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Cyclooxygenase-2 Over-Expression Inhibits Liver Apoptosis Induced by Hyperglycemia

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ABSTRACT

Increased expression of COX-2 has been linked to inflammation and carcinogenesis. Constitutive expression of COX-2 protects hepatocytes from several pro-apoptotic stimuli. Increased hepatic apoptosis has been observed in experimental models of diabetes. Our present aim was to analyze the role of COX-2 as a regulator of apoptosis in diabetic mouse liver. Mice of C57BL/6 strain wild type (Wt) and transgenic in COX-2 (hCOX-2 Tg) were separated into Control (vehicle) and SID (streptozotocin induced diabetes, 200 mg/kg body weight, i.p.). Seven days post-injection, Wt diabetic animals showed a decrease in PI3K activity and P-Akt levels, an increase of P-JNK, P-p38, pro-apoptotic Bad and Bax, release of cytochrome *c* and activities of caspases-3 and -9, leading to an increased apoptotic index. This situation was improved in diabetic COX-2 Tg. In addition, SID COX-2 Tg showed increased expression of anti-apoptotic Mcl-1 and XIAP. Pro-apoptotic state in the liver of diabetic animals was improved by over-expression of COX-2. We also analyzed the roles of high glucose-induced apoptosis and hCOX-2 in vitro. Non-transfected and hCOX-2-transfected cells were cultured at 5 and 25 mM of glucose by 72 h. At 25 mM there was an increase in apoptosis in non-transfected cells versus those exposed to 5 mM. This increase was partly prevented in transfected cells at 25 mM. Moreover, the protective effect observed in hCOX-2-transfected cells was suppressed by addition of DFU (COX-2 selective inhibitor), and mimicked by addition of PGE_2 in non-transfected cells. Taken together, these results demonstrate that hyperglycemia-induced hepatic apoptosis is protected by hCOX-2 expression. J. Cell. Biochem. 114: 669–680, 2013.

KEY WORDS: COX-2; LIVER; APOPTOSIS; DIABETES

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COX (cyclo-oxygenase) enzymes catalyze the first step in the biosynthesis of prostaglandins (PGs) and thromboxanes [DeWitt, 1991]. The COX-1 isoform is constitutively expressed in many tissues [Pilbeam et al., 1993], whereas COX-2 is induced by growth factors, tumor promoters, and cytokines [DeWitt, 1991; Feng et al., 1995]. Recent studies have shown that PGs are implicated in the regulation of multifaceted pathophysiological processes, for instance, acute inflammatory events [Mutschler et al., 2003] oxygen-deprived brain injury [Basu et al., 2003] and chronic inflammatory diseases [Basu et al., 2001]. Additionally, the role of some PGs in several cardiovascular risk factors such as type 1 and type 2 diabetes, obesity, smoking, and possibly also in atherogenesis has recently been revealed [Basu et al., 2005; Helmersson et al., 2005].

On the other hand, type 1 diabetes mellitus (DM) is considered as an inflammatory process [Alexandraki et al., 2008] in which a significant increase of cytokines IL-1, IL-6, IL-18, and TNF-α was found in the blood of patients with this disease [Foss et al., 2007]. Hepatocytes are capable to respond to pro-inflammatory cytokines promoting the expression of genes that mediate the inflammatory response [Martin-Sanz et al., 2002]. Besides, in a relatively short time, hepatic injury has been recognized as a major complication of DM; in this regard, type 1 diabetes is associated with increased risk of chronic liver injury [Kim et al., 2009a].

We have demonstrated that the hydroxyl radical acts as a reactive intermediate leading to liver apoptosis in a model of STZ-mediated hyperglycemia [Frances et al., 2010]. Also, we have shown that the increase of TNF-α in the liver is likely a fundamental key leading to apoptotic cell death in the diabetes state [Ingaramo et al., 2011]. These relevant previous studies provide further knowledge about the mechanisms which may contribute to the complications that occur in the diabetic liver.

Apoptosis is mediated by activation of caspases, a family of cysteine proteases [Talanian et al., 2000]. Caspase activation occurs by at least two mechanisms: the extrinsic or death receptor pathway, initiated by agonists of the TNF (tumor necrosis factor) super family such as TNFα, Fas ligand, and Apo2 ligand/TRAIL (TNF-related apoptosis-inducing ligand) [Ashkenazi and Dixit, 1998], and the intrinsic or mitochondrial pathway [Green and Reed, 1998]. 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 (apoptotic protease-activating factor 1), ATP and pro-caspase 9 in a multiprotein complex called apoptosome. Once activated in the apoptosome, caspase 9 in turn activates downstream executioner caspases, such as caspase 3 and caspase 7 [Zimmermann and Green, 2001]. In the 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 [Kondo et al., 1997].

In this connection, in a previous study we demonstrated that hyperglycemia enhances hydroxyl radical levels, induction of Bax protein, and translocation of Bax from cytosol to the mitochondria, leading to the release of cytochrome *c* and consequent activation of caspase-3 with resultant apoptosis [Frances et al., 2010]. Also, we demonstrated that in the diabetes state increase of TNF-α occurs in the liver, leading to apoptotic cell death, through activation of caspase-8, nuclear factor-κB (NF-κB) and c-Jun activating kinase (JNK) pathways [Ingaramo et al., 2011].

The pro-inflammatory and cytoprotective functions of COX-2 in liver remain unclear, mainly because hepatocytes, but not other hepatic cells, fail to express COX-2 upon pro-inflammatory challenge [Callejas et al., 2000]. Different reports demonstrated that PGE2 administration protects against liver injury [Takano et al., 1998]. The PGE2–EP4 (PGE receptor 4) signaling pathway effectively protects against hepatic ischemia/reperfusion injury because EP4 agonists down-regulate the expression of several pro-inflammatory cytokines, chemokines, and adhesion molecules [Kuzumoto et al., 2005]. Studies with different Tg mouse models of COX-2 also demonstrated the important anti-apoptotic role for PGs. Tg mice expressing COX-2 in the mammary gland developed tumors, and these animals had reduced levels of the pro-apoptotic proteins Bax and Bcl-xS, and elevated levels of the anti-apoptotic protein Bcl-2 [Liu et al., 2001]. A different study demonstrated hyperplasic gastric tumors in Tg mice expressing COX-2 and mPGES [Oshima et al., 2004]. Previous results, obtained with stable expression of COX-2 in liver cell lines and with mouse hydrodynamically transfected with a COX-2-GFP expression vector, supported the view that PGs produced by COX-2 protected the liver against Fas-mediated apoptosis [Casado et al., 2007]. Also, it is known that PGs produced by COX-2 in liver increased the levels of Bcl-2, Mcl-1, TNFR2, and TRAF2, all the hallmarks of the apoptotic/anti-apoptotic pathway [Mayoral et al., 2008]. In this regard, Fernandez-Martinez et al. [2006] have shown that PGs produced by COX-2 in liver, both in vitro and in vivo, inhibit apoptosis dependent on the intrinsic mitochondrial pathway.

