Hexachlorobenzene induces deregulation of cellular growth in rat liver

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ABSTRACT

Hexachlorobenzene (HCB) is an organochlorine pesticide widely distributed in the biosphere. *The aim of the present study was to investigate* the effect of HCB on the homeostasis of liver cell growth, analyzing parameters of cell proliferation and apoptosis, in HCB (0.1, 1, 10 and 100 mg/kg body weight)-treated rats, during 4 weeks. Cell proliferation and ERK1/2 phosphorylation, associated with survival mechanisms, were increased at HCB 100 mg/kg. The pesticide increased the number of apoptotic cells, and the activation of caspase-3, -9 and -8, in a dose-dependent manner, suggesting that HCB-induced apoptosis is mediated by caspases. Increased Fas and FasL protein levels indicate that the death receptor pathway is also involved. This process is associated with decreased Bid, and increased cytosolic cytochrome c protein levels. Transforming growth factor-beta1 (TGF-β1) intervenes in apoptotic and/or proliferative processes in hepatocytes. TGF-β1 cDNA and protein levels are dose-dependently increased, suggesting that this cytokine might be involved in HCB-induced dysregulation of cell proliferation and apoptosis. In conclusion, this study reports for the first time that HCB induces loss of the homeostatic balance between cell growth and cell death in rat liver. Induced apoptosis occurs by mechanisms involving signals emanating from death receptors, and the mitochondrial pathway.

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1. Introduction

Hexachlorobenzene (HCB) is a persistent environmental pollutant, currently formed as a byproduct in several industrial processes. HCB exposure is associated with a wide variety of toxic effects in humans and experimental animals. Serious hepatotoxic, neurotoxic, reproductive, developmental and carcinogenic effects have been reported (ATSDR, 2002). It has also been demonstrated that HCB is an inducer of liver foci growth in rats (Ou et al., 2003). It is thought that nongenotoxic carcinogens, like HCB, act by interfering with molecules involved in cell growth and death (Mally and Chipman, 2002). We have previously demonstrated that HCB stimulates epidermal growth factor receptor (EGFR) signaling pathway and c-Src kinase activity in rat liver (Randi et al., 2008).

Whereas the cytotoxic and carcinogenic effects of HCB in the liver have been studied, there are not available reports on the mechanisms regulating cell proliferation/apoptosis balance in the liver of HCB-treated rats. Imbalances between cell proliferation and cell death in the liver, contributes to the pathogenesis of acute and chronic liver diseases (Guicciardi and Gores, 2005). A chronic apoptotic stimulus can predispose to cancer development due to the high rate of regeneration invoked in the tissue, which elevates the risk of mitotic errors (Nzeako et al., 2002; Pikarsky et al., 2004).

Caspases, the key effectors of apoptosis, can be activated by extrinsic and intrinsic stimulus. In both pathways, signaling results in the activation of a family of caspases resulting in nuclear degradation of DNA. Extrinsic or death receptor-mediated pathway is triggered by extracellular death ligands, such as the tumor necrosis factor (TNF) and Fas ligand (FasL). Binding of TNF-α and FasL to their respective receptors induce apoptosis through the apical caspase-10 and caspase-8 pathways (Wang et al., 2001). Active caspase-8 may induce apoptosis either directly following activation of caspase-3 or indirectly following cleavage of cytosolic factors, such as the pro-death Bcl-2 family protein, Bid, to a truncated Bid (tBid), which translocates to the mitochondrial membrane, leading to the release of cytochrome c (Li et al., 2002). When present in the cytoplasm, cytochrome c interacts with apoptotic activator factor-1 (Apaf-1) and, in concert with caspase-9, forms the apoptosome, and triggers the caspase cascade and subsequent apoptosis (Guicciardi and Gores, 2005).

Intrinsic apoptosis is initiated intracellularly, by various forms of cellular stress, such as generation of reactive oxygen species (ROS), and involves loss of mitochondrial trans-membrane potential and

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the release of cytochrome *c* (Newmeyer and Ferguson-Miller, 2003).

Members of the transforming growth factor-beta (TGF-β) family are fundamental signals regulating cell behavior in a variety of cellular contexts (ten Dijke and Hill, 2004). Expression of TGF-β1 may be a critical step in the growth control of normal and proliferating rat hepatocytes. High levels of expression of active TGF-β1 has been implicated as a mechanism to control hepatocyte growth by apoptosis (Schrum et al., 2001).

The aim of the present study was to investigate molecular alterations in liver from HCB-treated rats, to determine whether they induce an imbalance in the homeostasis of cell growth, analyzing parameters of cell proliferation and apoptosis. Furthermore, we evaluated the effect of HCB on TGF-β1 gene expression and protein levels, which may be involved in cell growth imbalance.

2. Materials and methods

2.1. Reagents

HCB (>99% purity, commercial grade) was purchased from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). The primary antibodies against active caspase-8 (D-8), Fas, FasL, Bid and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody, were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against active caspase-9, active caspase-3, full length caspase-10, phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2 and histone H2B were from Cell Signaling Technology, (Beverly, MA). Anti-cytochrome *c* was from BD Biosciences (San Jose, CA); anti-β-actin from Sigma–Aldrich (St. Louis, MO); anti-digoxigenine was from Biogenex (San Ramon, CA); and anti proliferating cell nuclear antigen (PCNA) antibody from *Dako Cytomation* (Denmark A/S); anti-TGF-β1 was from Abcam Inc. (Cambridge, MA), anti 5′-Bromo-2-deoxyuridine (BrdU) monoclonal antibody was from Biogenex (San Ramon, CA) and Texas Red dye conjugated AffiniPure goat antimouse IgG, was from *Jackson ImmunoResearch Laboratories*, Inc. (PA, California). Hoescht 33342 fluorocrom, TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were obtained from Invitrogen Life Technology (Carlsbad, CA). The random primers, enzymes, and cofactors for reverse transcription (RT) and PCR were purchased from Promega Corporation (Madison, WI). All reagents used were of analytical grade.

