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Regulation of Bcl-2 and Bcl-xL anti-apoptotic protein expression by nuclear receptor PXR in primary cultures of human and rat hepatocytes

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Abstract

The pregnane X receptor (PXR) plays a major role in the protection of the body by regulating the genes involved in the metabolism and elimination of potentially toxic xeno- and endobiotics. We previously described that PXR activator dexamethasone protects hepatocytes from spontaneous apoptosis. We hypothesise a PXR-dependent co-regulation process between detoxication and programmed cell death. Using primary cultured human and rat hepatocytes, we investigated to determine if PXR is implicated in the regulation of Bcl-2 and Bcl-xL, two crucial apoptosis inhibitors. In the present study we demonstrated that the treatment of primary cultured hepatocytes with PXR agonists increased hepatocyte viability and protects them from staurosporine-induced apoptosis. The anti-apoptotic capacity of PXR activation was correlated with Bcl-2 and Bcl-xL induction at both the transcriptional and protein levels in man and rats, respectively. The inhibition of PXR expression by antisense oligonucleotide abolished PXR activators Bcl-xL induction. Accordingly, PXR overexpression in HepG2 cells led to *bcl-2* induction upon clotrimazole treatment and protects cells against Fas-induced apoptosis. Our results demonstrate that PXR expression is required for Bcl-2 and Bcl-xL up-regulation upon PXR activators treatment in human and rat hepatocytes. They also suggest that PXR may protect the liver against chemicals by simultaneously regulating detoxication and the apoptotic pathway.

Keywords: Primary human and rat hepatocyte; PXR; Bcl-2; Bcl-xL; CYP3A

1. Introduction

Organisms have developed an adaptative, regulatory mechanism to protect themselves against harmful xenobiotics. This process, which includes metabolism and elimination, results from the combined action of oxidative Phase I CYP enzymes, Phase II conjugated enzymes and drug transporters [1].

Since the nuclear pregnane X receptor (PXR, NR1I2) was discovered [2,3], a vast number of studies have identified it as the co-ordinate expression inducer of genes involved in all phases of drug metabolism, including those encoding CYPs (CYP3A, CYP2C, and CYP2B), GST, sulfotransferase, UDPGT as well as xenobiotic transporters

Abbreviations: AS, antisense oligonucleotides; CELL, non-treated control-cells; CLO, clotrimazole; CYP, cytochrome P450; DEX, dexamethasone; FBS, foetal bovine serum; GST, glutathione *S*-transferase; MDR1, multidrug resistance-1; MP, metyrapone; MRP, multidrug resistance-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OATP, organic anion-transporting polypeptide; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; PXR, pregnane X receptor; RP, rifampicin; Scr, scrambled AS control; SP, spironolactone; ST, staurosporine; TNF, tumor necrosis factor; UDPGT, UDP glucuronosyltransferase

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(MDR1, MRP2, MRP3, and OATP2) [4,5]. Overall, these findings postulate that PXR up-regulates the primary defence of the organism against chemicals by acting on detoxication genes regulation.

Another strategy of the organism to minimise the toxic effects of xenobiotic exposure is the apoptosis regulation process. Apoptosis [6], the cell-suicide programme executed by caspases, is critical for maintaining tissue homeostasis and is involved in a variety of biological events like development, remodelling and the removal of unwanted cells [7]. Whether a cell should live or die is largely determined by the Bcl-2 family of anti- and pro-apoptotic regulators [8]. Bcl-2 and Bcl-xL, two anti-apoptotic members of that subset, have been shown to protect cells from a broad range of apoptotic stimuli, thus regulating the intrinsic apoptotic pathway [9–11].

Our previous studies showed that the intrinsic mitochondrial apoptotic pathway plays a major role in regulating spontaneous apoptosis in hepatocytes [12]. It was also demonstrated that dexamethasone (DEX), a prototypical CYP3A inducer and PXR activator, was capable of inhibiting spontaneous apoptosis in hepatocyte primary cultures [13], up-regulating anti-apoptotic proteins Bcl-2 (in man) and Bcl-xL (in rat). All those facts led us to hypothesise that a PXR dependent co-regulation mechanism could exist between detoxication process and the anti-apoptotic regulation pathways. To analyse the role of PXR in Bcl-2 and Bcl-xL regulation, we used seven known human and/or rat PXR activators: dexamethasone (DEX), rifampicin (RP), phenobarbital (PB), clotrimazole (CLO), spironolactone (SP), pregnenolone-16α-carbonitrile (PCN) and methyrapone (MP) [14,15]. This report demonstrates that PXR activators protect against staurosporine-induced apoptosis by the up-regulation of Bcl-xL and Bcl-2 in rat and human hepatocytes. We found that the reduction of PXR expression by antisense technology in rat hepatocytes primary culture inhibited the Bcl-xL up-regulation induced by clotrimazole. Finally, the overexpression of PXR in HepG2 cells increased Bcl-2 expression upon clotrimazole treatment and protects them against Fas-induced apoptosis.

These results suggest that PXR plays a key role, not only in regulating detoxication processes (as previously described), but also in hepatocyte survival by up-regulating the Bcl-xL and Bcl-2 anti-apoptotic protein.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Williams' medium E, foetal bovine serum (FBS), SuperScript™ II, Platinum® *Taq* DNA Polymerase, Lipofectin and agarose were obtained from Invitrogen (Rockville, USA), and penicillin/streptomycin solution from Bio-Whittaker (CAMBREX Company, Walkersville, USA). Collagenase was from Roche Applied Science (Meylan, France); insulin from Nova Nordisk (Nova Nordisk A/S, Bagsvaerd, Denmark); and RiboGreen from Molecular Probes (Eugene, Oregon, USA). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.1.2. Antibodies (Abs)

Monoclonal Abs to Bcl-2 (clone Ab-1) was from Oncogene (Boston, USA); polyclonal Abs to Bcl-xL was from Transduction Laboratories (Lexington, KY, USA); polyclonal and monoclonal Abs to human CYP3A4 and rat CYP3A23 (clone 2.13.1) were from Oxford Biomedical Research (Oxford, MI); polyclonal Abs to PXR was from Santa Cruz Biotechnology Inc; and polyclonal Abs to Bax was from Cell signaling (Beverly, MA). Blots were reprobed with monoclonal Abs to human and rat β Tubulin (clone 2.1) from Sigma.

