TOXICOLOGICAL SCIENCES **108(2)**, 301–310 (2009)
doi:10.1093/toxsci/kfp016
Advance Access publication January 30, 2009

Hexachlorobenzene Triggers Apoptosis in Rat Thyroid Follicular Cells

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Received October 9, 2008; accepted January 22, 2009

Hexachlorobenzene (HCB) is a widespread environmental pollutant. Chronic exposure of humans to HCB produces a number of effects, such as triggering of porphyria, increased synthesis of liver microsomal enzymes, neurological symptoms, immunological disorders and thyroid dysfunctions. In rats, HCB induced hepatic porphyria, neurotoxic effects, and toxic effects on the reproductive system, thyroid function, and immune system. HCB is also known to cause tumors of the liver, thyroid and mammary gland in laboratory animals. The aim of this study was to investigate parameters of thyroid growth regulation, mainly cell proliferation and apoptosis in thyroid tissue from HCB (0.1, 1, 10, 100, and 500 mg/kg body weight)–treated female Wistar rats. The current study demonstrates that only the exposure to the highest HCB dose for 30 days, has adverse effects on thyroid endpoints examined related to thyroid gland morphology, and 3,3′5,5′-tetraiodothyronine (T_4, thyroxine) serum levels, without changes in thyroid-stimulating hormone concentrations or in thyroid gland weight. Morphological changes, included flattened epithelium and increased colloid size compared with control tissue. Transforming growth factor (TGF-β1) mRNA levels, evaluated by RT-PCR, revealed a significant upregulation after exposure to HCB (1, 10, 100 mg/kg body weight). Cell proliferation evaluated by 5′-Br deoxiuridine incorporation into DNA, was not altered at any dose. HCB (1, 10, 100 mg/kg body weight) induces apoptosis, evaluated by *in situ* end labeling of fragmented DNA, terminal deoxynucleotidyl transferase-mediated deoxy uridine triphosphate nick-end labeling, in rat thyroid glands. This process is associated with dose-dependent increases in cytochrome *c* release from the mitochondria and procaspase-9 processing to its active product. Caspase-8 was not activated. These studies indicate that doses of HCB that do not disrupt thyroid economy induce TGF-β1 expression and apoptosis in the thyroid gland, involving the mitochondrial pathway.

Key Words: **hexachlorobenzene; apoptosis; rat thyroid.**

Hexachlorobenzene (HCB) is a persistent environmental pollutant with toxic effects in humans and laboratory animals. Although the use of HCB was discontinued in most countries in 1970′s, it is still released into the environment as a byproduct in several industrial processes. Alterations in thyroid metabolism and a risk excess of thyroid cancer have been reported in human populations exposed to organochlorinated compound mixtures with a high content of HCB (Grimalt *et al*., 1994; Meeker *et al*., 2007). Increased incidence of goiter was noted two to three decades later among survivors of Turkish people after accidental ingestion of grain contaminated with HCB (Peters *et al*., 1987). Exposure of various strains of rats to high doses of HCB (1000 mg/kg body weight) causes hypothyroxinemia by elevated excretion of thyroxine (T_4) into bile after the induction of T_4-uridine diphosphate glucuronosyltransferase-1 (UDPGT1) (Alvarez *et al*., 2005; van Raaij *et al*., 1993). In some instances, these effects are accompanied by compensatory increases in thyroid weight and circulating levels of thyroid-stimulating hormone (TSH) (Alvarez *et al*., 2005; Kleiman de Pisarev *et al*., 1989; Rozman *et al*., 1986; van Raaij *et al*., 1993). HCB is a thyroid carcinogen in hamsters (Cabral and Shubik, 1986) and acts as a tumor promoter of rat liver foci growth (Gustafson *et al*., 2000; Ou *et al*., 2001) and rat mammary tumors (Randi *et al*., 2006). It is thought that nongenotoxic carcinogens act by interfering with molecules intimately involved in cell growth and cell death. Although many of the mechanisms of thyroid toxicity have been studied, little is known of the effects of HCB on thyroid-follicular cell proliferation, a measure of thyroid gland growth. Normal cell division in the thyroid seems to be affected by an interplay among several mitogenic factors namely TSH, insulin-like growth factor-1, insulin, epidermal growth factor (EGF), and transforming growth factor β1 (TGF-β1). TGF-β1 is the prototype of a large family of cytokines that regulate a wide variety of cellular processes including cell proliferation, cell differentiation, cell motility, apoptosis and extracellular matrix production (Khosla *et al*., 1994). TGF-β responses can be cell-type specific and are dependent on the activity of other signal transduction pathways, which can either synergize with or antagonize the TGF-β pathway (Massagué *et al*., 2000). It has been shown that UDPGT-inducing chemicals may increase the expression of TGF-β1 and apoptosis in the thyroid (Kolaja *et al*., 1999).