According to the preceding results, here we analyze whether the over-expression of COX-2 in the liver in the diabetes state is sufficient to inhibit apoptosis, an event largely implicated in the hepatic complications of this pathology [Frances et al., 2010; Ingaramo et al., 2011; Manna et al., 2010]. We have used two approaches to evaluate the role of COX-2 in liver cells in order to analyze the possible mechanism implicated in PGs-dependent inhibition of apoptosis induced by hyperglycemia. The first approach involves the use of transgenic mice that over-express COX-2 from human origin (hCOX-2 Tg). In the second one, we generated liver cell lines expressing COX-2 protein by stable transfection with a vector containing the human COX-2 cDNA.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Antibodies were from Santa Cruz Laboratories, BD Biosciences, Cayman Chemical, and Cell Signaling Technologies. Streptozotocin (STZ) was from Sigma Chemical Co. Fluorescent probes were from Molecular Probes (Invitrogen) and Calbiochem. Electrophoresis reagents were obtained from Bio-Rad, and other reagents were from Roche Diagnostics or Sigma Chemical Co. DFU [5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(*5H*)-furanone]

was from Merck. Prostaglandin E2 (PGE_2) was from Calbiochem EMD Biosciences.

ANIMALS

Hepatocyte COX-2 transgenic mice on a C57BL6JXDBA background were used in this study, along with corresponding age-matched Wt mice [Casado et al., 2007]. The 8- to 12-week-old male mice were housed on a 12-h light/dark cycle in an air conditioned room at 25°C with food and water available ad libitum and were treated according to the Institutional Care Instructions (Bioethical Commission, Spanish National Research Council—CSIC). To induce diabetes, mice were intraperitoneally given a single dose of streptozotocin (STZ, 200 mg/kg body wt) freshly dissolved in citrate buffer pH 4.5. Animals were divided into four groups: Control wild type (**Wt**), COX-2 transgenic (**COX-2 Tg**), STZ-induced diabetic wild type (**SID Wt**), and STZ-induced Diabetic COX-2 transgenic (**SID COX-2 Tg**). Animals were euthanized 7 days after STZ injection between 8 and 10 a.m. and liver tissues were snap-frozen in liquid nitrogen, stored at −80°C, and collected in a solution containing 30% sucrose in PBS or fixed in 10% buffered formalin. Plasma was obtained by cardiac puncture.

CELL LINES AND TREATMENTS

The human liver cell line CCL-13 [Chang liver (CHL)], an immortalized non-tumor cell line derived from normal liver, was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown on Falcon tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 μg/ml each of penicillin, streptomycin, and gentamicin) at 37°C in a humidified, 5% CO_2-enriched atmosphere.

Attached CHL cells at 50% confluence were exposed for 24 h to Fugene 6 reagent (Roche Applied Science, Indianapolis, IN) containing pPyCAGIP-hCOX-2-GFP or control vector pPyCAGIP-GFP.

Cells stably expressing hCOX-2-GFP or GFP proteins were obtained as previously described [Llorente et al., 2011], and termed CHL-C and CHL-V, respectively. Cells were cultured in DMEM containing 10% FBS, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, and 2 mM L-glutamine in a humidified atmosphere in 5% CO_2 at 37°C. Cells were plated for 72 h at different concentrations of glucose. Untreated cells in the first group served as controls. Cells treated with 5 mM D-glucose for 72 h replicated normoglycemic condition. Cells treated with 25 mM D-glucose for 72 h were considered as the hyperglycemic group. As an osmotic control, cells were treated with 5 mM or 25 mM of L-glucose for 72 h.

In some experiments, the cells were plated for 72 h at different concentrations of glucose and were incubated with 10 μM of [5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(*5H*)-furanone] (DFU) to inhibit COX-2 [Fernandez-Martinez et al., 2006] or with 10 μM PGE_2.

DETERMINATION OF METABOLITES

PGE_2 levels were determined in liver extracts by specific immunoassay (GE Healthcare) [Casado et al., 2007]. Serum glucose levels were tested by means of the glucose oxidase method (BioSystems SA, Barcelona, Spain), successful induction of diabetes was defined as a blood glucose level >13.2 mmol/L.

PROTEIN ANALYSIS: CELL EXTRACTS AND WESTERN BLOTTING

Cells [$(1–2) \times 10^6$] or tissue samples (100 mg) were homogenized in a lysis buffer containing 10 mM Tris/HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, 0.5% (w/v) CHAPS, 1 mM 2-mercaptoethanol, and 0.1 mM PMSF. Extracts were vortex-mixed for 30 min at 4°C and centrifuged for 20 min at 13,000*g*, the supernatants were stored at −20°C. Cytosolic and mitochondrial extracts were prepared as described previously [Frances et al., 2011]. For Western blot analyses, equal amounts of protein (20–30 mg) were loaded onto a 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The relative amounts of each protein were determined with the corresponding polyclonal or monoclonal primary antibodies. After incubation with the corresponding secondary antibody, blots were developed by the enzymatic chemiluminescence's protocol (Amersham–GE Healthcare, Chalfont St. Giles, UK). The blots were revealed, and different exposition times were performed for each blot with a charged coupling device camera in a luminescent image analyzer (Gel-Doc; Bio-Rad) to ensure the linearity of the band intensities. Densitometric analysis was expressed in arbitrary units.

PI 3-KINASE ACTIVITY

PI3-kinase activity was measured in the immunoprecipitates by in vitro phosphorylation of PI as previously described [Valverde et al., 1997].

FLOW CYTOMETRY

For the analysis of apoptosis by PI (propidium iodide)-positive distribution, cells were resuspended in PBS and fixed in 70% ethanol. PI staining was performed after incubation of the cells with 0.05% PI and 0.1 mg/ml RNase for 30 min which were then analyzed in a flow cytometer (FC 500 Series; Beckman-Coulter, Hialeah, FL) as described previously [Fernandez-Martinez et al., 2006].

The double staining with annexin V–propidium iodide was used to identify viable cells, apoptotic and necrotic cells which allows the identification of three different cell populations: living cells are negative/negative, apoptotic cells are positive/negative, and necrotic cells are positive/positive, respectively. Cells were incubated with annexin V–Alexa Fluor 488 conjugated and PI at room temperature for 15 min and then analyzed by flow cytometry (FC 500 Series; Beckman-Coulter), measuring the green fluorescence of annexin V–Alexa Fluor 488 conjugated in the FL1 channel, and the red fluorescence of PI in the FL3 channel.

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 dithiothreitol, 0.5 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml Tos-Lys-CH2Cl ("TLCK," tosyl-lysylchloromethane), 5 mM NaF, 1 mM $NaVO_4$, 10 mM Na_2MoO_4, and 0.5% Nonidet P-40. After centrifugation of the cell lysate at 15,700*g* for 5 min, 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 and 9 in cytosolic extracts were determined with the fluorogenic substrates *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin, and *N*-acetyl-Leu-Glu-His-Asp-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.

DETERMINATION OF APOPTOTIC INDEX (AI)

Light microscopic analysis of hematoxylin- and eosin-stained slides was used to quantify apoptotic cells, which were identified by morphological criteria (increased eosinophilic cytoplasm, darkened nucleus, and pycnotic separation of cytoplasmic membrane from neighboring cells). The number of apoptotic hepatocytes was assessed by systematically scoring at least 1,000 hepatocytes per field in 10 fields of tissue sections at a magnification of 400× [Gold et al., 1994; Klainguti et al., 2000].

