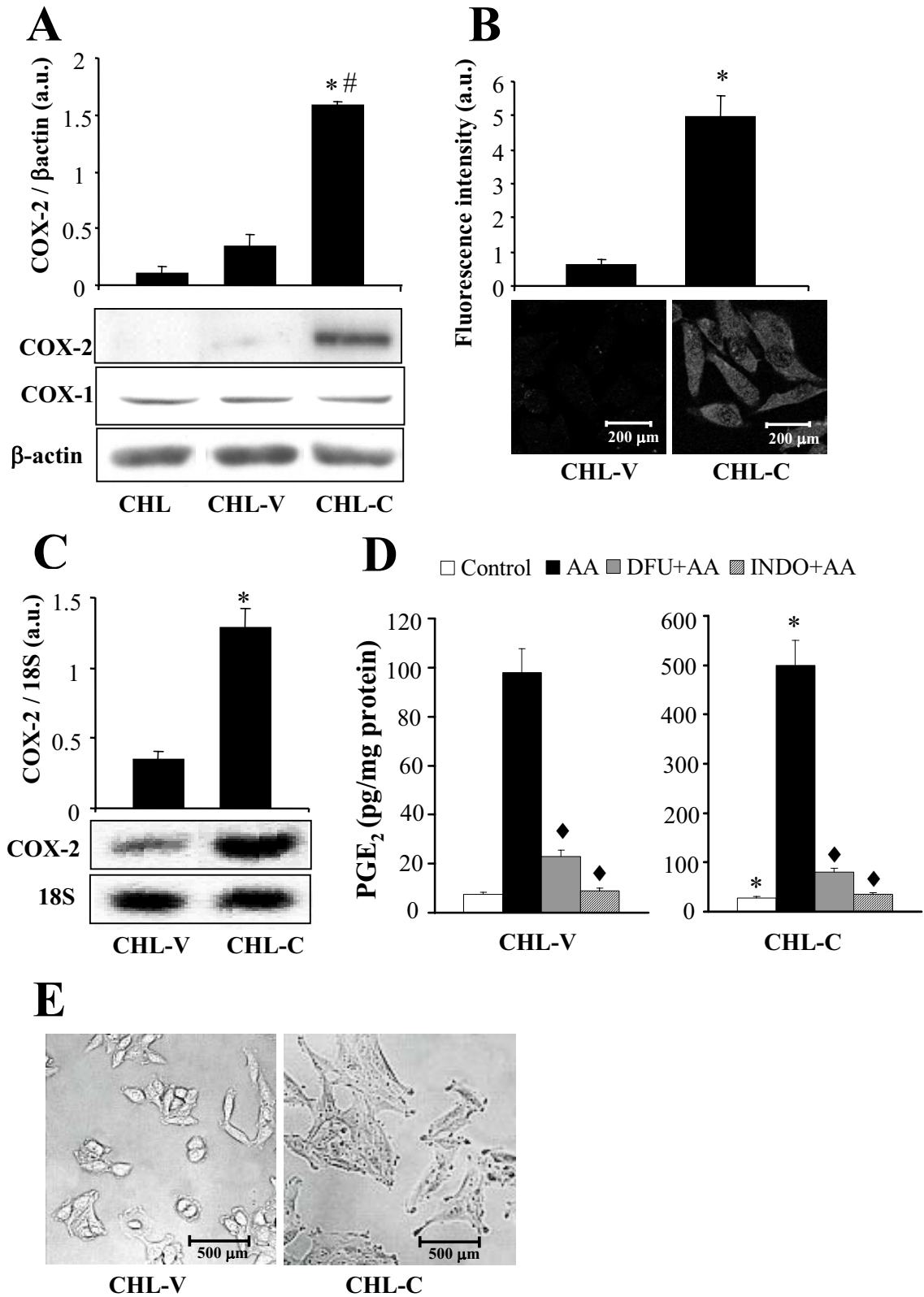


Figure 2