Programmed cell death or apoptosis is an important cellular process that eliminates unwanted cells during normal

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development or damaged cells after exposure to toxic chemicals (Lee *et al*., 2003). There are two major pathways through which apoptosis are induced; one involves extrinsic death [TNF/Fas (CD95)] receptors signaling pathway and is exemplified by caspase-8 activation. Active caspase-8 may induce apoptosis either directly following activation of caspase-3 or indirectly following cleavage of cytosolic factors, such as Bid, leading to involvement of mitochondria.

Another pathway is commonly associated with cytotoxic agents, radiation, increased reactive oxygen species (ROS) and growth factor withdrawal and involves cytochrome *c* release into the cytosol (Jiang and Wang, 2000). When present in the cytoplasm, cytochrome *c* interacts with apoptotic activator factor-1 (Apaf-1) and, in concert with the initiator caspase-9, forms the apoptosome. Caspase-9 is then activated at this complex and triggers the caspase cascade and subsequent apoptosis (Cain, 2003). Both pathways converge on caspase-3, -6, and -7 activation, resulting in nuclear degradation and cellular morphological change.

To our knowledge, the effect of HCB on thyroid-follicular cell proliferation and apoptosis has not been reported. In normal thyroid tissue total cell mass is maintained by a balance between cell proliferation and apoptosis. In this study, we hypothesize that HCB, an UDPGT inducer, will increase TGF-β1 expression in the thyroid. Increases in TGF-β1, we further hypothesize, will induce apoptosis. The objectives of the present investigation were to examine parameters of thyroid growth regulation, cell proliferation and apoptosis, in thyroid tissue from HCB-treated rats. We sought to identify the apoptosis pathway involved.

MATERIALS AND METHODS

Reagents. HCB (> 99% purity, commercial grade) was purchased from Aldrich-Chemie GmbH & Co, Steinheim, Germany. Methimazole (MMI) was a gift of Gador Laboratories, Argentina. Polyvinylidene difluoride (PVDF) membranes were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Anti-caspase-9 rabbit polyclonal antibody was from Cell Signaling Technology, Inc. (Danvers, MA); anti-caspase-8 mouse monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-cytochrome *c* mouse monoclonal antibody was obtained from (BD Biosciences, San Jose, CA); anti-β-actin mouse monoclonal antibody was from Sigma-Aldrich (St Louis, MO); anti-digoxigenine and monoclonal 5-Bromo-2-deoxiuridine (BrdU) antibodies were from Biogenex (San Ramon, CA). TGF-β1 primer was obtained from Invitrogen Life Technology (Carlsbad, CA). The random primers were obtained from Biodynamics, Argentina. Enzymes and cofactors for reverse transcription (RT) and PCR were purchased from Promega Corporation (Madison, WI). All reagents used were of analytical grade.

Animals and treatment. Female Wistar rats (160–180 g at the start of the experiment) 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, temperature 20–24°C, and 45–75% humidity. Following a 7-day acclimation period, HCB was administered daily by gavage for 5 days of each week during 4 weeks, at doses of 0.1, 1, 10, 100, and 500 mg/kg body weight, as indicated in the text. HCB was administered as a suspension in water, containing Tween 20 (0.5/100 ml). Control animals received equal volumes of the solvent by the same route. The lowest dose used (0.1 mg/kg body weight) did not disrupt thyroid hormone metabolism, or malic enzyme activity, a thyroid hormone responsive liver enzyme, as we have previously demonstrated (Kleiman de Pisarev *et al*., 1989, 1990). The maximum dose of HCB employed (500 mg/kg body weight) altered thyroid hormone metabolism in experiments involving up to 30 days of intoxication (Kleiman de Pisarev *et al*., 1995).