STATISTICAL ANALYSIS

Data are expressed as means ± SE. Student's *t*-test was applied wherever necessary, and statistical analysis of differences between groups was performed by one-way ANOVA followed by Tukey's method. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

EVALUATION OF INDUCED DIABETES

Diabetes was confirmed in streptozotocin-injected mice by monitoring weight loss and significant increase in blood glucose levels. These results are consistent with the STZ model described by others [Manna et al., 2010; Ingaramo et al., 2011]. Table I shows body weight and blood glucose levels in control and diabetic wild-type (Wt) mice, and also in control and diabetic transgenic (COX-2 Tg) mice.

As expected, streptozotocin treated mice (SID) showed a significant increase in blood glucose levels and a diminution of body weight (b.w.) (Table I). Prior to STZ injection, body weight of Wt and COX-2 Tg mice was similar, and there were no significant differences.

TABLE I. Evaluation of Induced Diabetes

	Wt	Tg	SID Wt	SID Tg
Body weight (g)	30.4 ± 0.9	31.3 ± 0.3	25.7 ± 1.4*	27.1 ± 0.7*
Blood glucose (mmol/L)	6.7 ± 2.2	7.1 ± 3.2	21.3 ± 7.3*	27.7 ± 8.3*

Wt, control wild type (vehicle); Tg, control COX-2 transgenic (vehicle); SID Wt, streptozotocin-induced diabetic wild type (STZ: 200 mg/kg); SID Tg, streptozotocin-induced diabetic COX-2 transgenic (STZ: 200 mg/kg). Values are mean ± SE (n = 4) animals per group.
*$P < 0.05$ versus Wt.

CHARACTERIZATION OF hCOX-2 Tg MICE: CONTROL AND DIABETIC STATE

First, we analyzed the expression of hCOX-2 in liver tissue of Wt and COX-2 Tg mice. We found hCOX-2 was significantly expressed in COX-2 Tg animals but undetectable in Wt (Fig. 1A). We confirmed the functional activity of COX-2 by measuring the hepatic levels of PGE_2, which showed a fivefold increase in comparison with Wt (Fig. 1B). The induction of a diabetes state did not significantly change the hepatic levels of PGE_2 (Fig. 1B).

EFFECT OF EXPRESSION OF hCOX-2 ON THE ACTIVITY OF MITOGEN-ACTIVATED PROTEIN KINASE (MAPKs) IN VIVO

MAPKs are critical upstream signaling proteins involved in several processes. There are three distinct subfamilies of MAPKs: ERK, p38-MAPKs, and JNK. To assess the effects of STZ-induced diabetes and the increase of expression of hCOX-2 on the activation of MAPK subfamilies, liver tissue homogenates were analyzed for phosphorylated forms of JNK, p38-MAPKs, and ERK by immunoblotting. Figure 2 shows that in the Wt-diabetic animals, phosphorylated JNK, p38, and ERK were activated. The increased expression of hCOX-2 (SID Tg animals) was able to prevent the hyperglycemia-induced phosphorylation of both JNK and p38, showing SID Tg animals values of normoglycemic Tg mice for this phospho-proteins. For Erk, a marked increase in its phosphorylated form was observed in the SID COX-2 Tg when compared to SID-Wt mice.

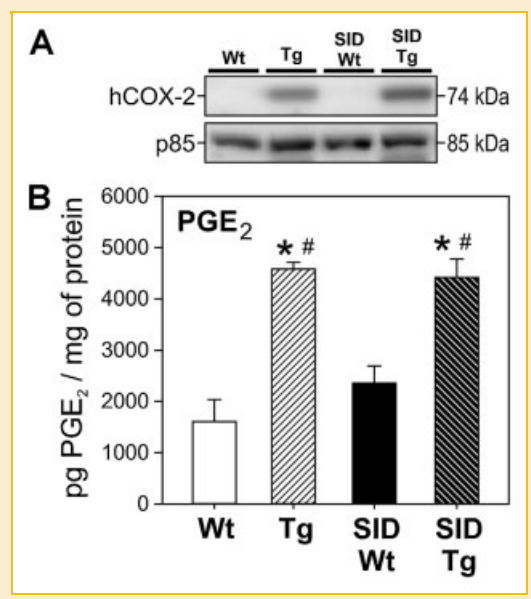

Fig. 1. Expression and function of human COX-2 (hCOX-2) in COX-2 Tg Control and STZ-induced diabetic mice. A: Human hCOX-2 protein expression in liver homogenates from Wt and COX-2 Tg animals with or without STZ treatment detected by Western blot and normalized with p85. B: Intrahepatic PGE_2 concentrations were determined by ELISA in liver homogenates of Control and SID animals from both, Wt and COX-2 Tg group. Values are the means ± SE of four animals per condition. *$P < 0.05$ versus Wt animals. #$P < 0.05$ versus SID-Wt animals.

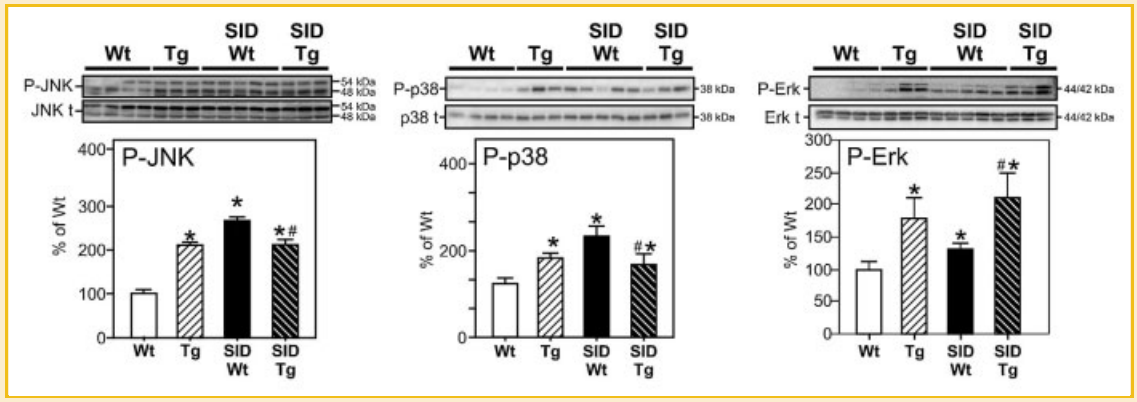

Fig. 2. Effect of diabetic state and over-expression of hCOX-2 on MAPKs signaling pathway in vivo. Phosphorylated-JNK, phosphorylated-p38 and phosphorylated-ERK in liver tissue. Typical example of Western blot is shown in top panel for each experimental group. The accompanying bars represent the densitometry expressed as percentage of Wt group. Data are expressed as means ± SE for at least four mice of each experimental group: Wt, COX-2 Tg, SID Wt, SID COX-2 Tg. $^{*}P < 0.05$ versus Wt; $^{\#}P < 0.05$ versus SID Wt.