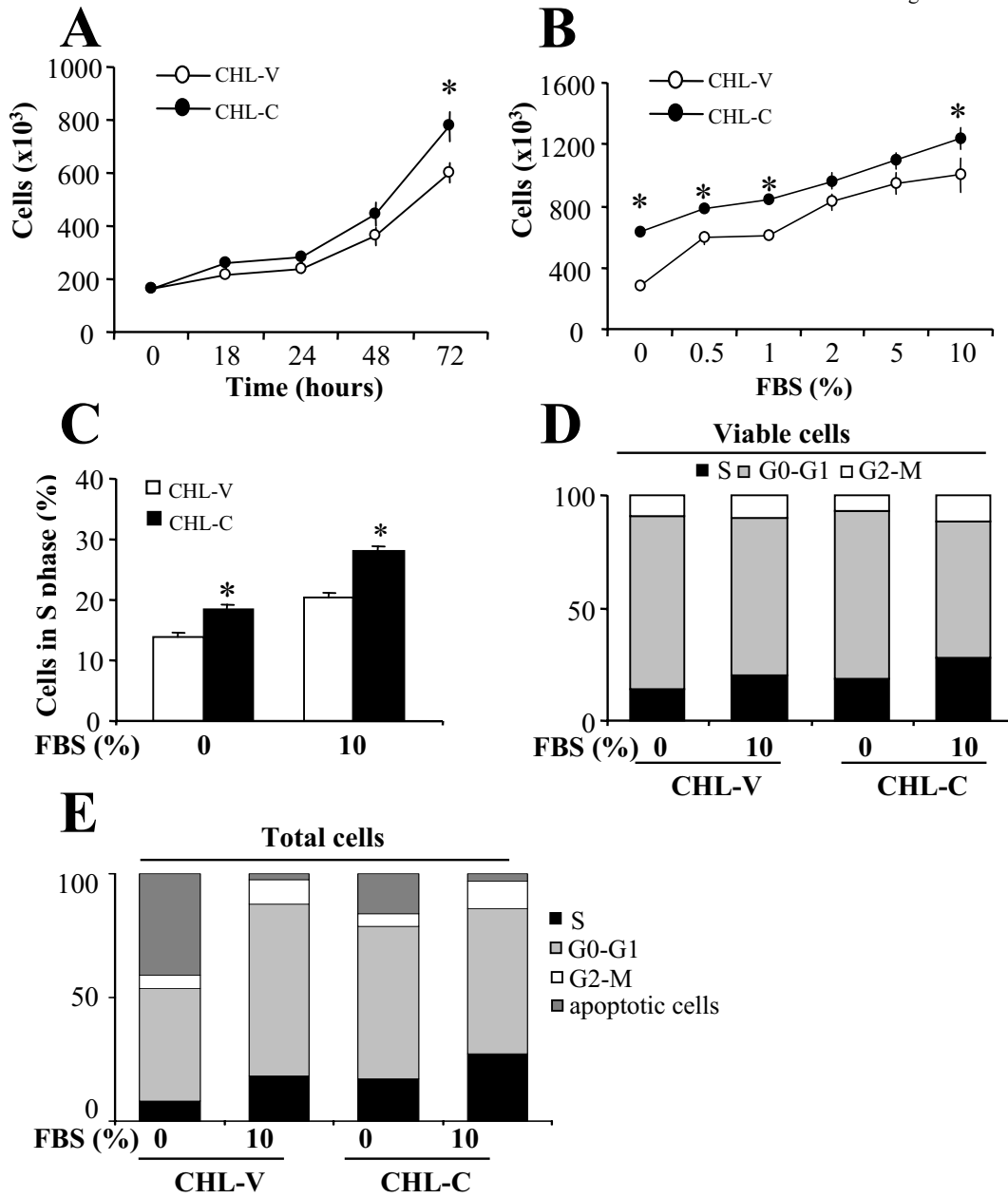


Figure 3

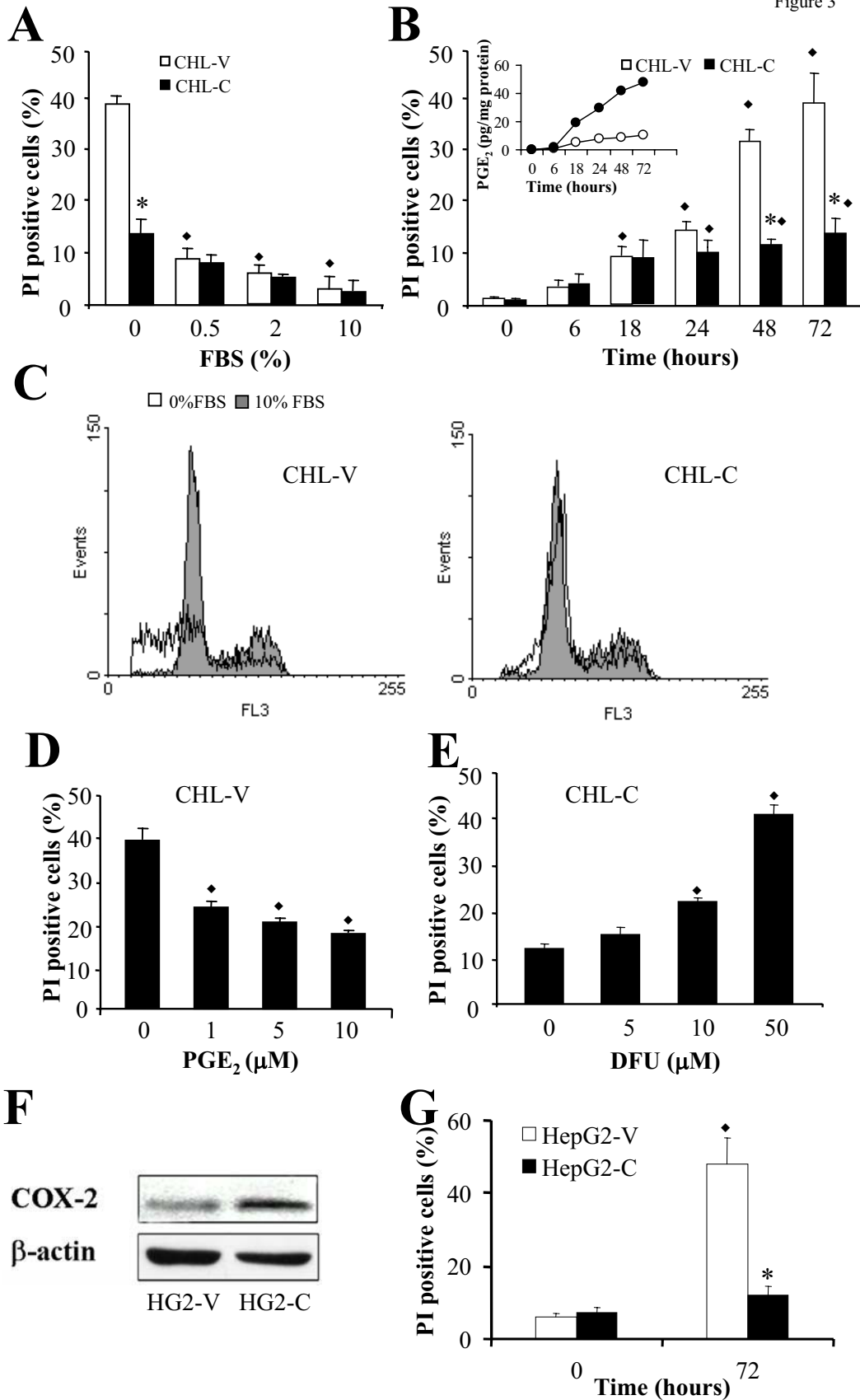