2.2. Experimental animals and treatment

Female Wistar rats (160–180 g) were purchased from the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina. The rats were fed Diet 3 rat chow (Asociación Cooperativas Argentinas, División Nutrición Animal) and water *ad libitum*. Environmental conditions consisted of a 12-h light/12-h dark cycle, 20–24 °C, and 45–75% humidity. HCB was administered daily by gavage for 5 days a week during 4 weeks, at doses of 0.1, 1, 10, and 100 mg/kg body weight (bw). HCB was administered as a suspension in water, containing Tween 20 (0.5 ml/100 ml). Control animals received equal volumes of the solvent by the same route. The general health of the animals was not affected by the doses of HCB employed, as evaluated by the behavior and appearance of the rats, including examination of the coat, mucous membranes, and body weights, and their food and water consumption. The animals were killed by decapitation, blood was obtained, serum prepared, and frozen at −80 °C. All animal procedures were undertaken in accordance with the Institutional Guidelines for Animal Care and Research.

2.3. Subcellular fractionation

Livers were homogenized in homogenization buffer (0.25 M sucrose, 10 mM NaCl, 5 mM MgCl$_2$, 50 mM Tris, 0.2% Triton, pH 7.5, 10 mM NaF, 2 mM Na$_3$VO$_4$, 1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A) (1:3, w/v). The homogenate was centrifuged at 1220 × g for 4 min to obtain the nuclear pellets. The supernatant layer was centrifuged for 20 min at 14 000 × g to remove mitochondria. The resulting supernatant was used for cytosolic and membrane proteins assays. The nuclear pellets were resuspended in homogenization buffer without Triton, and layered over a 2 M sucrose cushion prepared in homogenization buffer, and centrifuged at 100 000 × g for 60 min. The nuclear pellet was resuspended in lysis buffer (20 mM Tris, 0.3 M KCl, 1 mM MgCl$_2$, 2 mM DTT, pH 8), and incubated at 4 °C for 1 h. The resuspended pellet was centrifuged at 1220 × g for 15 min, to yield a supernatant fraction containing nuclear proteins. Protein concentration was determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

2.4. Western blotting

Rat liver cytosolic and nuclear fractions were subjected to 12% SDS-PAGE, transferred to polivinylidene difluoride membranes (PVDF) (Millipore, Bedford, MA),
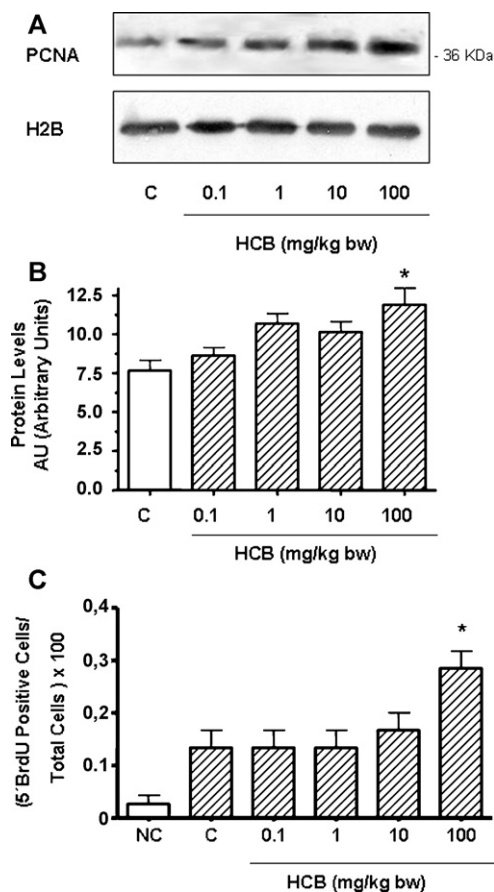

Fig. 1. Effect of HCB on liver cell proliferation. (A) Western blot analysis of PCNA in the nuclear fraction of rat liver tissue from rats treated with HCB (0.1, 1, 10, and 100 mg/kg bw) during 4 weeks. A western blot from one representative experiment is shown in the upper panel. H2B: Histone H2B. (B) Quantification of the PCNA protein levels by densitometric scanning of immunoblots. (C) BrdU incorporation assay. Number of BrdU labeled cells over total number of cells (LI). Values are means ± SEM of three independent experiments of five rats per group. *Significantly different from control rats ($p < 0.05$).

and immunodetected using appropriate primary and secondary antibodies, and visualized by enzyme-linked enhanced chemoluminescence kit (Amersham Biosciences, Inc., UK) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3. The antibodies used for Western blotting were: rabbit polyclonal anti-cleaved caspase-9, anti-cleaved caspase-3, anti-FasL, anti-phospho ERK1/2 (Thr-202/Tyr 204), anti-ERK1/2, and anti-histone H2B at (1:500) dilution. Rabbit polyclonal anti-Bid was used at (1:200) dilution. Mouse monoclonal anti-cleaved caspase-8, anti-caspase-10, anti-cytochrome *c*, anti-PCNA, and anti-TGF-β1 were used at (1:500) dilution and anti-β-actin at (1:2000) dilution. The suitable horseradish peroxidase-conjugated anti-species-specific antibodies (1:1000) were used for protein detection.