MMI, a goitrogeneous product, was used as a positive control for cell proliferation. MMI (10 mg per rat) was administered by daily ip injections for 30 days. 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. On the day following the final dose, blood was obtained, and serum was collected by centrifugation (3000 × g for 15 min), aliquoted and frozen at −80°C until analysis. Body weight and wet weight of the excised thyroid lobes were recorded. All experiments were performed in compliance with institutional and international guidelines for laboratory animal care.

Biochemical determinations. Concentrations of plasma thyroid hormones were determined in duplicate by RIA using commercially available kits for tri-iodothyronine (T_3) and T_4, Eleccys (Electro Chemiluminescence reactions). TSH was measured according to the RIA kit and the procedure for rat TSH provided by the National Pituitary Agency. Values were expressed as arithmetical mean ± SEM (ng/dl for T_3, μg/dl for T_4 and ng/ml for TSH).

Thyroid gland histomorphology. A portion of the trachea, with intact thyroid gland attached, was dissected free of connective tissue and fixed in phosphate buffered saline (PBS), containing 10% neutral formalin, for more than 24 h, dehydrated in ethanol and embedded in paraffin. Sections of thyroid glands, 5 μm thick, were cut parallel to the longitudinal axis of the trachea and stained with hematoxilin-eosin or Periodic-Schiff acid (PAS) for the morphologycal studies. A subset of 200 follicles from the thyroids of three rats, were selected for parametric measures of follicle morphology and analyzed at a magnification of ×400. The morphometric measurements were performed using digitalized images obtained directly from the light microscope via a video camera. Measurements were processed using Image-Pro Plus v4.5-Media (Cybernetics, Inc., Silver Spring, MD). Once selected, the follicular colloid longest and shortest axes were automatically calculated for every complete follicle cross section visible in a digital image. Epithelial height was estimated according to Wade *et al*. (2002). Follicle colloid area was identified for the follicles that had no evidence of any artifactual distortion in each image and was calculated according to Behrends *et al*. (2005).

Assessment of thyroid-follicular cell proliferation. Thirty minutes prior to sacrifice, the animals were given an intraperitoneal injection of a solution (5 mg/ml) of BrdU in saline solution at a dose of 0.08 mg BrdU/g body weight. BrdU incorporation was stained in tissue sections by an indirect immunohistochemical technique. Briefly, the histological sections mounted on silane-coated slides were dewaxed and incubated in methanol–hydrogen peroxide (100:1) for 30 min to inhibit the activity of endogenous peroxidase. Antigen retrieval was performed in a microwave oven with citrate buffer, pH 6. Nonspecific blocking was performed by washing in 0.5M Tris–saline buffer, 0.3% bovine albumin. The sections were incubated with the mouse anti-BrdU antibody (1:40), and washed with the same buffer. Afterward they were incubated at room temperature for 1 h in a moist chamber with the second biotinylated antibody (1:200), antimouse IgG (Multilink, Biogenex). Finally, the sections were incubated in the same conditions with the streptavidin-peroxidase complex. Horseradish peroxidase was visualized with 3,3′-diaminobenzidine (DAB) (Sigma Chemical Co.). Sections were then counterstained using PAS and hematoxylin. As negative controls, adjacent sections were processed omitting incubation with the primary antibody. A dark brown precipitate 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 in the selected area (magnification: ×400), employing an image analysis software developed *ad hoc* to distinguish between positive nuclei (with DAB

deposits) and hematoxylin stained nuclei. Two to five fields were evaluated for each category. One thousand nuclei (labeled plus unlabeled) of each thyroid gland were counted.

Detection of apoptosis. Apoptotic nuclei in tissue sections of thyroid glands 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, for digesting protein in tissue, followed by quenching of endogenous peroxidase with 3% H_2O_2 in PBS. 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 DAB and counterstained with methyl-green. The mean percentage of positive staining of nuclei was calculated after counting positively or negatively stained nuclei of 1000 thyrocytes in several fields under light microscope. For negative controls we performed TUNEL method by the omission of the TdT reaction step. The apoptotic index was determined as the percentage of apoptotic cells in the total number of follicular nuclei observed. 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.