2.2. Cell culture and stable cell lines

Male Sprague–Dawley rats (200–250 g) were obtained from IFFA Credo France. All animals received humane care in compliance with institutional guidelines. All experiments on human tissue were in accordance with ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration. Hepatocytes from human and rat liver were isolated and cultivated as previously described [16]. Briefly, after isolation, cells were seeded onto type-1-collagen-coated dishes. Hepatocytes were cultured in medium I (William's medium E, 10% FBS, 50 UI/ml penicillin, 50 μg/ml streptomycin and 0.1 UI/ml insulin) for 4 (rat) or 18 (man) h. Then, medium I was replaced by a similar one that did not contain serum and was supplemented with hydrocortisone hemisuccinate (1 μM) and bovine serum albumin (240 μg/ml). The generation and maintenance of HepG2 and stably transfected HepG2 cells with hPXR (HepG2-hPXR) have been established in our laboratory and described [17]. HepG2-hPXR were cultured in DMEM, 10% FBS and maintained in a G418-active drug (800 μg/ml).

2.3. Cell treatment

Hepatocytes were treated with pre-defined concentrations of PXR-activated compounds: dexamethasone, DEX (10, 25, and 50 μM); phenobarbital, PB (0.5, 1, and 2 mM); rifampicin, RP (10, 25, and 50 μM); clotrimazole, CLO (1, 2.5, and 5 μM); spironolactone, SP (1, 10, and 25 μM); pregnenolone-16α-carbonitrile, PCN (1, 5, and 10 μM); and metyrapone, MP (0.1, 0.25, and 0.5 mM) for identified durations. All these compounds were prepared as a dimethylsulfoxide (DMSO) stock solution and were directly added to the cultures. The final DMSO concentration was 0.25% (v/v). HepG2 and HepG2-PXR cells were treated with DMSO, 2.5 or 5 μM CLO for 4 h (real-time RT-PCR) or 24 h (immunoblotting).

2.4. Induction of apoptosis

For apoptosis induction, rat hepatocytes were treated with 100 nM staurosporine for 16 h, afterwards 48 h treatment with PXR-activated compounds. Apoptosis was induced in HepG2 and HepG2-PXR cells using an agonistic anti-Fas antibody (CH11, clone 02, Beckman Coulter, Marseille, France). Briefly, cells were cultured until they reached 80–100% confluence. The confluent cells were rinsed three times in PBS, cultured in DMEM lacking FBS and treated with PXR-activated compounds for 24 h and then exposed to 150 ng/ml CH11 for 36 h.

2.5. Viability assay in vitro

Cell viability was determined by MTT assays. After treatment with various concentrations of PXR-activated compounds, MTT dissolved to 0.5 mg/ml in William's medium E was added to each plate well, and then the plate was incubated for 2 h at 37 °C. Absorbance at 550 nm was measured with a microplate reader (MR 7000, Dynatech Laboratories, Inc. USA).

2.6. Western blot analysis

After treatment, cells were lysed in a hypotonic buffer (25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A) supplemented with 0.1% SDS. After concentration determination (BCA Protein Assay kit), proteins were loaded onto 12% SDS-polyacrylamide gel and transferred onto PVDF membrane (Amersham Life Science, Buckinghamshire, UK). The membrane was incubated with the corresponding primary antibodies. After incubation with horseradish peroxidase-conjugated secondary antibodies (anti-mouse immunoglobulin G or anti-rabbit immunoglobulin G, Promega, Madison, WI, USA), immunodetection was performed using the ECL detection kit (Amersham Life Science).

2.7. RNA extraction and Northern blot analysis

After treatment, the medium was removed and cells were washed once with cold PBS. Total RNAs were isolated by acidic phenol extraction [18]. The total RNAs extracted were quantified with RiboGreen and 20 μg of them were size-fractionated on 1% agarose gel containing 10% formaldehyde, then were transferred onto a nylon membrane (Roche Applied Science, Meylan, France). Probes for Bcl-2 and Bcl-xL were generated by PCR with a digoxygenin conjugated-dUTP (Roche Applied Science). Prehybridisation and hybridisation were carried out in Dig Easy Hyb buffer (Roche Applied Science) at 50 °C. The blot was washed, blocked and incubated with an anti-digoxygenin antibody (Roche Applied Science) in blocking reagent. After washing, the blot was developed using the CDP Star detection kit (Roche Applied Science).

2.8. Enzymatic assay for caspase-3 activity

Caspases activity was determined by measuring the release of aminotrifluoromethylcoumarin (AFC) from the caspase tetrapeptide substrate DEVD. Briefly, cells grown in 6-well culture dishes were lysed on ice with hypotonic buffer. Protein concentrations were determined by the BCA Protein Assay kit (Pierce, Rockford, Illinois, USA) and mixed with buffer B (312.5 mM HEPES pH 7.5, 31.25% sucrose, 0.3% CHAPS, 50 μM of DEVD-AFC). The fluorescence of free AFC was measured in a fluorometer (λ_{ex}=390 nm; λ_{em}=530 nm [Fluorolite 1000, Dynatech Laboratories]).

2.9. Real-time RT-PCR

To measure the relative amount of defined mRNA, quantitative real-time PCR analysis was performed using a DNA Engine Opticon 2 system (MJ Research, Inc., Reno, NV). All primers (Table 1) were entered into the NCBI Blast program to ensure specificity. Total RNA was isolated as described before and reverse transcribed using SuperScript™ II. The resultant cDNA was diluted 10-fold and real-time PCR was performed using the ABsolute™ QPCR SYBR® Green (ABgene, Blenheim Road, Epsom, Surrey, KT19 9AP, UK) according to the manufacturer's protocol. Amplification was performed with the following PCR reaction profiles: 95 °C for 15 min and 40 cycles of 95 °C for 15 s, 57 °C for 30 s and 72 °C for 30 s. After PCR, melting curves were acquired by stepwise increases in the temperature from 65 to 95 °C to ensure that a single product was amplified in the reaction. Samples from three inde-

Table 1
Sequences of the different primers used

mRNA	Sense primers (5′–3′)	Antisense primers (5′–3′)
EFI-α	CTGGTTGGAATGGTGACA	GGTTCAGGATAATCACCTGA
fPXR	TGTTGGCCTTGTACAACG	GCCTCAATCTTTTCCCTCT
hPXR	CTTTGAAGGTCTCTCTGCA	ACCTGCAGTGTCTTCCAA
CYP3A4	CTAGCACATCATTTGGACTG	ACAGAGCTTTGTGGGACT
CYP3A23	GTGAACGTGGATTCCCTT	TTCTGTGTATCTGGGTGAG
Bcl-2	GATTGTGGCCTTCTTTGAG	CAAACTGAGCAGAGTCTTC
Bcl-xL	CAGAGCTTTGAACAGGTAG	GCTCTCGGGTGCTGTATTG

pendent time courses were analysed. All mRNA data were normalised to EF-1α mRNA.