2.5. Cell proliferation measured by 5′-Bromo-2-deoxyuridine (BrdU) incorporation assay

Thirty minutes prior to sacrifice, the animals were given an i.p. injection of a solution (5 mg/ml) of BrdU at a dose of 0.08 mg BrdU/g bw. BrdU incorporation was stained in tissue sections by an indirect immunohistochemical technique. Briefly, the histological sections were dewaxed and antigen retrieval was performed in a microwave oven with citrate buffer, pH 6. Endogenous peroxidase was inhibited, incubating with methanol–hydrogen peroxide (3%, v/v). Nonspecific blocking was performed by washing in saline Phosphate buffer (PBS) containing 0.3% Triton X-100 and 1% BSA. The sections were incubated overnight, with the mouse anti-BrdU antibody (1:40), and washed with PBS three times. After incubation with the secondary antibody at room temperature, for 1 h in a moist chamber, slides were counterstained with Hoechst 33342 (1 μg/ml in PBS) to locate nuclei. Adjacent sections incubated without the primary antibody were used as negative controls. A red fluorescent mark indicated the presence of BrdU. The labeling index (LI) was calculated as the number of labeled cells with BrdU over the total number of cells employing image analysis software developed *ad hoc* to distinguish between positive nuclei
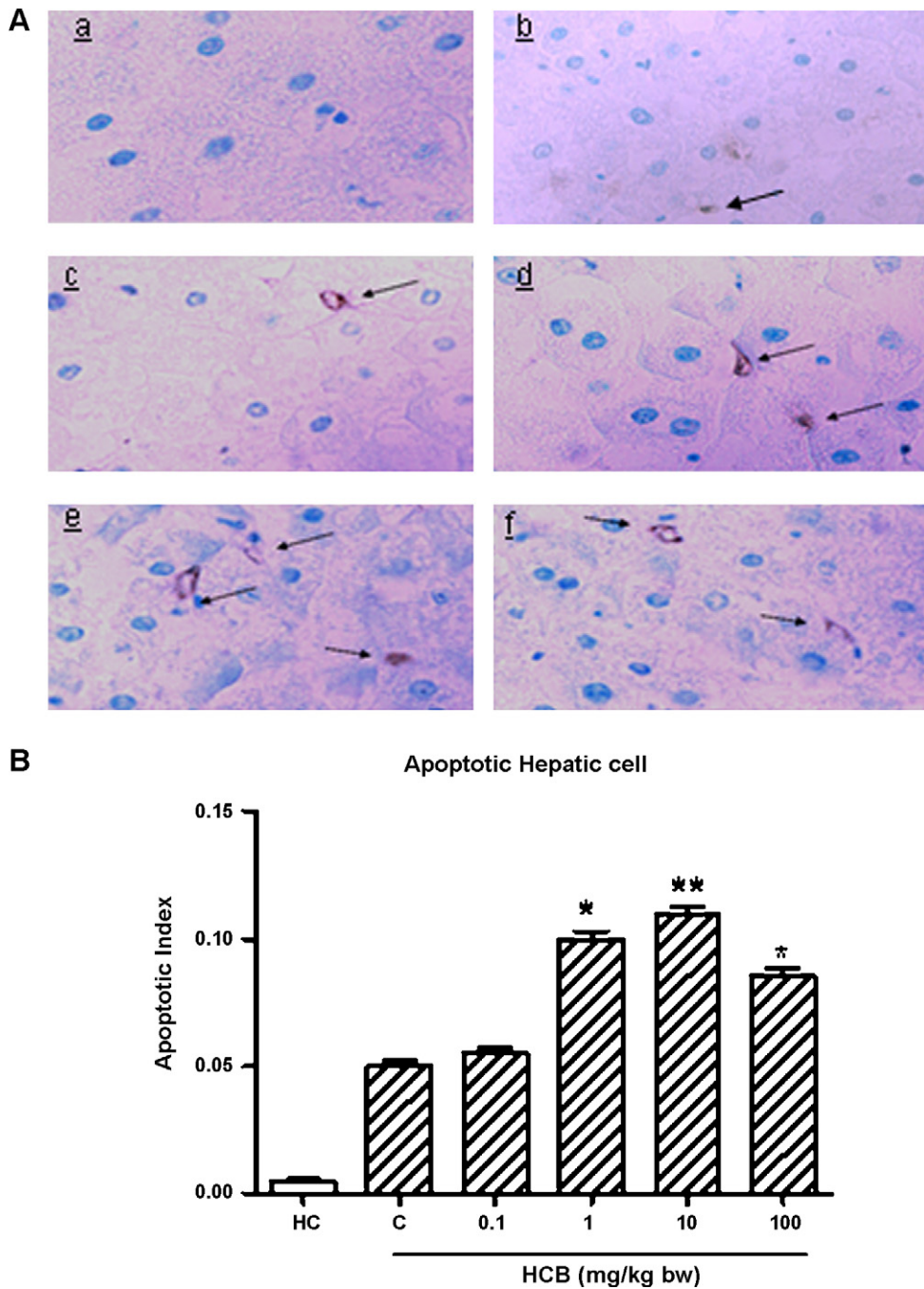
Fig. 2. Detection of *in situ* DNA breaks by TUNEL staining. (A) TUNEL-positive cells from control and HCB-treated rats. (a) Negative control (NC), (b) control, (c) 0.1 mg/kg, (d) 1 mg/kg, (e) 10 mg/kg and (f) 100 mg/kg. Magnification 40×. Arrows point to brown stained apoptotic cells. (B) Values represent the mean ± SEM of TUNEL-positive cells (n: 1000 cells per rat) from three independent experiments of five rats per group. * and ** significantly different from control rats ($p < 0.05$ and $p < 0.01$), respectively.

and Hoescht stained nuclei. Two to five fields were evaluated for each category. One thousand nuclei (labeled plus unlabeled) of each group were counted.

2.6. DNA-fragmentation studies

Apoptotic nuclei in tissue sections of rat liver were identified by detection of the DNA breaks with the terminal deoxynucleotidyl transferase-mediated deoxy uridine triphosphate nick-end labeling technique (TUNEL). In brief, the paraffin-embedded tissue sections were deparaffinized by xylene and ethanol. Sections were then incubated with 20 μg/ml proteinase K in PBS, followed by quenching of endogenous peroxidase with 3% H_2O_2. Next, digoxigenin-dUTP was added to the 3′-OH ends of DNA fragments by the enzyme terminal deoxynucleotidyl transferase (TdT). After incubating with anti-digoxigenin antibody conjugated with horseradish peroxidase, the sections were stained with hydrogen-peroxidase-activated diamine benzidine (DAB) and counterstained with methyl-green. Negative control was performed by

the omission of the TdT reaction step. The apoptotic index (AI) was determined as the percentage of positively stained apoptotic cells in the total number of nuclei observed (n: 1000 nuclei per rat). Image-Pro Plus v4.5 (Media Cybernetics Inc., Silver Spring, MD) software was used for image capturing and cell counting. Photographic figures show a representative section of part of one field of view.

2.7. Immunohistochemistry

The liver slices were fixed in 4% paraformaldehyde in PBS for 24 h. Subsequently, the slices were incubated in 0.1% Triton X-100 in PBS for 15 min. Then a primary mouse monoclonal anti-Fas antibody (1:50) in PBS/1.0% BSA, and a FITC-conjugated goat anti-mouse IgG secondary antibody (1:100) in PBS/1.0% BSA, were added, and incubated overnight at 4 °C. Finally, the slices were rinsed in PBS/1.0% BSA for 10 min and nuclei were assessed after staining with 8 μM Hoescht 33342 (1:1000) in PBS/1.0% BSA. After incubation, slices were washed twice with PBS/1.0%

BSA for 10 min. Negative controls were prepared, replacing both primary antibodies with normal serum. The immunohistochemical staining was analyzed according to the staining intensity using a fluorescence confocal Nikon C1 (Plan Apo 40x/0.95) microscope with lasers at excitation wavelengths of 488 nm. The digital images were transferred to Adobe PhotoShop 5.5 software for color channel analysis and figure assembly.