EFFECT OF THE EXPRESSION OF hCOX-2 ON THE EXPRESSION OF BOTH Bcl-2 FAMILY PROTEINS AND THE X-LINKED INHIBITOR OF APOPTOSIS PROTEIN (XIAP) IN VIVO

The Bcl-2 family of proteins plays a major role in regulating apoptosis. Mammalian anti-apoptotic members include Bcl-2, Bcl-x_L, or Mcl-1 and display sequence conservation throughout four Bcl-2 homology domains (BH1–4). They oppose the multi-domain pro-apoptotic proteins such as Bax, Bak, Bid, Bim, Puma, Bad, and Noxa [Puthalakath and Strasser, 2002]. Also, it is known that XIAP, a member of the inhibitor family of apoptosis proteins (IAPs), can suppress apoptosis triggered by diverse stimuli by binding directly to procaspase-9 and activated caspase-3, preventing apoptosis [Roucou et al., 2001].

Immunoblot analyses followed by quantitative densitometry from four separate animal sets revealed that the diabetic state increased the mitochondrial levels of both Bax and Bad, two pro-apoptotic proteins. The increased expression of hCOX-2 prevented the hyperglycemia-induced pro-apoptotic protein levels (Fig. 3). Besides, it is known that Bax:Bcl-x_L ratio determines cell survival or death after apoptotic stimuli [Ronco et al., 2002]. Figure 3 shows that mitochondrial Bax:Bcl-x_L ratio was significantly increased in diabetic Wt mice, indicating that in the diabetic state, the liver is promoted to an apoptotic state. The increased levels of hCOX-2 present in COX-2 Tg animals, however, significantly repressed hyperglycemia-induced pro-apoptotic state. The anti-apoptotic Mcl-1 and XIAP protein levels were assessed. From the immunoblotting study, it was observed that in the diabetic state the expression levels of Mcl-1 did not change; in contrast, the levels of XIAP were significantly decreased. The protein levels of Mcl-1 and XIAP were significantly increased in COX-2 Tg diabetic mice compared with the Wt diabetic group (Fig. 3).

EFFECT OF THE OVER-EXPRESSION OF hCOX-2 ON THE CELL DEATH PATHWAY IN VIVO

It is well established that induction of Bax protein and its translocation from the cytosol to the mitochondria lead to the release of cytochrome *c*, which causes the activation of the caspases via (initiator caspase-9 and effector caspase-3), thus inducing apoptotic cell death [Zimmermann and Green, 2001]. Therefore, we assessed the level of cytochrome *c* in the cytosol, as well as the activation status of caspases. Immunoblot analyses (Fig. 4) showed that the diabetes state elevated the levels of cytosolic cytochrome *c* (Fig. 4A), together with up-regulating caspase-9 and caspase-3 (Fig. 4B) thus indicating the involvement of the mitochondrial pathway in this pathology. The over-expression of hCOX-2 significantly inhibited hyperglycemia-induced mitochondria-dependent cell death.

To assess whether cell death is primarily due to programmed cellular death, the occurrence of apoptosis was confirmed by hematoxylin and eosin staining in hepatic tissue section. Typical features of apoptosis, such as cellular shrinking with cytoplasmic acidophilia, condensation, and margination of the chromatin, are shown in Figure 4D. Apoptotic index (AI) was calculated for each sample (Fig. 4C). We observed that the diabetes state increased the apoptosis in the liver tissue, whilst the increase of COX-2 expression attenuated the apoptotic cell death. In no case, the careful histological analysis of liver sections stained with hematoxylin–eosin showed inflammatory foci or necrosis.

EXPRESSION OF hCOX-2 PROMOTES PI3K (PHOSPHOINOSITIDE 3-KINASE)/AKT PATHWAY IN VIVO

The PI3K (phosphoinositide 3-kinase)/Akt pathway plays a central role in integrating diverse survival signals [Fabregat, 2009; Kroczynska et al., 2009]. In our study, we determined the activity of PI3K by an in vitro activity assay and the levels of P-Akt using Western immunoblotting. We observed that the diabetes state decreased the PI3K activity and consequently, the levels of P-Akt (Fig. 5). The PI3K activity and the levels of P-Akt were significantly increased in COX-2 Tg diabetic mice compared with the wild-type diabetic group (Fig. 5), thus suggesting an activation of the PI3k/Akt pathway by hCOX-2 over-expression, a pathway known to be decreased in the diabetic state [Shepherd et al., 1998; Virkamaki et al., 1999; Frances et al., 2010].