2.10. Antisense inhibition of PXR expression

The PXR phosphorothioate (PTO) antisense oligonucleotides (AS) used in this study (5′PTO-[CTTGC]GG-AAGGGGCA-PTO[CCTCA]-3′) were synthesised by Invitrogen. Control oligonucleotide consisted of the scrambled (Scr) version of the specific PXR antisense, as follows: Scr-PXR, 5′PTO-[CTCCG]AAACGGACA-PTO[TCTGA]-3′. The oligonucleotides (300 nM) were added to the RPMI medium in the presence of lipofectin (2 μl/ml of RPMI). After vigorous shaking, the preparation was added to the cells just adhered, but not spread, on coated 6-well dishes. After 24 h, the transfection medium was replaced with William's complete medium containing 10% foetal bovine serum and the cells were maintained for 24 h. Then the cells were either untreated or treated with DMSO (0.25% v/v), or treated with 5 μM clotrimazole for 4 h (real-time PCR analysis) or 48 h (Western blot analysis).

2.11. Statistics

All experiments were repeated at least three times. Data are expressed as means ± standard deviations (S.D.). The statistical significance of the differences between various samples was determined by the Mann–Whitney *U*-test. The levels of probability are noted (*$P<0.05$ or **$P<0.001$).

3. Results

3.1. PXR activators protect hepatocytes from staurosporine-induced apoptosis

To asses the effect of PXR activators on primary cultured rat hepatocytes, cells were treated with seven known PXR activators (DEX, PB, RP, CLO, SP, PCN and MP) and viability was evaluated by MTT dye reduction assay [19]. Upon PXR agonists treatment, hepatocyte viability increased significantly as early as 24 h after the beginning of incubation (data not shown) and was marked after 48 h of treatment (Fig. 1A). Those results demonstrated that various unrelated chemicals pertaining to PXR activator compounds can improve hepatocyte life span.

In order to establish whether PXR activators exert their positive effect on cell viability through the inhibition of apoptosis process, we analysed their effects on induced apoptosis. Cells were pre-treated or not with each PXR activator and thus exposed to staurosporine (ST), a well known pro-apoptotic compound. After 100 nM ST exposure, viability was reduced to 44% and caspase-3 activity was increased by an average of 6 times, while PXR activators pre-treatment prevents hepatocytes survival drop off (Fig. 1B) and caspase-3 activation (Fig. 1C). Moreover, the pre-incubation of cells with PXR activators prevents ST-induced cell death, limiting thus the typical apoptotic morphological changes induced by ST, characterised by vacuolisation in the cytoplasm and condensation of the cytoplasm and nucleus (Fig. 1D). Taken together, these results clearly indicate that PXR activators protect hepatocytes from ST-induced apoptosis.

3.2. PXR activators induced Bcl-2 and Bcl-xL

In order to determine the mechanism by which PXR activators are able to protect hepatocytes from apoptosis, we analysed Bcl-2 and Bcl-xL expression, two well known apoptosis repressors. Northern blot analysis was performed using total RNA isolated from rat and human hepatocytes treated with PXR activators. A strong induction of *bcl-xL* mRNA expression was detected after 4 h following CLO, PCN and MP treatment in rat hepatocytes (Fig. 2A). This mRNA induction was transient and slightly declined after 24 h. With the same treatment duration (4 h), DEX, RP, SP and PB moderately affected *bcl-xL* while longer exposure (24 h) resulted in a significant induction of *bcl-xL* mRNA expression. Similar studies were performed in human hepatocytes to determine *bcl-2* mRNA expression upon PXR activator treatment (Fig. 2B). We found that all compounds, except PCN, up-regulated *bcl-2* mRNA after 48 h of treatment. These results indicate that PXR activators regulate *bcl-2* and *bcl-xL* at the transcriptional level.

Accordingly, Western blot analyses were performed in order to examine the effect of PXR activators on Bcl-2 and Bcl-xL protein expression. In addition, to asses PXR implication in this mechanism, we investigated to compare the expression level of Bcl-2 and Bcl-xL to CYP3A, a known PXR target gene and indicator of PXR activation [3,20]. As shown in Fig. 3A, PXR activators result in a parallel increase in Bcl-xL and CYP3A23 protein expression level. Indeed, Bcl-xL and CYP3A23 were strongly up-regulated upon DEX, CLO, MP and PCN. In contrast, SP slightly induced Bcl-xL expression consistent with a weak CYP3A23 up-regulation. As expected, RP treatment has no effect on CYP3A23 expression and, interestingly, resulted in a modest increase in Bcl-xL expression. Similar results were obtained after 24 or 72 h of treatment (data not shown). To determine whether Bcl-2 expression responded in a similar manner in humans, we incubated primary human hepatocytes with the same PXR activators (Fig. 3B). Bcl-2 protein level increased significantly in human hepatocytes after 72 h of treatment. As observed with Bcl-xL regulation in rat hepatocytes, Bcl-2 expression was correlated to CYP3A4 up-regulation. No Bcl-2 or Bcl-xL specific immunoreactive bands were detected in either rat or human hepatocytes, respectively. Moreover, PXR