2.8. RT-PCR analysis of TGF-β1 mRNA

Rat livers were dissected out, frozen and stored at −80 °C, prior to RNA extraction according to Chomczynski and Sacchi (1987). An aliquot of 2 μg of total RNA was used to synthesize first-strand cDNA with the random primers, deoxynucleotide triphosphates, transcriptase reverse (RT), and transcriptase reverse buffer. Reaction mixture for TGF-β1, and GAPDH amplification contained GoTaq reaction buffer (1.5 mM MgCl$_2$, 0.2 mM dNTP's mix, 1.25 U GoTaq DNA polymerase, 0.5 μM of each forward and reverse primers, and 1 μl of RT products). PCR was performed as follows: reaction mixture for TGF-β1 amplification was incubated at 94 °C for 5 min and then amplified at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Reactions were repeated for 27 cycles. Reaction mixture for GAPDH amplification was incubated at 94 °C for 5 min and then amplified at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Reactions were repeated for 27 cycles. The sequences of forward and reverse primers were as follows:

TGF-β1: 5′-CTGCTGGCAATAGCTTCCTA-3′, 5′-CGAGCCTTAGTTGGACAGGAT-3′.
GAPDH: 5′-TGAACGGGAAGCTCACTGG-3′, 5′-TCCACCACCCTGTTGCTGTA-3′.

GAPDH cDNA was used as a loading control. PCR products were detected as a single band on 2% agarose gel, containing 0.05% (v/v) ethidium bromide. Bands were detected, and intensity was quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

2.9. Statistical analysis

Data are expressed as mean ± SEM. Differences between treated and control groups were analyzed by one-way ANOVA, at a 95% confidence interval, followed by Tukey post hoc test to identify significant differences between samples and their respective controls, after testing homogeneity of variance using Barlett's procedure. Differences between control and treated animals were considered significant when p values were <0.05.

3. Results

3.1. Effect of HCB on liver cell proliferation

Because it has been shown that organochlorine pesticides or their metabolites could induce cell proliferation in rat hepatocytes (Okoumassoun et al., 2003), we hypothesized that HCB could increase cell proliferation in liver. PCNA was evaluated as a marker of cell proliferation in liver cells of control and HCB (0.1, 1, 10 and 100 mg/kg bw)-treated rats. Western blot analysis using anti-PCNA antibody, showed an increase in cell proliferation (53%) in HCB 100 mg/kg (Fig. 1A and B) compared to control group. We also evaluated BrdU incorporation into DNA of replicative liver cells of control and HCB-treated rats, followed by immunohistochemical detection. As shown in Fig. 1C, the LI was significantly increased (83%, $p < 0.05$) only at the highest HCB dose (100 mg/kg body weight).

3.2. In situ detection of DNA fragmentation

Induction of apoptosis was detected by immunohistochemical detection of fragmented DNA (TUNEL) in response to HCB (0.1, 1, 10 and 100 mg/kg bw) treatment. Representative tissue sections of each treatment are depicted in Fig. 2A. In normal liver, apoptotic nuclei were rarely found (<1%). The mean percentage of apoptotic cells increased (80, 115 and 70%) in HCB (1, 10 and 100 mg/kg bw)-treated rats, respectively, compared with control rats (Fig. 2B).

3.3. HCB induced ERK1/2 phosphorylation

Because ERK1/2 signaling could modulate cell proliferation or induction of apoptosis, we investigated the effect of HCB (0.1, 1, 10
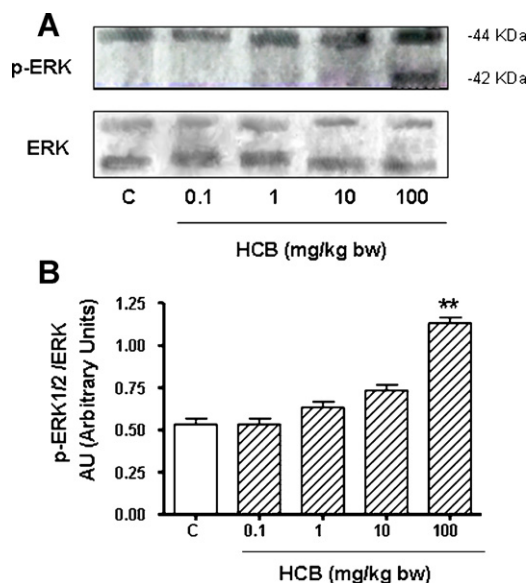

Fig. 3. HCB induces ERK1/2 phosphorylation in rat liver. Rats were treated with HCB (0.1, 1, 10 and 100 mg/kg bw), during 4 weeks. (A) The activation of ERK1/2 was determined by Western blotting using anti-phospho specific antibodies for p-ERK1/2 and total ERK1/2. (B) Quantification of p-ERK1/2/ERK1/2 ratio by densitometric scanning of the immunoblots is shown in the lower panel. Data are indicated as mean ± SEM of three independent experiments of four rats per group. **Significantly different from control rats ($p < 0.01$).

and 100 mg/kg bw), on ERK1/2 phosphorylation in liver. As shown in Fig. 3A and B, phospho-ERK1/2 protein levels were induced (115%, $p < 0.05$) in HCB 100 mg/kg. HCB-treatment did not change total non-phosphorylated forms.

3.4. HCB triggered activation of several caspases

To detect if proteolytic processing of caspases were involved in the apoptotic cell death induced by HCB, we have performed dose-dependent analysis of caspase-3, - 8, and -9 active forms, and pro-caspase-10, by Western blot. Caspase-3 is a key apoptotic executive caspase, being activated by proteolytic cleavage due to caspase-8, -10 and -9. Western blotting analysis of caspase-3 was performed using a polyclonal antibody, known to recognize the p17 and p19 activated forms. As shown in Fig. 4A and B, HCB (1, 10 and 100 mg/kg bw), increased (210, 270 and 240%) active caspase-3 protein levels. Activation of caspase-8 was analyzed by detection of the p18 fragment of processed caspase-8 by Western blotting (Qiu et al., 2002). Exposure to HCB (1, 10 and 100 mg/kg bw) resulted in the increase of active caspase-8 (115, 150 and 146%), respectively, compared to control rats (Fig. 4C and D).