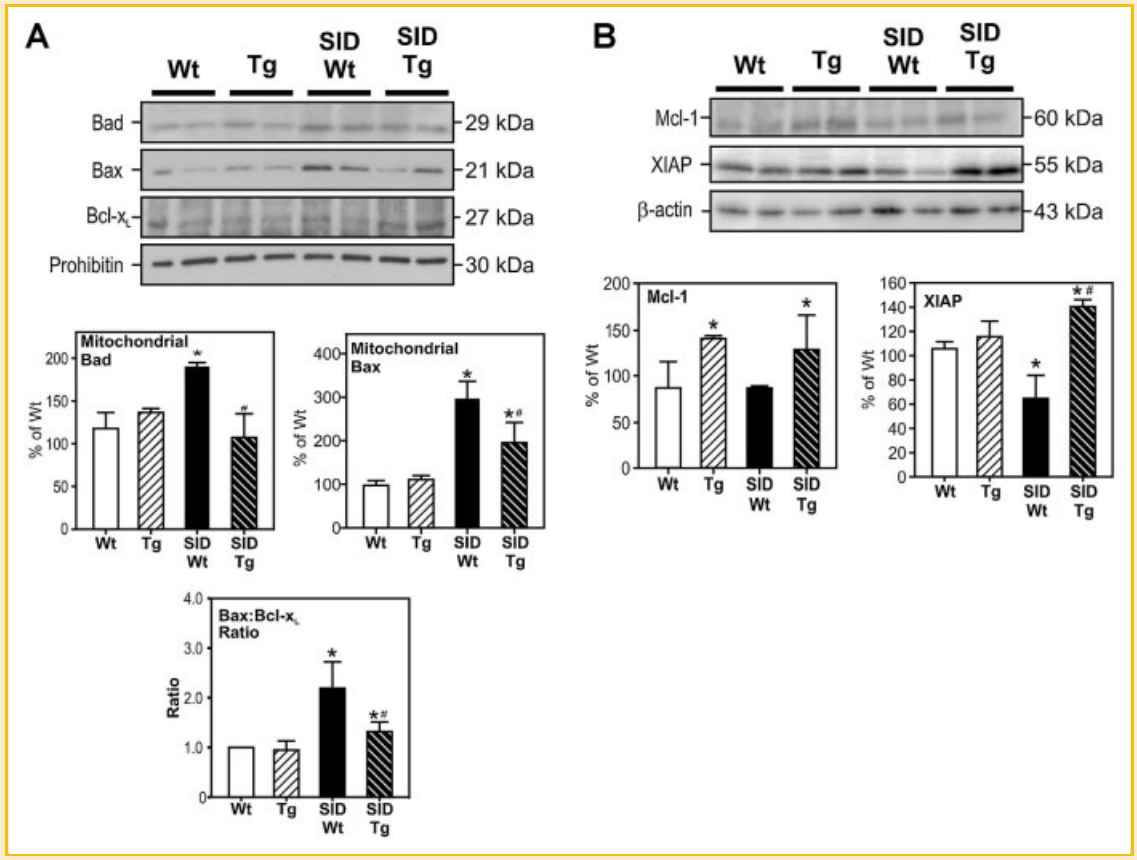

Fig. 3. Effect of diabetic state and expression of hCOX-2 on Bcl-2 protein family member expression. Immunoblot analysis of pro-apoptotic and anti-apoptotic proteins in liver subcellular fractions. A: Mitochondrial Bax, Bad, Bcl-x_L protein expression and Bax:Bcl-x_L ratio expressed as percentage of the Wt group of the densitometry obtained for Bax and Bcl-x_L. Typical examples of Western blots are shown in top panel for each experimental group. The accompanying bars represent the densitometry expressed in percentage from four separate animal sets, considering Wt as 100%. Data are expressed as means ± SE. B: Mcl-1 and XIAP expression levels. Typical examples of Western blots are shown in top panel for each experimental group. The accompanying bars represent the densitometry expressed in percentage from four separate animal sets, considering Wt as 100%. Data are expressed as means ± SE for at least four mice for each experimental group: Wt, COX-2 Tg, SID Wt, SID COX-2 Tg.* $P < 0.05$ versus Wt; # $P < 0.05$ versus SID Wt.

COX-2 EXPRESSION IN CHANG LIVER TRANSFECTED CELLS

We addressed whether liver cells, which are constantly exposed to high glucose in the diabetic state in vivo, responds to exposure to elevated glucose with augmentation of apoptosis. Besides, we examined whether the increase of hCOX-2 expression is able to inhibit liver cell apoptosis induced by hyperglycemia.

The CHL-V cells and CHL-C cells were incubated in a medium containing 5 mM glucose (normoglycemic condition) or 25 mM glucose (hyperglycemic condition). Both solutions were of equal osmolarity (n = 4/group). Figure 6A shows exclusive hCOX-2 expression in CHL-C cells in both glucose concentration conditions, and GFP-dependent fluorescence confirming plasmid integration and expression.

Studies performed in a different cell line, human umbilical vein endothelial cells (HUVECs), demonstrated that JNK activation mediates the apoptosis induced by hyperglycemia [Ho et al., 2006]. Also, other authors have demonstrated that hyperglycemia induces p38 MAPK-mediated renal proximal tubular cell (RPTC) apoptosis [Rane et al., 2010]. In the present study we analyzed both JNK and p38 MAPKs activation in CHL-V and CHL-C cells, incubated at 5 and 25 mM glucose. Results revealed that the treatment with high glucose in CHL-V cells leads to an increased activation of both JNK and p38 MAPKs, whilst a moderate increase of P-JNK was observed in CHL-C cells at 25 mM glucose when compared to CHL-C at 5 mM glucose. As regards to p38 MAPK activation in CHL-C cells, no differences were observed between high glucose and 5 mM glucose situations (Fig. 6B).

Besides, we analyzed PI-stained cells in CHL-C and CHL-V cell cultures at various times of incubation (8, 12, 24, and 72 h). When CHL-V cells were incubated with 25 mM glucose for 72 h, there was an increase of 30% in cell death compared with CHL-V cells incubated with 5 mM glucose. The incubation of the CHL-C cells with high glucose did not show any increase in cell death, suggesting a protective role of hCOX-2 (Fig. 6C). This protective effect of COX-2 expression was even more evident when the cells were incubated with 50 mM glucose (data not shown). The prevention of cell death was mimicked in CHL-V cells incubated with 25 mM glucose and treated with exogenous PGE_2. The role of COX-2 metabolites in the protective effect in CHL-C cells was confirmed by treatment with the potent and selective COX-2

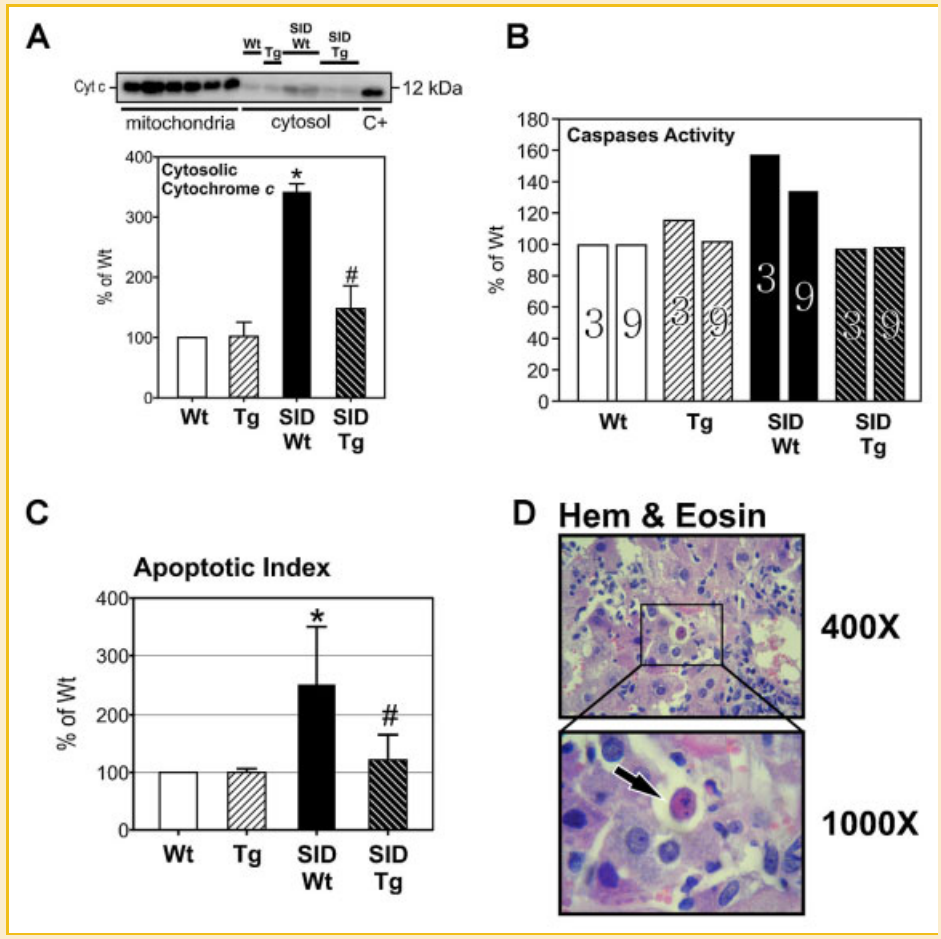

Fig. 4. Effect of diabetic state and expression of hCOX-2 on liver apoptosis. A: Mitochondrial and cytosolic cytochrome c expression. Typical example of Western blot is shown in top panel for each experimental group and a positive control (C+). The accompanying bars represent cytosolic cytochrome c levels in all experimental groups, expressed as percentage of Wt group of the densitometry obtained by Western Blot analysis. Data are expressed as means ± SE for at least four mice of each experimental group: Wt, COX-2 Tg, SID Wt, SID COX-2 Tg. $^{*}P < 0.05$ versus Wt; $^{\#}P < 0.05$ versus SID Wt. B: The activity of both caspases-3 and -9 were determined in all experimental groups by means of a fluorometric assay. The bars represent activity expressed in percentage, considering Wt as 100%. C: Apoptotic index (AI) was expressed as percentage of apoptotic cells scored per 10,000 hepatocytes per slide at a magnification of 400×. The bars represent AI considering Wt as 100%. Data are expressed as means ± SE for at least four mice of each experimental group. $^{*}P < 0.05$ versus Wt; $^{\#}P < 0.05$ versus SID Wt. D: Representative photograph of apoptotic cells in SID Wt liver is shown stained with hematoxylin–eosin for the morphological analysis.

inhibitor DFU, which increased the number of apoptotic cells (Fig. 6C).