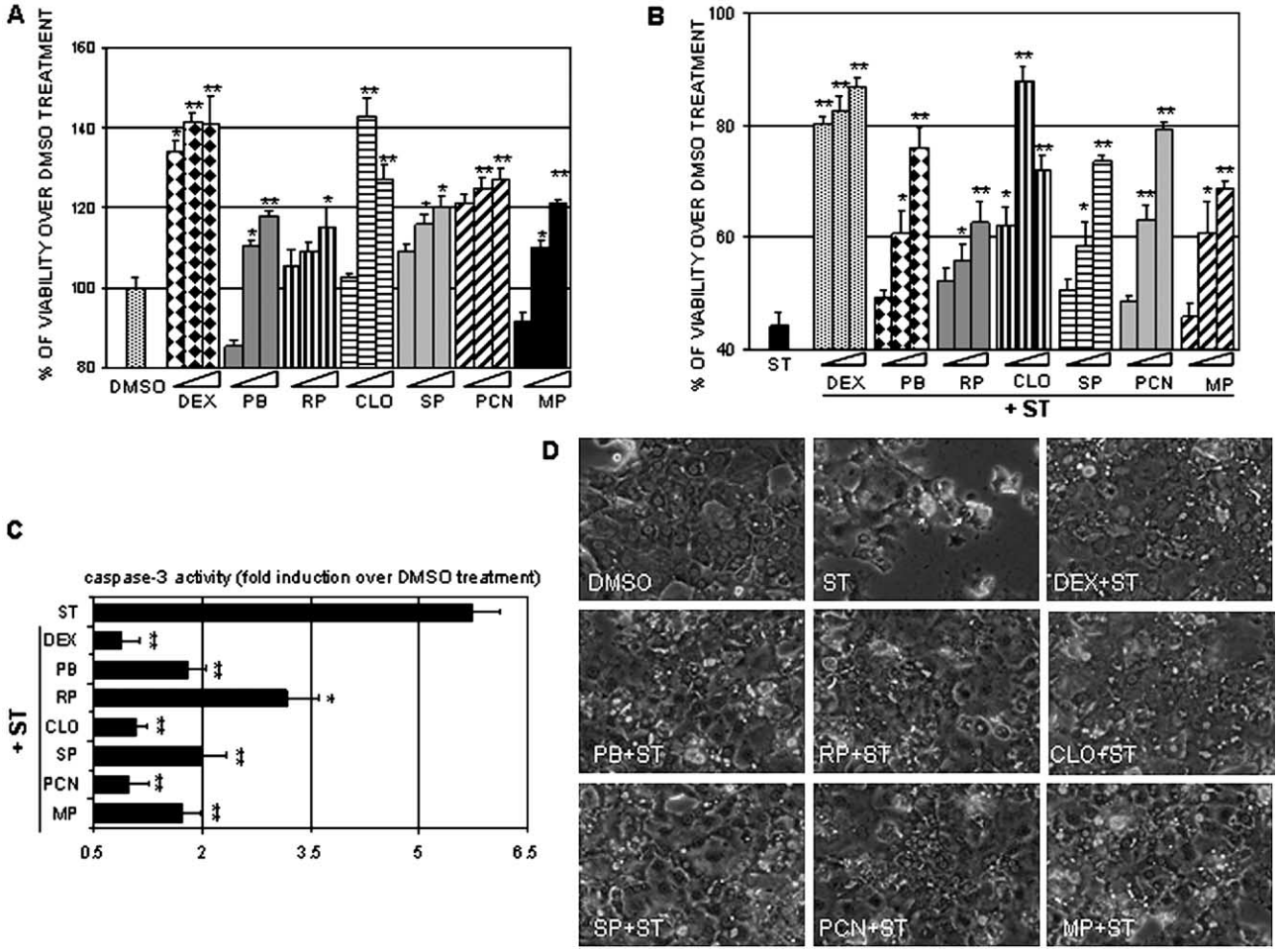

Fig. 1. PXR activators maintain rat hepatocyte viability and prevent staurosporine-induced apoptosis. (A) Rat hepatocyte viability was assessed by MTT test after 48 h treatment with increasing concentrations of DEX (10, 25, 50 μM), PB (0.5, 1, 2 mM), RP (10, 25, 50 μM), CLO (1, 2.5, 5 μM, SP (1, 10, 25 μM), PCN (1, 5, 10 μM), MP (0.1, 0.25, 0.5 mM), or DMSO (0.25%). (B) The same experiment was performed and hepatocyte viability was assessed after the induction of apoptosis by 100 nM staurosporine (ST) for 16 h. All MTT results are presented as a percentage of viability over DMSO treatment and each value is the mean ± S.D. of three separate experiments with 4 replicates (note: means ± S.D., $*P<0.05$ and $**P<0.001$ when compared to DMSO (A) or ST (B) treatment). (C–D) Cells were pre-treated with a single concentration of each PXR activator (25 μM DEX, 1 mM PB, 25 μM RP, 2.5 μM CLO, 10 μM SP, 5 μM PCN, and 25 mM MP) and then exposed to 100 nM ST for 16 h. (C) Caspase-3-like protease activities were assayed as described in the experimental procedure and the results are defined as the ratio between the mean fluorescence of treated cells versus DMSO-treated cells. Each value is the mean ± S.D. of three separate experiments with 4 replicates (note: means ± S.D., $*P<0.05$ and $**P<0.001$ when compared to ST treatment). (D) The morphological changes of rat hepatocytes were observed under phase-contrast microscopy. Cells exposed to ST exhibit morphological characteristics of apoptosis such as vacuolisation (full arrow) and nuclear shrinkage (empty arrow).

activating compounds had no effects on Bax expression level in human and rat hepatocytes.

Taken together, our results demonstrate that PXR activation induced Bcl-2 and Bcl-xL expression in both human and rat hepatocytes, protecting them from apoptosis. These data provide support for the hypothesis that PXR is implicated in the regulation of anti-apoptotic protein.

3.3. Effects of PXR-antisense oligonucleotides on PXR expression

To address the implication of PXR in regulating Bcl-xL expression, we inhibited PXR expression by using an antisense oligonucleotide directed against rPXR. Rat hepatocytes were transfected with PXR phosphorothioate antisense oligonucleotides (AS-PXR) or the corresponding scrambled oligonucleotide (Scr-PXR) and maintained for 24 h to permit PXR decrease. Transfected hepatocytes were then treated or not with DMSO or CLO for 4 h (mRNA analysis) or 24 h (protein level). Transfection with AS-PXR reduces by ~75% the PXR mRNA and protein levels, as measured by real-time RT-PCR (Fig. 4A) and immunoblotting (Fig. 4B), respectively. Scrambled oligonucleotides had no effect whatsoever. To confirm the efficiency of the PXR depletion, we analysed the CYP3A23 mRNA level, a gene known to be regulated by the rPXR. Fig. 4C shows that AS-PXR treatment decreased the induction of CYP3A23 mRNA by clotrimazole. This data indicated that the oligonucleotides used in this study are well efficient and allowed us to analyse their effect on Bcl-xL induction.