In order to evaluate if caspase-10 was involved in HCB-induced apoptosis, we evaluated the decreases in the pro-caspase-10 form, which correlates with expression of active caspase-10, as reported by Kalli et al. (2003). Pro-caspase-10 protein levels were not affected, indicating that HCB-induced apoptosis is independent of caspase-10 activation in liver (Fig. 4E and F). Upon apoptotic stimulation, cytochrome c released from mitochondria associates with procaspase-9/Apaf 1. This complex processes procaspase-9 into large fragments comprising the prodomain plus large fragment (40 kDa and 38 kDa) and a 17 kDa active large fragment (Marques et al., 2003). Our results show that exposure to HCB led to a significant increase (100, 108 and 133%) in caspase-9 active fragment (p17), at HCB (1, 10 and 100 mg/kg bw), respectively, with the maximum increase at HCB 100 mg/kg (Fig. 4G and H). These results indicate that HCB activates initiator and
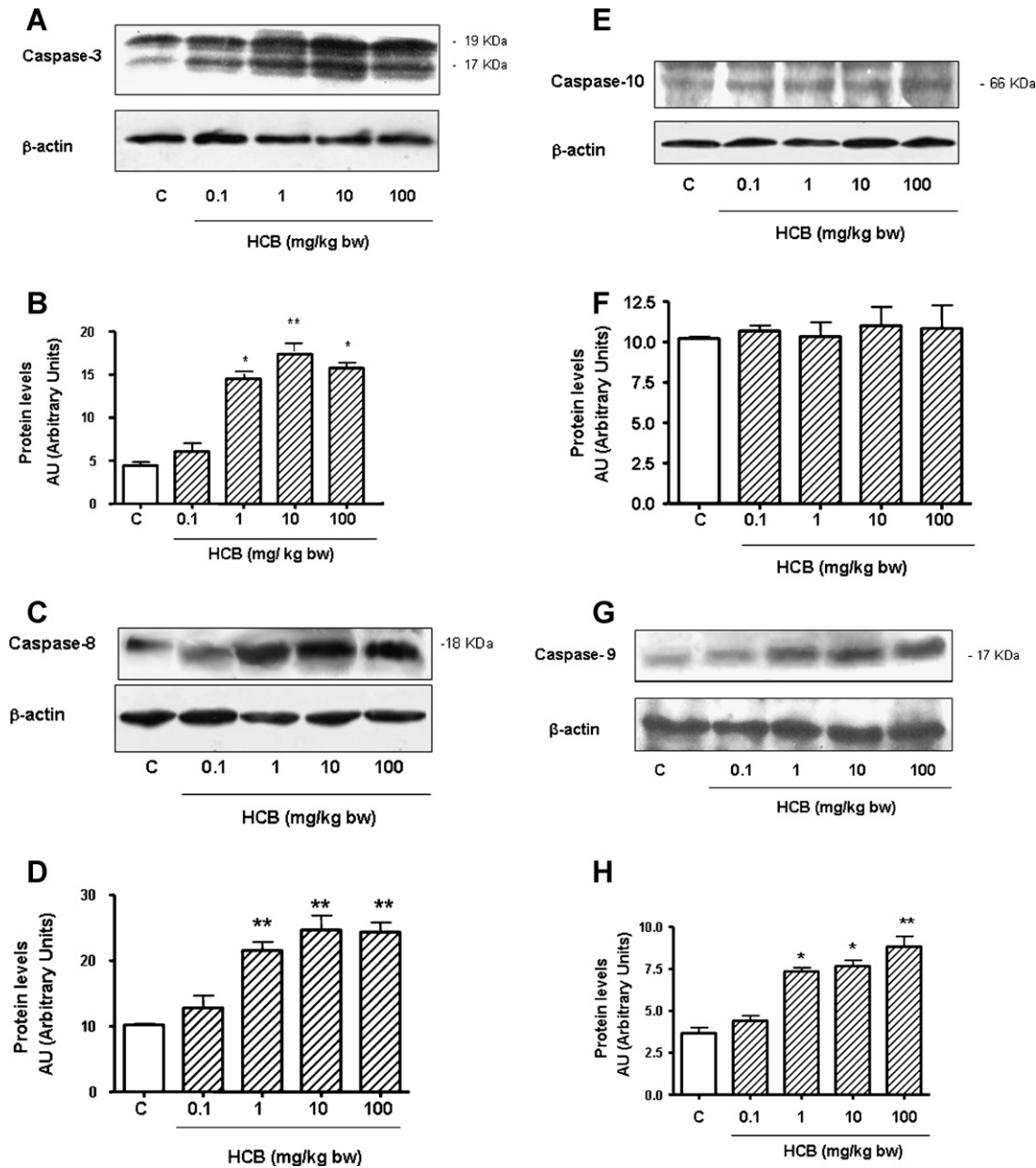
Fig. 4. HCB effect on caspases-3, -8, -10 and -9 activation. Active caspase-3, -8, and -9, and procaspase-10 protein levels, were determined by immunoblotting in the cytosolic fraction of liver tissue from HCB-treated rats during 4 weeks. Western blots of: (A) active caspase-3, (C) active caspase-8, (E) pro-caspase-10 and (G) active caspase-9 in HCB (0.1, 1, 10 and 100 mg/kg bw)-treated rats. (B, D, F, and H) Quantification of cytosolic caspases- 3, -8, -10, and -9 by densitometric scanning of the immunoblots. The same samples were probed with β-actin antibody as a loading control. Values are means ± SEM of three independent experiments of four rats per group. * and ** significantly different from control rats ($p < 0.05$ and $p < 0.01$), respectively.

executioner caspases involved in both the extrinsic and the intrinsic pathways.