To confirm apoptotic cell death, we performed the double staining with annexin V–propidium iodide. Specifically, high glucose led to a 60% increase of apoptosis in comparison with cells exposed to basal glucose in CHL-V cells; moreover, this increase was partially prevented in CHL-C cells, suggesting a role for hCOX-2 in the protection from apoptotic death (Fig. 6C).

When we analyzed the levels of the anti-apoptotic proteins XIAP and Mcl-1, we found no differences between high glucose and 5 mM glucose treatments in the CHL-V cells as well as in the CHL-C cells. The increased levels of both XIAP and Mcl-1 proteins observed in CHL-C cells as compared to CHL-V cells, for either glucose concentration, suggest that this augmentation is associated to over-expression of COX-2 (Fig. 6D).

To explore the role of endogenous Akt activity in high glucose-induced cell death, we measured the activated (phosphorylated) form of Akt in both CHL-V and CHL-C cells. The immunoblotting study using an antibody specific to Akt phosphorylated at Ser 473 showed a significantly higher expression of this protein in CHL-C cells treated with high glucose compared to CHL-V cells. The over-expression of hCOX-2 in CHL-C cell leads to an increase in the level of Akt in both treatments (5 and 25 mM glucose) (Fig. 6E).

DISCUSSION

The data presented so far provide convincing evidence that PGE_2 synthesized by COX-2 suppresses hyperglycemia-induced apoptotic signals in the liver. To investigate the mechanisms through which this occurs we have used both in vivo and in vitro models of constitutive COX-2 expression and elevated PGE_2 synthesis in liver cells.

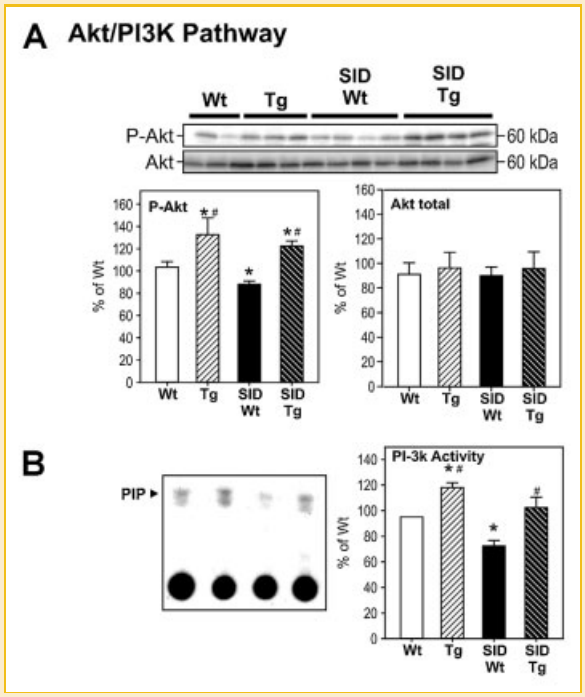

Fig. 5. PI3k/Akt pathway. A: Total and phosphorylated Akt was examined by immunoblotting. Typical example of Western blot is shown in top panel for each experimental group. The accompanying bars represent protein levels in all experimental groups, expressed as percentage of Wt group of the densitometry obtained by Western blot analysis. Data are expressed as means ± SE for at least four mice of each experimental group: Wt, COX-2 Tg, SID Wt, SID COX-2 Tg. $^{*}P < 0.05$ versus Wt. $^{\#}P < 0.05$ versus SID Wt. B: Samples were prepared as previously reported [Valverde et al., 1997]. The immune complexes were washed and immediately used for an in vitro phosphatidylinositol kinase assay as described in the Materials and Methods Section. The conversion of phosphatidylinositol to phosphatidylinositol phosphate (PIP) in the presence of [γ-^{32}P] ATP was analyzed by TLC. Results are representative of three independent experiments. Values are the means ± SE of four animals per condition. $^{*}P < 0.05$ versus Wt animals. $^{\#}P < 0.05$ versus SID Wt animals.

In a previous work, we indicated that diabetes-induced apoptosis in the rat liver is mediated by TNF-α/TNFR1/JNK/caspase 3 pathway [Ingaramo et al., 2011]. JNK is one of the mitogen-activated protein (MAP) kinases, and it is a mandatory signal for apoptosis induced by high glucose in many cells types [Ho et al., 2006; Manna et al., 2010]. Erk, JNK, and p38 contribute to COX-2 expression, and the anti-inflammatory activity of many compounds is a function of COX-2 inhibition via JNK and p38 MAPK inactivation [Tanabe and Tohnai, 2002]. The effect of COX-2-dependent PGs on these signaling pathways have been recently studied using cell lines stably over-expressing COX-2 [Kim et al., 2009b]. Our data obtained in COX-2 Tg animals and in hepatocyte-like cells confirm basal activation of JNK, Erk, and p38.

Importantly, in this study we found that the over-expression of COX-2 can prevent the increase of JNK activity induced by high glucose (Fig. 2). Besides, we found that p38 MAPK, which plays an essential role in inflammation and apoptosis [Yang et al., 2007], is induced by high glucose, and also that the over-expression of COX-2 was able to prevent such increase (Fig. 2). Unlike both JNK and p38 MAPK, Erk is considered important in differentiation and proliferation, as well as in cell survival [Seger and Krebs, 1995; Yang et al., 2007]. In this regard, we found that in the diabetic state there is a slight increase in P-Erk, whereas over-expression of COX-2 leads to a significant increase in the levels of phosphorylated Erk (Fig. 2).

An early study had demonstrated that the activation of JNK is associated with an increase of the apoptosis induced by TNF in hepatocytes [Wullaert et al., 2006]. In this connection, our previous results demonstrated that diabetes in the rat liver leads to the activation of JNK, and finally induces an increase of the apoptotic index [Ingaramo et al., 2011]. Prior investigations attributed the mechanism of this JNK effect to phosphorylation events that activates pro-apoptotic Bcl-2 family members Bad or Bax [Tsuruta et al., 2004]. In the present study, we observed a pro-apoptotic state in the STZ-induced diabetic wild-type mice, which is evidenced by an increase in the expression of the pro-apoptotic proteins Bad and Bax (Fig. 3A). Besides, it is known that the Bax:Bcl-x_L ratio determines cell survival or death after apoptotic stimuli [Ronco et al., 2002]. We observed an increase in Bax:Bcl-x_L ratio in the liver of STZ-induced diabetic wild-type mice which adds to the pro-apoptotic state (Fig. 3A). Of note, STZ-induced diabetic COX-2 Tg mice exhibited an important decrease of the apoptotic cell death as a result of a decreased expression of Bad and Bax (Fig. 3A). Moreover, STZ-induced diabetic COX-2 Tg mice exhibited an important increase in the anti-apoptotic proteins Bcl-x_L, XIAP, and Mcl-1, which contributes to a decrease in the apoptotic index (Fig. 3B).