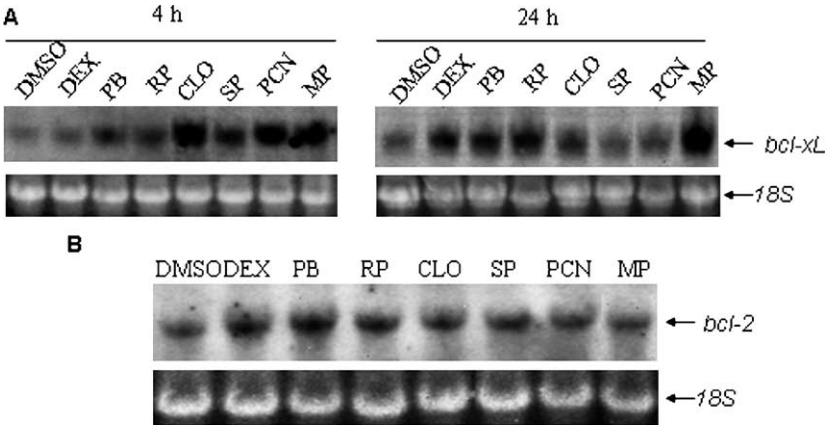

Fig. 2. PXR activators increased *bcl-2* and *bcl-xL* mRNA level in the primary culture of rat and human hepatocytes. Cells were treated with a single concentration of each PXR activator: 25 μM DEX, 1 mM PB, 25 μM RP, 2.5 μM CLO, 10 μM SP, 5 μM PCN, and 25 mM MP. (A) After 4 or 24 h treatment, 20 μg of total RNA extracted from rat hepatocytes was size-fractionated on 1% formaldehyde gel, transferred to a nylon membrane and hybridised to *bcl-xL* probes as described. (B) Human hepatocytes were treated with the same compound concentrations as rat hepatocytes. The *bcl-2* mRNA level was analysed by Northern blotting following 48 h treatment. Results are those from one experiment representative of three independent experiments. Ribosomal RNA (*18S*) was used as loading control.

3.4. Effects of PXR-antisense oligonucleotides on Bcl-xL expression

Rat hepatocytes were transfected with AS-PXR for 24 h and maintained for 24 h to permit PXR mRNA and protein levels to decrease. Cells were then either treated or not with DMSO or with CLO for 4 h or 48 h, and processed in real-time PCR and Western blotting analysis, respectively. As shown in Fig. 5A, CLO induction of *bcl-xL* was drastically reduced by AS-PXR (~80%), as observed under scrambled control oligonucleotides treatment. Accordingly, the up-regulation of Bcl-xL protein expression following clotrimazole treatment was abolished in the presence of AS-PXR (Fig. 5B). Similar results were obtained with PCN (data not shown). These results show that PXR expression is essential for Bcl-xL up-regulation under PXR activators treatment.

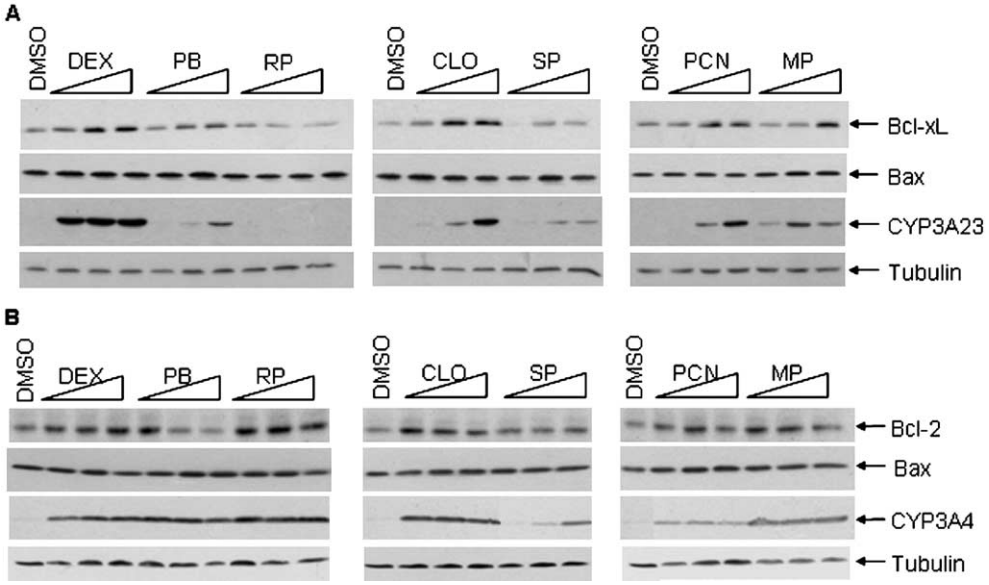

Fig. 3. Parallel up-regulation between Bcl-xL or Bcl-2 and CYP3A23 or CYP3A4 by PXR activators in cultured rat and human hepatocytes. (A) Rat hepatocytes were treated with DMSO (0.25%) and increasing concentrations of DEX (10, 25, and 50 μM), PB (0.5, 1, and 2 mM), RP (10, 25, and 50 μM), CLO (1, 2.5, and 5 μM), SP (1, 10, and 25 μM), PCN (1, 5, and 10 μM), and MP (0.1, 0.25, and 0.5 mM). After 48 h treatment, cells were harvested and lysed as described for the examination of Bcl-xL and CYP3A23 expression by immunoblotting. (B) Human hepatocytes were treated with the same compound concentrations as rat hepatocytes. After 72 h treatment the Bcl-2, Bax and CYP3A4 protein level was examined by immunoblotting. The immunoblots shown here represent the typical results from four independent experiments. The detection of Tubulin proteins was included as a control for loading and membrane transfer.