3.5. Effects of HCB on FasL and Fas protein levels

It has been previously demonstrated that death receptor induced hepatocytes apoptosis, contributes to the development of a number of liver diseases (Yin and Ding, 2003). FasL and Fas were used as indicators for activation of the death receptor mediated pathway. Western blotting analysis of the postmitochondrial fraction showed that HCB (1, 10 and 100 mg/kg bw) increased FasL protein levels (100, 145 and 35%), respectively (Fig. 5A and B). Fas protein, detected both on the membrane and in the cytoplasm, was significantly increased (80, 150 and 120%), in HCB (1, 10 and 100 mg/kg bw)-treated rats, respectively (Fig. 5C and D).

3.6. Involvement of mitochondrial pathway in HCB-induced apoptosis

Bid activity and the release of cytochrome *c* from the mitochondria to the cytosol are important steps in the apoptotic signaling pathway which activates the caspase cascade in hepatocytes (Li et al., 2002). We investigated the effect of HCB on Bid and cytosolic cytochrome *c* protein levels. Because Bid is cleaved by caspase-8 after Fas activation, reductions in full-length Bid are concomitant with increases in truncated Bid as reported by Breitschopf et al. (2000). Our results demonstrate a dose-dependent decrease (30, 55 and 52%) in cytosolic Bid protein levels with HCB (1, 10 and 100 mg/kg bw), respectively (Fig. 6A and B), which indicate cleavage of the protein. On the contrary, HCB increased cytosolic cytochrome *c* protein levels (60, 105 and 120%) in a dose-dependent manner,
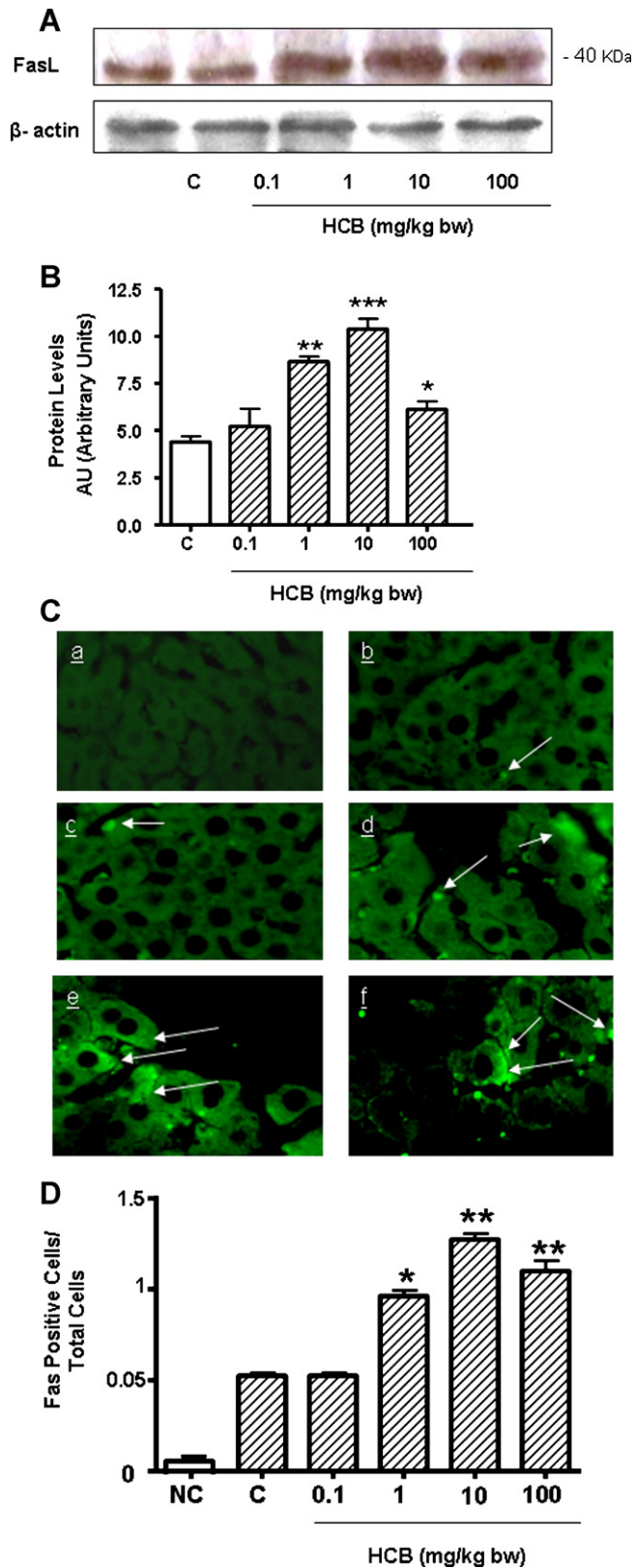
Fig. 5. Effects of HCB on FasL and Fas protein levels. (A) Western blot analysis of FasL protein levels in the postmitochondrial fraction of rat liver homogenate. (B) Quantification of FasL protein levels by densitometric scanning of the immunoblots. Values are means ± SEM of three independent experiments of four rats per group. *, ** and *** significantly different from control rats ($p < 0.05$, $p < 0.01$ and $p < 0.001$), respectively. (C) Detection of Fas by inmunohistochemical staining from control and HCB-treated rats. (a) Negative control (NC), (b) control, (c) HCB 0.1 mg/kg, (d) 1 mg/kg, (e) 10 mg/kg and (f) 100 mg/kg bw. Magnification 40×. Arrows point to
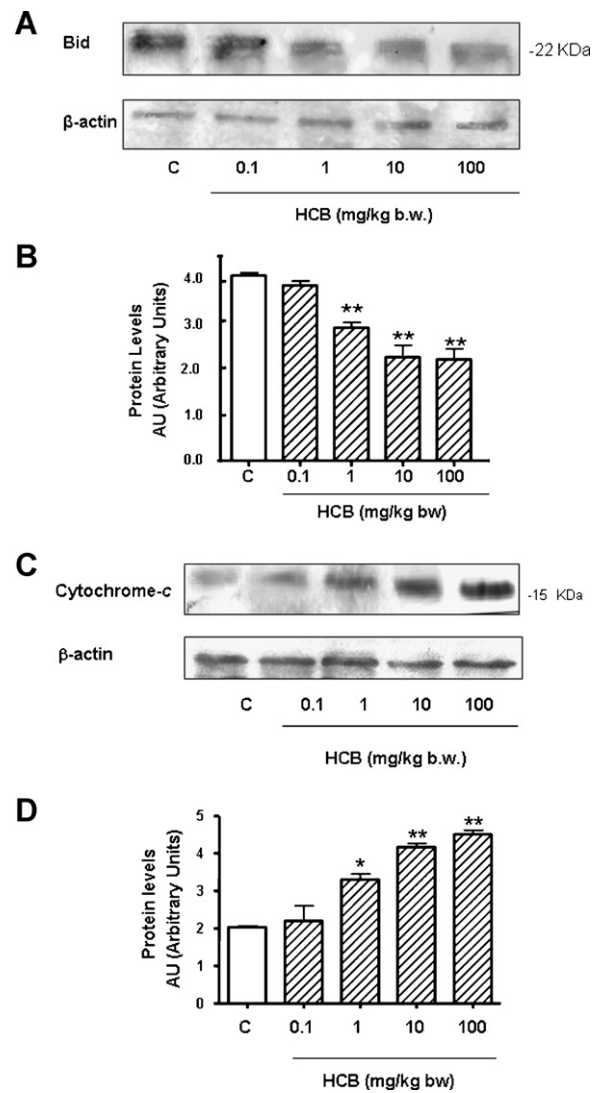