It is known that the pro-apoptotic members of the Bcl-2 family physically interact to form oligomers that can move onto the mitochondrial membrane and release cytochrome *c* from mitochondria to the cytosol leading to activation of the cell death executioner caspases (caspase-3 and caspase-9) that ultimately induce apoptosis [Zhao et al., 2001; Verzola et al., 2002]. Our results clearly show an increased release of cytochrome *c* from mitochondria to cytosol leading to the activation of both caspase-3 and -9, with the consequent increase in the apoptotic index in the liver of STZ-induced diabetic wild-type mice (Fig. 4B). Besides, we demonstrate that caspase-3 and -9 levels are decreased in mice over-expressing COX-2, as a consequence of the absence of cytochrome c in the cytosol which clearly implies that COX-2 derived PGE_2 products act on the mitochondrial pathway to prevent apoptosis in the diabetic state (Fig. 4A,B).

Akt has been implicated in the suppression of apoptosis through inactivation of several components of the cell death machinery such as Bad [Datta et al., 1997; Galetic et al., 1999; Verzola et al., 2002]. In other studies, as in our own, a diminution of Akt has been observed in the liver of insulin-deficient rats [Nawano et al., 1999; Katso et al., 2001; Frances et al., 2010]. Akt is required to maintain the pro-apoptotic protein Bad inactive [Fernando and Wimalasena, 2004]. Phosphorylated Bad is sequestered away from the site of action in the mitochondria by binding to cytosolic 14-3-3 proteins [Datta et al., 1997; Yano et al., 1998]. Besides, it is known that PI3K and Akt/PKB (protein kinase B) pathways are targets of PGs and that Akt phosphorylation is enhanced compared with Wt in COX-2 Tg mice liver, thus indicating a reinforcement of survival pathways [Schulze-Bergkamen et al., 2004; Fernandez-Martinez et al.,