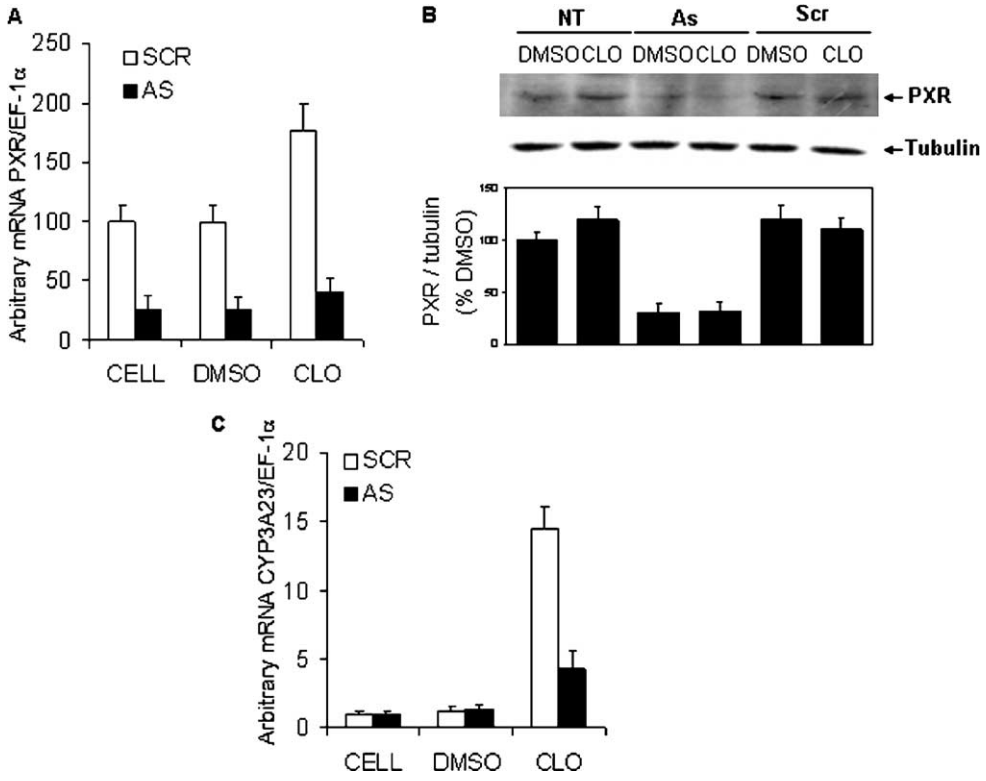

Fig. 4. The effect of PXR antisense oligonucleotide treatment on PXR mRNA and protein expression in rat hepatocytes. Rat hepatocytes were untreated (NT) or treated with AS-PXR or a corresponding scrambled (Scr) oligonucleotide (300 nM each) for 24 h and maintained for 24 h. Cells were then either untreated or treated with 0.25% DMSO or 5 μM CLO. The relative mRNA levels of PXR (A) and CYP3A23 (C) were determined after 4 h treatment by real-time RT-PCR and normalised against EF-1α RNA. The ratio (means ± S.D. for three independent experiments) was expressed as a ratio over untreated Scr cells (CELL). (B) Total proteins were submitted to immunoblotting with anti PXR antibody. The quantification of chemiluminescence was performed after the acquisition with a CCD camera and ratios (means ± S.D. for three independent experiments) were expressed as a percentage of the mean value in DMSO-treated cells without AS treatment (NT) normalised by tubulin.

3.5. Overexpression of PXR in hepatoma cell lines leads to Bcl-2 up-regulation

To further confirm the role of PXR in Bcl-2 induction, we used HepG2 stably transfected with PXR (HepG2-PXR). The PXR protein levels in HepG2 and HepG2-PXR were monitored by Western blotting and are represented in Fig. 6A. According to the stable expression, PXR protein was detectable only in HepG2-PXR. Moreover, and as expected, CLO increased the expression of CYP3A4 at both mRNA and protein levels (Fig. 6B and C, respectively) in HepG2-PXR whereas only a slight expression level was observed in

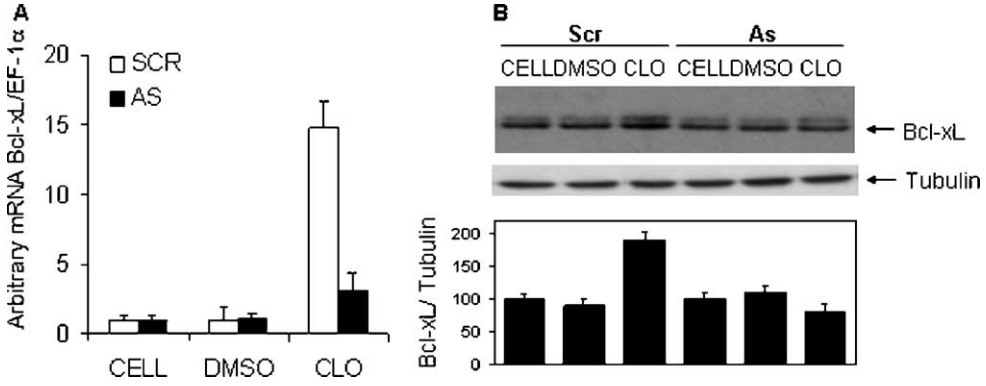

Fig. 5. The effect of PXR antisense oligonucleotide treatment on Bcl-xL mRNA and protein expression in rat hepatocytes treated with clotrimazole. Cells were transfected with AS-PXR or a corresponding scrambled (Scr) oligonucleotide (300 nM each) for 24 h and maintained for 24 h. Cells were then either untreated or treated with 0.25% DMSO or 5 μM CLO. (A) Relative mRNA levels of Bcl-xL after 4 h treatment were determined by real-time RT-PCR and normalised against EF-1α RNA. The ratio (means ± S.D. for three independent experiments) was expressed as a ratio over untreated Scr cells (CELL). (B) Bcl-xL expression was examined by immunoblotting upon 24 h treatment. Protein bands (means ± S.D. for three independent experiments), acquired using a CCD camera, were expressed as a percentage of the mean value in untreated Scr cells (CELL) normalised by tubulin.