Figure 4

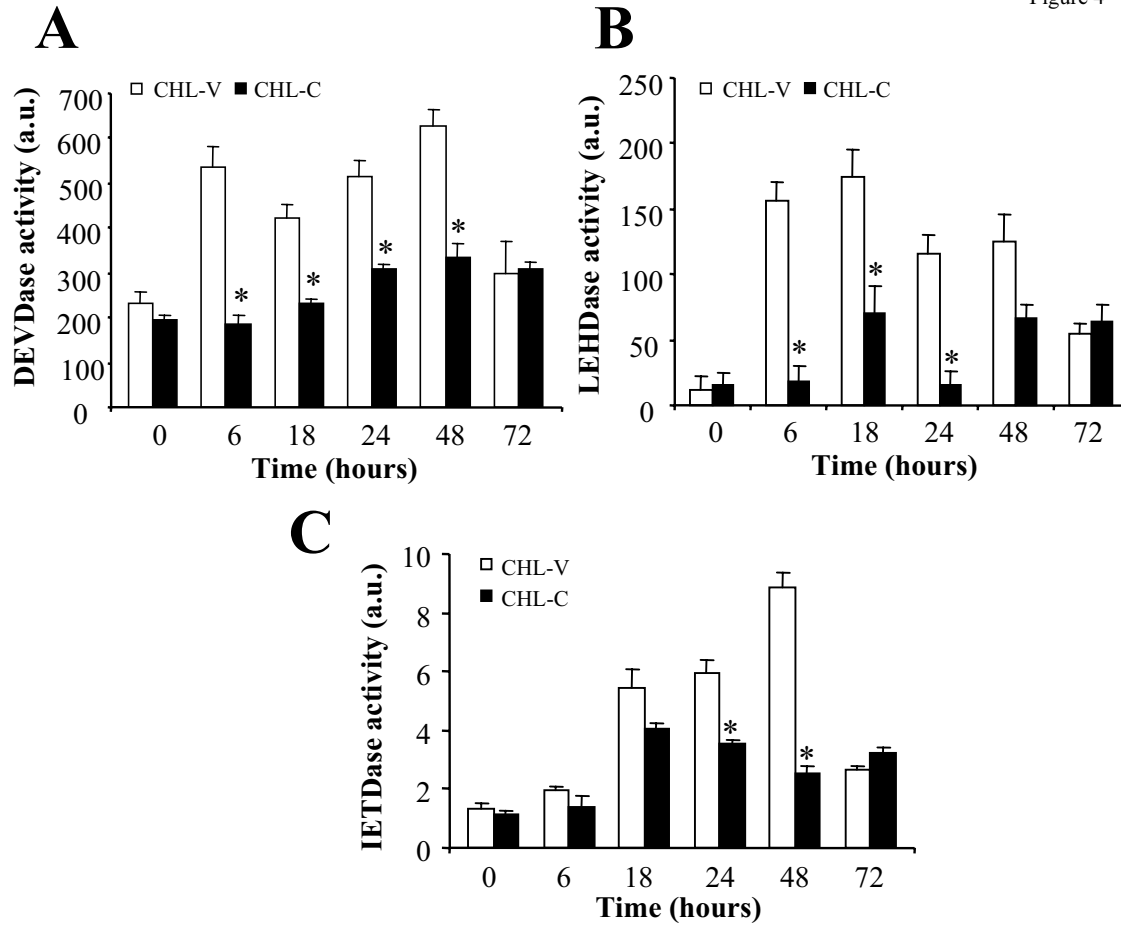