Subcellular fractionation. Thyroids were removed, weighed and immediately homogenized in a Dounce-type glass homogenizer with a Teflon pestle, in three volumes of homogenization buffer (0.32M sucrose, 1mM ethylenediaminetetraacetic acid in 10mM Tris-HCl pH 7.4). Homogenates were used for preparation of mitochondria and cytosol. Homogenates were centrifuged at 1300 × g for 10 min. The supernatant layer was centrifuged at 14,000 × g for 15 min to yield a crude mitochondrial pellet and a supernatant layer. The resulting supernantant fraction was used for the assay of cytosolic proteins. Protein concentration was determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

Western blotting. Rat thyroid cytosolic fraction adjusted protein concentration (50 μg/lane), were electrophoresed in 12% sodium dodecyl sulfate–polyacrylamide gels and then transferred to PVDF membranes. The blots were blocked for overnight in TBST (10mM Tris, pH 8.0, 150mM NaCl, 0.05% vol/vol Tween 20) containing 2% nonfat dry milk and 2% BSA. Blocked membranes were incubated with anti-caspase-9 rabbit polyclonal antibody (1:500), anti-caspase-8 mouse monoclonal antibody (1:500), anti-cytochrome *c* mouse monoclonal antibody (1:1000), anti-β-actin (1:2000) in TBST overnight at 4°C. The membranes were washed with TBST and incubated with the suitable horseradish peroxidase-conjugated anti-species-specific antibody (1:1000) in TBST. Immunoblots were then washed five times with TBST and once with TBS. Proteins were visualized employing the enzyme-linked enhanced chemoluminescence kit (Amersham Biosciences, Inc., UK) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

RT-PCR analysis of TGF-β1 mRNA. The thyroids were dissected out and were frozen and stored at −80°C prior to RNA extraction. An aliquot of 2μg of total RNA was used to synthesize first-strand cDNA with the random primer, deoxynuclotide triphosphates, transcriptase reverse, and transcriptase reverse buffer. The RT mixture (1 μl) was amplified with hot starting PCR in a 50-μl reaction using GoTaq DNA polymerase (Promega Corporation) for TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control to correct for differences between lanes in the amount of RNA. 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 40 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 PCR mixture (50 μl) contained GoTaq reaction buffer with 1.5mM $MgCl_2$, 0.2mM dNTP's mix, 1.25 U GoTaq DNA polimerase (Promega Corporation), 0.5μM each forward and reverse primer, and 0.5 μl of RT products. PCR products were detected as a single band on 2% agarose gel, containing 0.05% vol/vol ethidium bromide. Bands were detected by Fotodyne (Foto/Analyst) equipment, and intensity was quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

Statistical analysis. Results are presented as mean ± SEM. Differences between treated and control animals 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, as described in each legend. Differences between control and treated animals were considered significant when p values were < 0.05.

RESULTS

Effect of HCB on Serum Total T_4, T_3, TSH, and Thyroid Weight/Body Weight Ratio

In the present study, thyroid endpoints (thyroid weights and serum hormone concentrations) have been evaluated for their utility in detecting thyroid-modulating effects after 30 days of treatment with HCB (1, 10, 100, and 500 mg/kg body weight).

Serum total T_4 was reduced 30% in rats treated with HCB (500). In contrast, treatment of rats with lower doses of HCB did not affect total T_4 after 30 days of treatment. T_3 serum levels were not altered at any dose. No differences in serum TSH were observed at any dose of HCB. The thyroid relative weight, thyroid weight (mg)/body weight (g) x 100 (TW/BW), was not increased by HCB (Table 1). A positive control for increased serum TSH levels was included, treating rats with the goitrogeneous agent MMI for 30 days. In all animals, MMI administration elicited a significant increase in TSH serum levels and at the same time the TW/BW of the thyroid glands was increased.