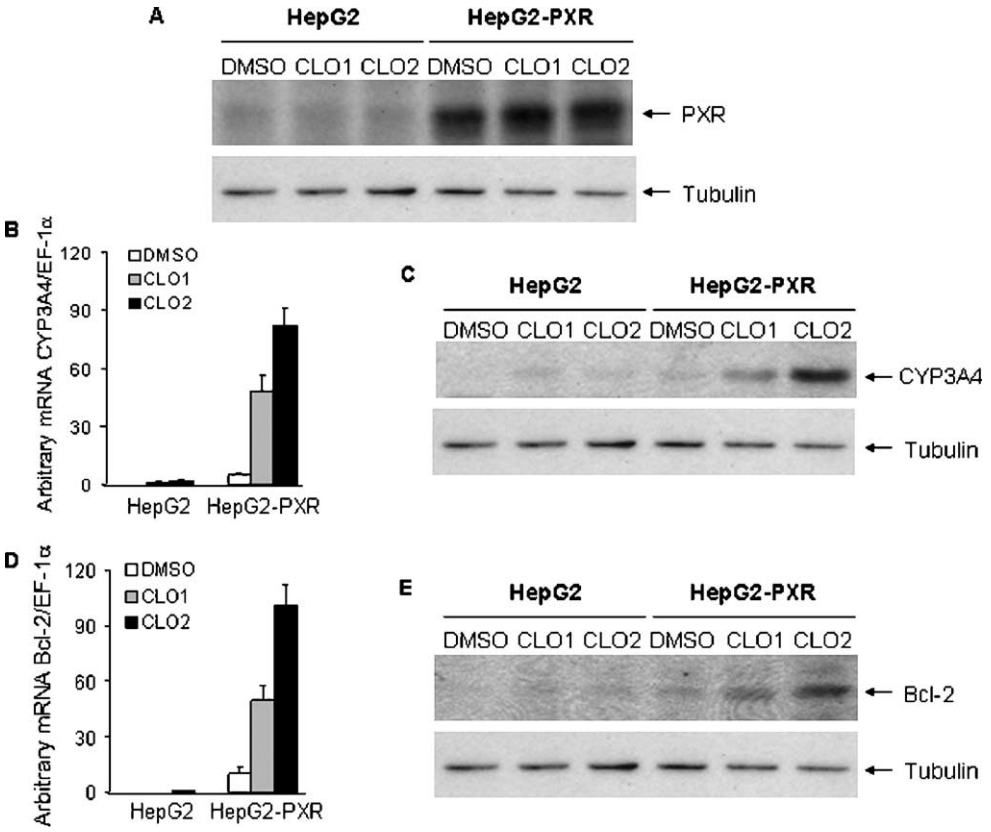

Fig. 6. PXR overexpression and activation-induced Bcl-2. HepG2 or HepG2-PXR were treated with vehicle (DMSO) or CLO (CLO1 = 2.5 μM, CLO2 = 5 μM). (A, C, E) Cells were treated for 24 h and subjected to immunoblotting to PXR (A), CYP3A4 (C) and Bcl-2 (E) protein level examination. The detection of Tubulin proteins was included as a control for loading and membrane transfer. (B, D) After 4 h treatment, relative CYP3A4 (B) and Bcl-2 (D) mRNA levels were measured by real-time PCR analysis and normalised against EF-1α mRNA. The ratio (means ± S.D. for three independent experiments) was expressed as a ratio over CLO1 treatment (B) or CLO2 treatment (D).

HepG2. This experiment showed that the PXR protein stably expressed in HepG2 transfected cells is able to be activated by PXR activation.

Bcl-2 mRNA (Fig. 6D) and protein level (Fig. 6E) were up-regulated in HepG2-PXR upon 4 h and 24 h CLO treatment respectively in a dose-dependent manner, while no significant expression level was detectable in HepG2. These data demonstrate that PXR activation in HepG2-PXR leads to Bcl-2 induction.

3.6. PXR activators protect HepG2-PXR but not HepG2 cells against Fas-induced apoptosis

In order to determine whether PXR activation and the subsequent Bcl-2 induction could protect cells from apoptosis, we analysed the effect of PXR activators on spontaneous and Fas-induced apoptosis in HepG2 as well as HepG2-PXR cells. Cells were pre-treated or not with each PXR activator and thus exposed to CH11, an agonistic anti-Fas antibody. After 200 ng/ml CH11 exposure, caspase-3 activity was increased by an average of 4.5 and 2.5 times in HepG2 and HepG2-PXR cells, respectively (Fig. 7). Thus, it appears that the parental cell line is more sensitive to Fas

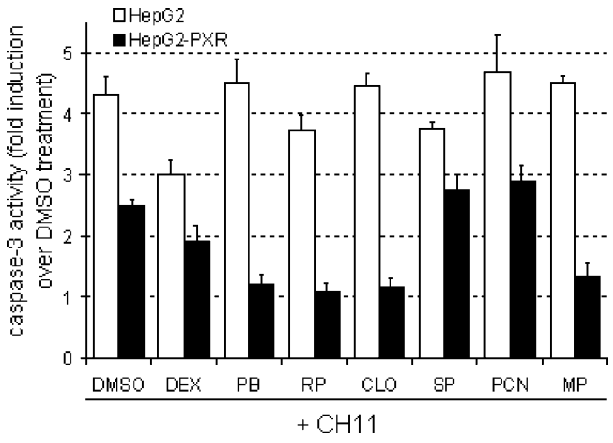

Fig. 7. PXR overexpression and activation protected HepG2 from Fas-induced apoptosis. HepG2 and HepG2-PXR cells were pre-treated for 24 h with a single concentration of each PXR activator: DEX (50 μM), PB (1 mM), RP (25 μM), CLO (2.5 μM), SP (10 μM), PCN (5 μM), MP (0.25 mM), or DMSO (0.25%) and then exposed or not to 200 ng/ml CH11 for 36 h. Caspase-3-like protease activities were assayed as described in the experimental procedure and results are defined as the ratio between the mean fluorescence of treated cells exposed to CH11 versus DMSO-treated cells. Each value is the mean ± S.D. of three separate experiments.

activation than HepG2-PXR. PB, RP, CLO and MP pretreatment prevents HepG2-PXR caspase-3 activation after Fas activation (Fig. 7) whereas no effect was observed in HepG2. This experiment showed that PXR overexpression and Bcl-2 induction are able to suppress Fas-induced apoptosis.

The sum of ours results demonstrates that PXR is essential for the induction of Bcl-2 and Bcl-xL in human and rat primary cultured hepatocytes upon PXR agonist treatment and can protect cells against apoptosis.

4. Discussion

This study identifies PXR activators as modulators of apoptosis in the liver. They are able to protect hepatocytes against spontaneous and staurosporine-induced programmed cell death by increasing the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL through PXR activation without modifying Bax expression, a pro-apoptotic member of the Bcl-2 family. Under normal culture conditions, isolated hepatocytes lose their liver-specific functions and spontaneously die as a result of apoptosis modulating the mitochondria pathway with Bad translocation, cytochrome *c* release and processing of caspase-9 and -3. The Bcl-2 protein family constitutes a critical intracellular checkpoint in the intrinsic pathway [21] and protein concentration may act as a rheostat for the suicide program. Indeed, it is now established that anti-apoptotic members of the Bcl-2 family prevent a wide variety of cells from undergoing apoptosis, as induced by apoptotic stimuli. Furthermore, Bcl-2 and Bcl-xL overexpression blocks apoptotic liver injury in transgenic mice [22–24]. Indeed, these two pro-survival proteins are known to confer resistance against hepatic apoptosis. Notably, Bcl-2 overexpression can prevent fulminant liver apoptosis [25,26] and antisense oligonucleotide directed against Bcl-2 caused spontaneous apoptosis in human hepatocellular carcinoma cells [11]. Similarly, Bcl-xL expressed in hepatoma cells play a determinant role in suppressing apoptosis [27]. These data argue the importance of Bcl-2 and Bcl-xL in the inhibition of apoptosis.