Figure 5

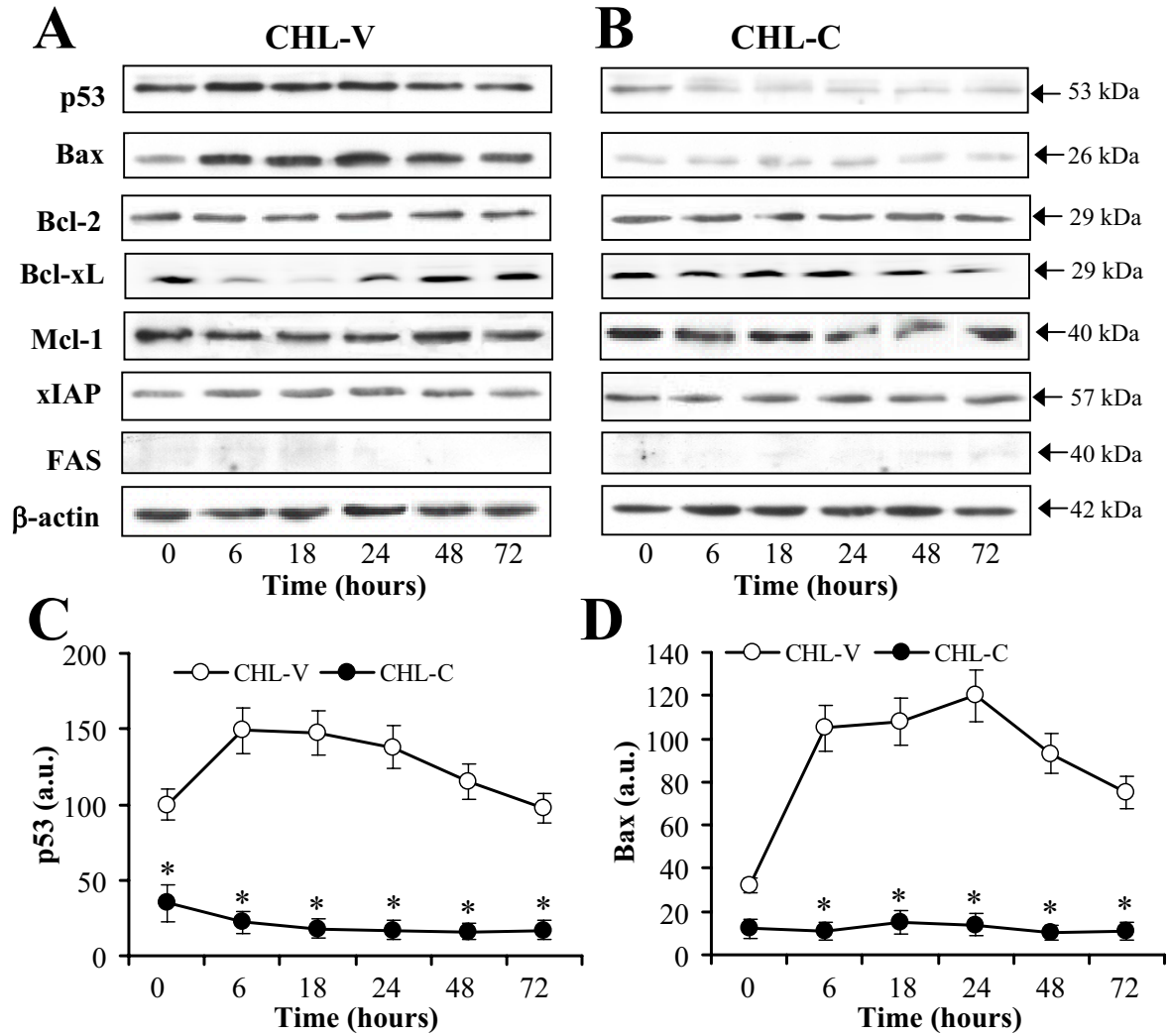