Fig. 6. Dose-dependent effect of HCB on Bid and cytoplasmic cytochrome c protein levels. After HCB (0.1, 1, 10, and 100 mg/kg bw)-treatment for 4 weeks, Bid and cytochrome c were analyzed in the post-mitochondrial fraction. (A and C) Westernblot analysis of Bid and cytosolic cytochrome c protein levels. (B and D) Quantification of Bid and cytochrome c protein levels by densitometric scanning of immunoblots. Values are means ± SEM of three independent experiments of four rats per group. * and ** significantly different from control rats ($p < 0.05$ and $p < 0.01$), respectively.

after treatment with HCB (1, 10 and 100 mg/kg bw), respectively (Fig. 6C and D). Taken together, these data suggest that mitochondrial dysfunction may play a pivotal role in HCB-mediated apoptosis in liver.

3.7. Expression of TGF-β1 in liver of HCB-treated rats

Provided that one of the most relevant cytokines that intervene in apoptotic and/or proliferative processes is TGF-β1 in hepatocytes, we evaluated its mRNA content and protein levels in livers of HCB-treated rats. The RT-PCR of TGF-β1 mRNA levels, revealed a significant dose-dependent upregulation (60, 126, 92%), after HCB (1, 10 and 100 mg/kg bw) exposure, respectively (Fig. 7A and B). In addition, Western blot analysis showed dose-dependent increases

green stained Fas positive cells. (D) Values represent the mean ± SEM of stained cells (n: 2000 cells per rat) from three independent experiments of five rats per group. * and ** significantly different from control rats ($p < 0.05$ and $p < 0.01$), respectively.
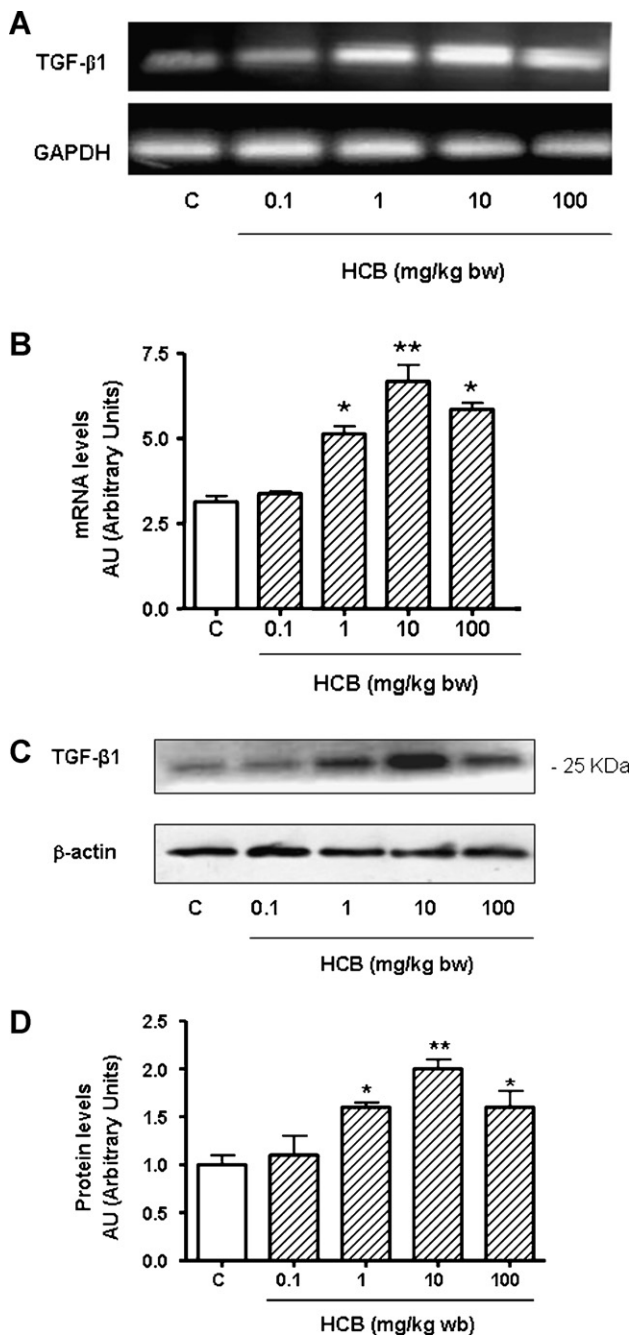
Fig. 7. Analysis of TGF-β1 expression in liver from HCB-treated rats. (A). Rats were treated with HCB (0.1, 1, 10 and 100 mg/kg bw) for 4 weeks. Representative pattern of RT-PCR amplification of TGF-β1 cDNA from control and HCB-treated rats, synthesized from total RNA. GAPDH was used as a loading control. (B) Quantification of cDNAs, after correction with GAPDH cDNA. Ethidium bromide stained gels were photographed, scanned and the band intensities determined. (C) Westernblot analysis of TGF-β1 protein levels. (D) Quantification of TGF-β1 protein levels by densitometric scanning of immunoblots. Values are means ± SEM of three independent experiments of four rats per group. * and ** significantly different from control group ($p < 0.05$ and $p < 0.01$), respectively. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

(80, 115, 70%) in TGF-β1 protein levels at the same HCB concentrations (Fig. 7C and D).