We found that rifampicin is an effective inducer of Bcl-2 in human hepatocytes but had virtually no action on Bcl-xL expression in rat hepatocytes, consistent with the observation that rifampicin activated hPXR but had virtually no activity on rPXR [3,20,28]. However, this compound is able, in a slight but effective manner, to prevent staurosporine-induced apoptosis in rat hepatocytes probably according to a rPXR-independent pathway. Along the same lines, PCN, a known potent rPXR rather than a hPXR activator [14], induced much more Bcl-xL than Bcl-2. However, we noted an appreciable amount of Bcl-2 protein in response to PCN treatment while no mRNA induction was observed, consistent with a post-translational PXR-independent mechanism. PXR was remarkably divergent between species, sharing only 76% amino acid identity in ligand-binding domains (LBD) between rat and human sequences [14]. It is now well established that this sequence divergence constitutes the molecular basis for the species-related differences noted in CYP3A regulation by xenobiotics. In this way, we proposed that Bcl-2 and Bcl-xL could be differently regulated in human and rat respectively depending on the PXR activator structure, consistent with the LBD divergence between human and rat PXR.

Our studies underscore an intriguing relationship between Bcl-2/Bcl-xL induction and PXR activation, as monitored by CYP3A expression. These observations lead us to test the implication of PXR in the regulation of Bcl-2 anti-apoptotic proteins. In this way, we used specific PXR phosphorothioate antisense oligonucleotides to determine whether PXR was responsible for PXR-activators-induced Bcl-xL expression. We found that clotrimazole-induced (and PCN-induced, data not shown) Bcl-xL up-regulation was reduced by antisense oligonucleotides targeted against the rPXR in the primary cultures of rat hepatocytes. So, it appears that PXR is involved, either directly or indirectly, in Bcl-xL regulation as induced by PXR-activating compounds. To further investigate the necessity of hPXR in regulating *bcl-2* expression, we used HepG2 hepatoma cells stably transfected with PXR. Importantly, this PXR overexpression leads to the induction of Bcl-2 by clotrimazole, demonstrating that PXR plays an important role in the regulation of Bcl-2. Moreover, we found that HepG2 cells are more sensitive than HepG2-PXR to Fas-induced apoptosis, suggesting a positive effect of PXR expression on hepatoma cell

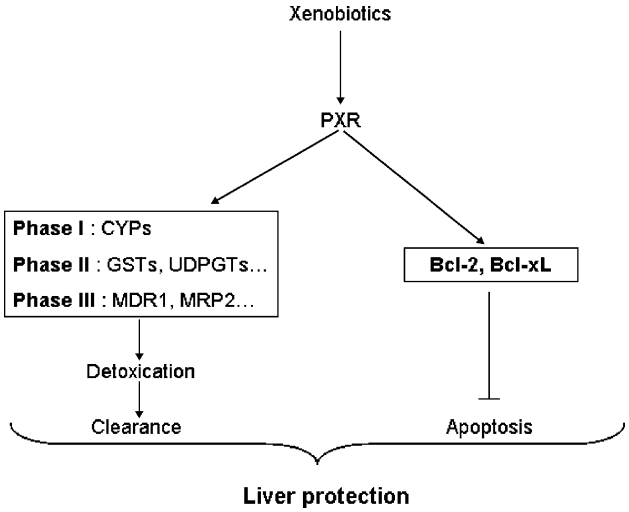

Fig. 8. A model of liver-protective effect of PXR. We suggested that the protective effect of PXR in the liver results in the combinatory effect of the (1) transcriptional stimulation of genes implicated in the detoxication process (Phases I, II and III), responsible for the clearance process of xenobiotics, and (2) increased concentration of Bcl-2 and Bcl-xL, two major apoptosis-inhibitory proteins and pro-survival guardians. The cumulative resultant lead to promotion of liver cells survival.

survival. It is reported that agonistic anti-Fas antibody induces apoptosis in HepG2 cells through the activation of caspase-8 and caspase-3 [46], and that Bcl-2-overexpressed HepG2 cells resist apoptosis induced by CH11 clone antibody [11]. These finding are compatible with our results that show that PXR activators could regulate Bcl-2 and Bcl-xL expression and protect cells against mitochondrial and Fas apoptosis.

Our results were consistent with several reports which described PXR activators as protective compounds against intoxication [29–31]. It is well known that rifampicin induced the remission of cholestasis [32] in some instances, although the molecular basis of that clinical effect remains unclear. In this liver syndrome, hepatocytes die by apoptosis [33] through Fas direct activation [34,35], secondary to hydrophobic and toxic bile salt retention. It has also been shown that bile-acid-induced apoptosis is dependent on Bid [36], the pro-apoptotic member of the Bcl-2 family, whose cleavage and translocation to the mitochondria are associated with cytochrome *c* release [37,38]. Bcl-xL has been shown to antagonise Bid-induced apoptosis [39] and can prevent Fas- and TNFα-induced cell death and inhibit downstream caspase activation [40,41]. We therefore suggest that PXR activators could notably protect the liver against cholestasis by up-regulating Bcl-xL and so limiting apoptosis. Moreover, PCN treatment in vivo causes hepatomegaly [42] through cellular hypertrophy and hyperplasia in mice [43]. These effects were absent in PXR-null mice [44]. Constitutive expression of an active form of human PXR also led to hepatomegaly in PXR-null mice [45]. This phenomenon could be due either to a proliferative effect of PXR or to apoptosis inhibition. In any case, apoptosis is viewed as a hepatocyte renewal process under normal conditions, even if the percentage of apoptotic hepatocytes is low. However, one possible cause of hepatomegaly could be apoptosis inhibition secondary to the up-regulation of anti-apoptotic proteins belonging to the Bcl-2 family. These facts and our results suggested biological pathways other than the PXR-regulated detoxication process.

In summary, we found that PXR was implicated in Bcl-2 or Bcl-xL regulation and promoted cell survival. We proposed a model (Fig. 8) where PXR coordinately regulates Phase I, II, III and anti-apoptotic proteins, so as to protect hepatocytes from the harmful effects of endo- and xenobiotics. Based upon the present results and literature, we suggest that PXR serves as a physiological sensor that coordinately regulates gene expression in the liver to promote its survival.

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