Figure 6

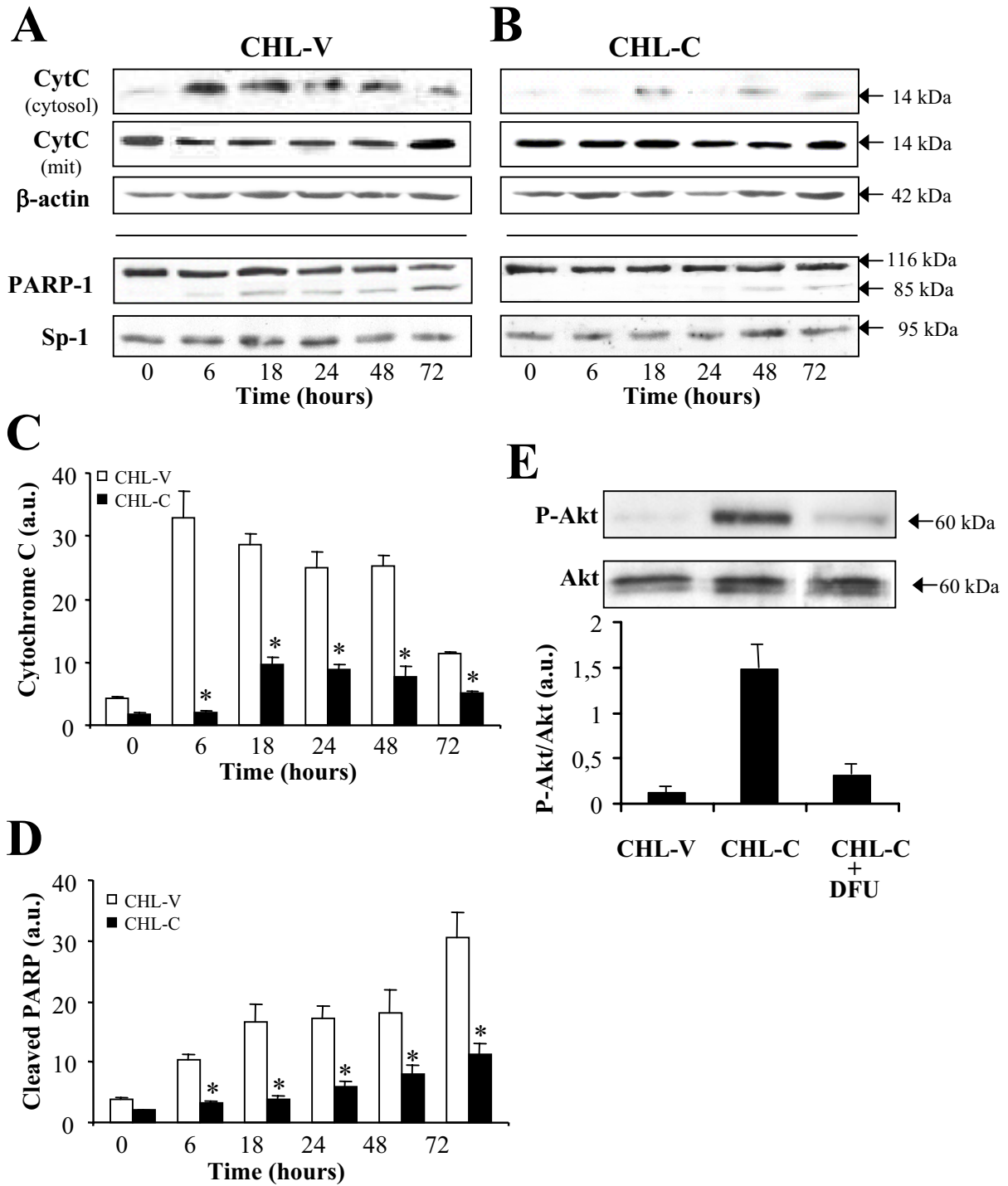


Figure 7