4. Discussion

The results obtained from this study demonstrate that HCB administration causes an imbalance between cell proliferation and cell death in the liver. Increased cell proliferation may be related to the induction of specific genes transcription, such as c-jun and c-myc, downstream of mitogen-activated protein kinase (MAPK) signaling pathways. We have previously demonstrated that HCB increased c-Myc, c-Jun and c-Fos protein levels in rat liver (Randi et al., 2003). These results are relevant because cell proliferation may increase the risk of mutations within target cells, and may also be important in selective clonal expansion of initiated cells to form preneoplastic foci and eventually tumors (Ou et al., 2003). On this respect, we have previously demonstrated that HCB is a tumor co-carcinogen in rat mammary gland (Randi et al., 2006).

In the present paper we have also shown that HCB increased the number of apoptotic cells even at HCB doses that did not alter cell proliferation. It is noteworthy that the apoptotic index tended to decrease at HCB 100 mg/kg, concomitantly with an increase in cell proliferation and activation of ERK1/2, at the same dose. These results suggest that the increase in cell proliferation may be a response to the increase in ERK1/2 signaling pathway, observed at the highest dose. On this respect, several lines of evidence support a role for ERK in DNA synthesis and cell proliferation (Nakagami et al., 2001). The decrease in the apoptotic index at the highest HCB dose may be a consequence of the increase in cell proliferation. It has also been reported that heptachlor, an organochlorine compound used as an insecticide, triggers proliferation in rat hepatocytes both by the induction of ERK1/2 phosphorylation and the inhibition of apoptosis (Okoumassoun et al., 2003).

The concomitant increase in the percentage of *apoptotic cells* and the levels of active *caspase-3*, suggest that HCB induces apoptosis by a caspase-dependent pathway in rat liver. From the present data we could not rule out the involvement of caspase-7 on the cleavage of PARP to its apoptotic fragments. Our results showed that HCB increased caspase-3 upstream regulators, such as active caspase-8 and caspase-9 protein levels. Because the hierarchic activation of caspases has not been determined in this study, we cannot discard that the significant activation of caspase-3 may lead to the activation of caspase-8, as other authors have suggested (Herrera et al., 2001).

In search of possible mechanisms that may contribute to HCB-induced apoptosis, we measured key signaling events related to particular pro-apoptotic mechanistic pathways. Increased Fas and FasL protein levels, as well as the active form of caspase-8, suggest that the Fas-mediated signaling pathway is involved in HCB-induced apoptosis in rat liver. It has been previously shown that Fas-mediated signaling is recognized as an important pathway of caspase activation and subsequent apoptotic cell death in the liver (Ogasawara et al., 1993). Caspase-8 mediated cleavage of Bid increases its pro-death activity and results in translocation to mitochondria, where it promotes cytochrome *c* release (Guicciardi and Gores, 2005). Consistent with these reports, our work demonstrates that HCB induces dose-dependent decreases of Bid protein levels, as well as increases of cytosolic cytochrome *c*, and active caspase-9, providing evidence that the mitochondrial pathway is involved in HCB-induced apoptosis. It has been reported that the Bid mediated pathway is critical in hepatocytes apoptosis induced by Fas/TNF-R1 engagement, where direct activation of cytosolic caspase cascade seems inefficient (Yin and Ding, 2003).

The extrinsic and intrinsic pathways are not mutually exclusive, as some cells, including hepatocytes and cholangiocytes, have been shown to require mitochondrial involvement to amplify the apoptotic signal from death receptors (Chen et al., 2001). We have previously demonstrated that HCB triggers apoptosis in rat thyroid through a mitochondrial-mediated pathway involving caspase-9, without alterations in caspase-8 activation. In addition, the pesticide did not produce any difference with respect to control animals on thyroid-follicular cell proliferation (Chiappini et al., 2009). Altogether, these data indicate that the mechanism of action of HCB on

the regulation of cell growth is tissue-specific. A role of mitochondrial dysfunction and oxidative stress elicited by endosulfan, an organochlorine pesticide, has also been reported in human T-cells (Kannan et al., 2000). Many authors have reported that ROS generation may contribute to mitochondrial damage and lead to cell death by acting as an apoptotic signaling molecule (Simon et al., 2000). The association between an *in vivo* oxidative stress condition in liver from HCB-treated rats, has been reported (Almeida et al., 1997; Ezendam et al., 2004).

Numerous observations suggest that TGF-β plays an important biological role mediating hepatocyte apoptosis (Oberhammer et al., 1992; Schrum et al., 2001). In the normal quiescent liver, hepatocytes rarely divide and TGF-β transcripts are low expressed. However, following chemical or physical injury, growth is strictly controlled by this cytokine. TGF-β-induced apoptosis in primary rat hepatocytes can be inhibited with a pancaspase inhibitor, suggesting that hepatocyte apoptosis is the major mechanism of TGF-β1-induced hepatocyte growth control (Schrum et al., 2001). In this study we demonstrate for the first time that HCB increases TGF-β1 gene expression and protein levels, in the rat liver. Similarly, we reported that HCB induced TGF-β1 expression and apoptosis in the rat thyroid gland (Chiappini et al., 2009). Thus, the increased apoptosis found in the present study, may be induced by enhancing the cellular response to TGF-β. It has been reported that, in mouse hepatocytes, there is a connection between the apoptotic Fas signaling and the TGF-β pathway. The connection is mediated by Daxx, a Fas receptor-associated protein that can interact directly with TGF-β type II receptor (Perlman et al., 2001). Further studies to unravel the involvement of the TGF-β dependent pathways in HCB-induced imbalance of cell growth, are necessary to avoid speculative interpretations.

Altogether, our results are relevant because it has been reported that disruption of the delicate balance between cell proliferation and cell death, contributes to the pathogenesis of acute and chronic liver diseases (Castilla et al., 1991; Miwa et al., 1997). In conclusion, this study reports for the first time that HCB induces cell growth imbalance in rat liver. Induced apoptosis occurs by mechanisms involving signals emanating from death receptors, and the mitochondrial pathway.

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Conflict of interest statement

None.

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