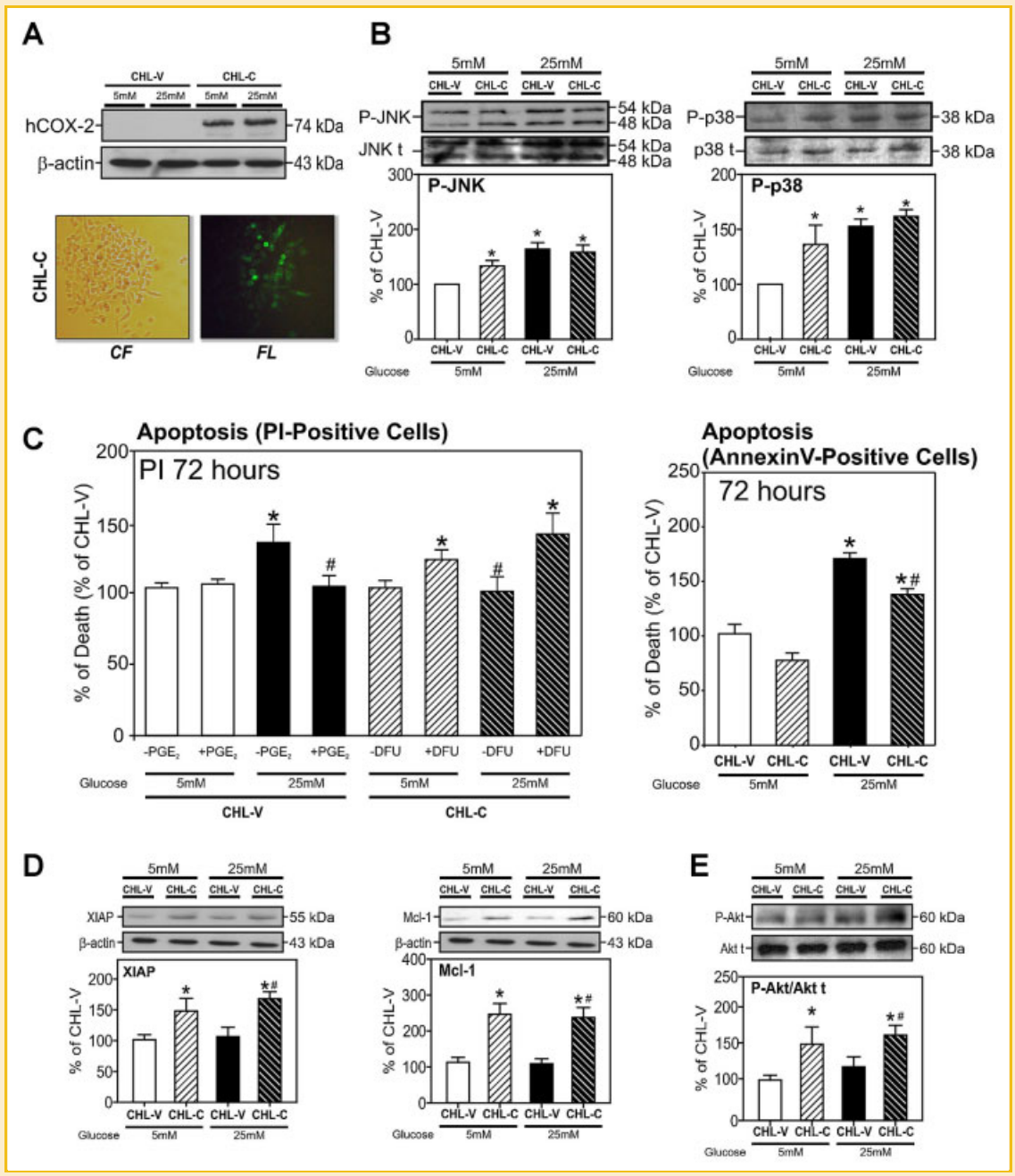

Fig. 6. Effect of over-expression of hCOX-2 in Chang liver transfected cells on cell death pathway. A: Assessment of hCOX-2 expression in Chang liver transfected cells. Human hCOX-2 protein expression in cell homogenates from CHL-C and CHL-V cells at glucose 5 and 25 mM conditions detected by Western blot and normalized with β-actin. Example microphotography in clear field (CF) and green fluorescence (FL) showing GFP-dependent fluorescence. B: Effect of hyperglycemia and over-expression of hCOX-2 on MAPKs signaling pathway in vitro. Phosphorylated-JNK and phosphorylated-p38 in cell extract were performed. Typical example of Western blot is shown in top panel for each experimental group. The accompanying bars represent expression levels in all groups, expressed as percentage of CHL-V at glucose 5 mM. Data are expressed as means ± SE for at least four mice for each experimental group: CHL-V 5 mM, CHL-C 5 mM, CHL-V 25 mM, and CHL-C 25 mM. $^{*}P < 0.05$ versus CHL-V 5 mM. $^{\#}P < 0.05$ versus CHL-V 25 mM. C: Effect of hyperglycemia and over-expression of hCOX-2 on apoptosis and total cell death evaluated by flow cytometry. The double staining with annexin V–propidium iodide was used to identify viable cells, apoptotic and necrotic cells which allows the identification of three different cell populations: living cells are negative/negative, apoptotic cells are positive/negative, and necrotic cells are positive/positive. Apoptosis was expressed as percentage of CHL-V at glucose 5 mM. The prevention of apoptosis was mimicked in CHL-V cells incubated with 25 mM glucose and treated with exogenous PGE_2 (10 μM), and the action of COX-2 metabolites in the protection of apoptosis in CHL-C cells was confirmed by treatment with the selective COX-2 inhibitor DFU (10 μM). D: Effect of hyperglycemia and over-expression of hCOX-2 on Mcl-1and XIAP expression. Typical examples of Western blots are shown in top panel for each experimental group. The accompanying bars represent the densitometry expressed in percentage, considering CHL-V 5 mM as 100%. Data are expressed as means ± SE. $^{*}P < 0.05$ versus CHL-V 5 mM; $^{\#}P < 0.05$ versus CHL-V 25 mM. E: Effect of hyperglycemia and over-expression of hCOX-2 on Akt phosphorylation. Total and phosphorylated Akt were examined by immunoblotting. Typical example of Western blot is shown in top panel for each experimental group. The accompanying bars expressed as percentage of basal values. Data are expressed as means ± SE. $^{*}P < 0.05$ versus CHL-V 5 mM.

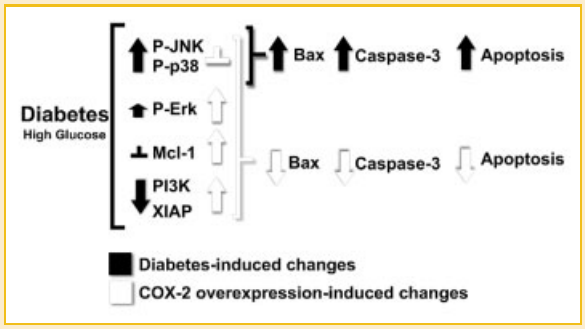

Fig. 7. Summary of high glucose-mediated signal transduction pathways for hepatocytes apoptosis and the anti-apoptotic effects produced by over-expression of COX-2. Hyperglycemia increases phosphorylation of both JNK and p38 proteins, consequently augmenting Bax protein mitochondrial levels, concomitantly with caspase 3 activation, thus leading to apoptosis. In hyperglycemia, the over-expression of COX-2 leads to sustained levels of P-JNK and P-p38, an increase of P-Erk (implicated in cell survival), and anti-apoptotic proteins (Mcl-1 and XIAP) levels, and an increase of PI3K/Akt pathway which plays a key role in the protection from cell death. Taken together, these events result in a decrease in liver apoptosis (↑ = increase; ↓ = decrease; ⊥ = no change).

2006; Mayoral et al., 2008]. In the present work, we demonstrate that the increase in COX-2 expression in STZ-induced diabetic COX-2 Tg mice produces an increase of PI3K activity when compared to STZ-induced diabetic Wt mice. Such an increase leads to the activation of Akt (Fig. 5), thus producing an anti-apoptotic signal.

To examine the extent to which COX-2 expression inhibiting apoptosis in liver cells in the whole animal is exclusively related to hyperglycemia, we performed in vitro studies in cultures of CHL-V and CHL-C cells. As shown in Figure 6A, hCOX-2 protein was significantly expressed in CHL-C cells, as determined by Western blot, but was not present CHL-V cells. When the cells were incubated with 25 mM glucose no changes in the expression of hCOX-2 were observed.

Our results show a decrease in apoptosis in CHL-C cells as compared to CHL-V cells when they were incubated with 5 mM glucose (normoglycemic condition) after 72 h of culture. At the same time of incubation, there was a 30% increase in apoptotic CHL-V cells compared to CHL-C cells when they were incubated with 25 mM glucose (hyperglycemic condition). This shows that COX-2 expression in liver cells is related to the inhibition of cellular apoptosis (Fig. 6B). Besides, the incubation of CHL-C cells in a medium containing 25 mM glucose supplemented with DFU (selective COX-2 inhibitor) leads to a recovery of the sensitivity of the cells to apoptosis (Fig. 6B). Importantly, when CHL-V cells cultured in 25 mM glucose were incubated with PGE_2, a decreased cellular apoptosis was observed, thus evidencing the protective effect of PGE_2 (Fig. 6B). This is in agreement with a number of experimental studies that have demonstrated a clear positive in vitro correlation between COX-2 expression and the inhibition of apoptosis [Tsujii and DuBois, 1995; Fernandez-Martinez et al., 2006; Casado et al., 2007; Mayoral et al., 2008].

To investigate the mechanism by which high glucose causes apoptosis in liver cells and COX-2 over-expression inhibits this effect, several proteins were studied.

Both JNK and p38 MAPK have been reported contribute to the COX-2 expression [Tanabe and Tohnai, 2002]. Our results show that the incubation with 5 mM glucose leads to a moderate increase in P-JNK levels in CHL-C cells compared to CHL-V cells. When CHL-V cells were incubated with 25 mM glucose, we found a significant increase in the expression of P-JNK as compared to that observed with 5 mM glucose. This indicates that activation is derived from high glucose levels. We did not find any significant changes in the expression of P-JNK between CHL-V and CHL-C when the cells were incubated with 25 mM glucose, failing to see a protective response of the COX-2-dependent PGs as happened in vivo. This is probably attributable to a different cytokine background (Fig. 6C).

In cholangiocarcinoma cells, it has been reported that the IAP2 protein (member of XIAP family) was involved in the anti-apoptotic action of PGE_2 synthesized by COX-2 in intestinal epithelial cells [Nishihara et al., 2003]. In this connection, our results show a significant increase in the expression of XIAP proteins in CHL-C cells when incubated both with 5 or 25 mM glucose (Fig. 6D), coinciding with the lowest percentages of apoptosis (Fig. 6B).

COX-2 has been reported to promote survival of human lung adenocarcinoma cells by up-regulating the levels of the anti-apoptotic protein Mcl-1, and activating the Akt-dependent pathway [Lin et al., 2001; Li et al., 2003]. Our results show a significant increase in the expression of Mcl-1 and an increase in Akt phosphorylation in CHL-C cells when incubated with 5 or 25 mM glucose (Fig. 6E). These results demonstrate that both Mcl-1 and Akt play an important role in the lowest percentages of apoptosis observed in CHL-C cells when incubated with either glucose concentration (Fig. 6B).

Thus, our findings suggest that apoptosis observed in CHL-V cells exposed to high glucose is inhibited in part by COX-2 over-expression in CHL-C cells. Figure 7 depicts a summary of our current findings.

Taken together, the results of the present study indicate that PGs produced by COX-2 in the liver are able to inhibit the apoptosis developed by the hyperglycemic state, dependent on the intrinsic mitochondrial pathway, both in vivo and in vitro. The goal of this study is to shed light on the anti-apoptotic and hepatoprotective effects of PGE_2 observed after acute liver injury induced by the diabetic state. Further studies with the COX-2 Tg diabetic mice might help to elucidate the precise role of COX-2 expression in mouse liver within the context of a chronic disease such as diabetes.

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