TABLE 1
Dose Response to HCB Administration on Serum Total T_4, T_3, TSH, and TW/BW Ratio

	T_4 (μg/dl)	T_3 (ng/dl)	TSH (ng/ml)	TW/BW
Control	3.67 ± 0.52	77.00 ± 5.00	1.83 ± 0.27	8.16 ± 0.30
HCB 1	3.55 ± 0.43	69.00 ± 7.00	2.01 ± 0.32	8.90 ± 0.45
HCB 10	3.88 ± 0.36	75.00 ± 3.00	2.08 ± 0.42	8.49 ± 0.48
HCB 100	3.32 ± 0.29	66.00 ± 5.00	1.89 ± 0.36	9.06 ± 0.52
HCB 500	2.21 ± 0.19*	73.00 ± 6.00	1.93 ± 0.29	8.96 ± 0.41
MMI	ND	ND	4.65 ± 0.17*	18.6 ± 0.75*

Note. The effect of HCB administration (1, 10, 100, and 500 mg/kg body weight) on serum total T_4, T_3, TSH, and thyroid weight/body weight ratio were measured at 30 days. T_4, T_3, and TSH serum levels were estimated as described in ''Materials and Methods.'' The values are mean ± SEM ($n = 4$). TW/BW [thyroid weight (mg)/body weight (g) × 100]. (ND): not determined. *Significantly different from control rats ($p < 0.05$). Statistical comparisons were made by analysis of variance (one-way ANOVA), with a 95% confidence interval followed by Tukey *post hoc* test to identify significant differences between mean values and indicated control.

Thyroid Gland Histomorphology

Direct measurements of colloid area and the epithelial height, provide information on structures involved in thyroid hormone synthesis, storage and secretion, and also on changes at the morphological and functional levels. Thyroids from vehicle treated rats (Fig. 1A) depict the normal thyroid architecture. Control follicles are oval shaped containing colloid of uniform color surrounded by orderly cuboidal epithelium. The epithelium of some larger follicles was flattened cuboidal to squamous. Examination of histological sections revealed that the size of the follicles was not at all homogeneous. Large follicles were located mainly on the outer edge of some glands. Our results show that only the highest dose of HCB (500 mg/kg body weight) caused a significant increase in the colloid area and a decrease in the epithelial height relative to control (Figs. 1B and 1C). Thus, morphofunctional characteristics of the thyroid gland, in HCB (500 mg/kg body weight), suggest the organ hypofunction. Animals exposed to lower doses of HCB did not show alterations neither in the colloid area nor in the heights of the follicular epithelium. The size of a follicle depends on the number of cells and the amount of colloid. These are interchangeable and vary according to biological activity. MMI-treatment induced hypertrophy of the thyroid-follicular cells (Fig. 1A, f). Follicular lumens were narrow, irregular and depleted of colloid and they were surrounded by a columnar epithelium.

Expression of TGF-β1 in Thyroids of HCB-Treated Rats

As TGF-β1 is increased by UDPGT inducers, we evaluated TGF-β1 thyroid mRNA levels in HCB-treated rats. The RT-PCR measurements revealed a significant upregulation of the mRNA content of TGF-β1 after exposure to HCB (1, 10, 100 mg/kg body weight) for 30 days (Fig. 2). As the lowest dose used in these experiments (HCB 1 mg/kg body weight) increased TGF-β1 expression, we have also included a lower dose (HCB 0.1 mg/kg body weight) that did not alter this parameter. HCB (500 mg/kg body weight) has not been included in this and the next experiments, with the purpose of excluding HCB doses that disrupt thyroid economy.

GAPDH mRNA was used as a loading control.

Effect of HCB on Thyroid-Follicular Cell Proliferation

In order to study the effect of HCB on thyroid-follicular cell proliferation, we evaluated BrdU incorporation into DNA of replicative thyroid-follicular cells at doses of HCB (0.1, 1, 10, 100 mg/kg body weight) that did not disrupt thyroid economy and morphology. The LI was not affected in rats treated with HCB at any dose. As expected, treatment with MMI induced a remarkable increase in thyroid-follicular cell proliferation (Fig. 3B). Proliferating cell nuclear antigen (PCNA) was also evaluated as a marker of cell proliferation. HCB did not produce any difference with respect to control animals in PCNA labeling (data not shown).

In Situ *Detection of DNA Fragmentation*

Induction of apoptosis was detected by immunohistochemical detection of fragmented DNA (TUNEL) in response to HCB treatment. In normal thyroid glands, apoptotic nuclei were rarely found (< 4%) (Fig. 4). Treatment with HCB increased the incidence of apoptosis, indicated as brown-

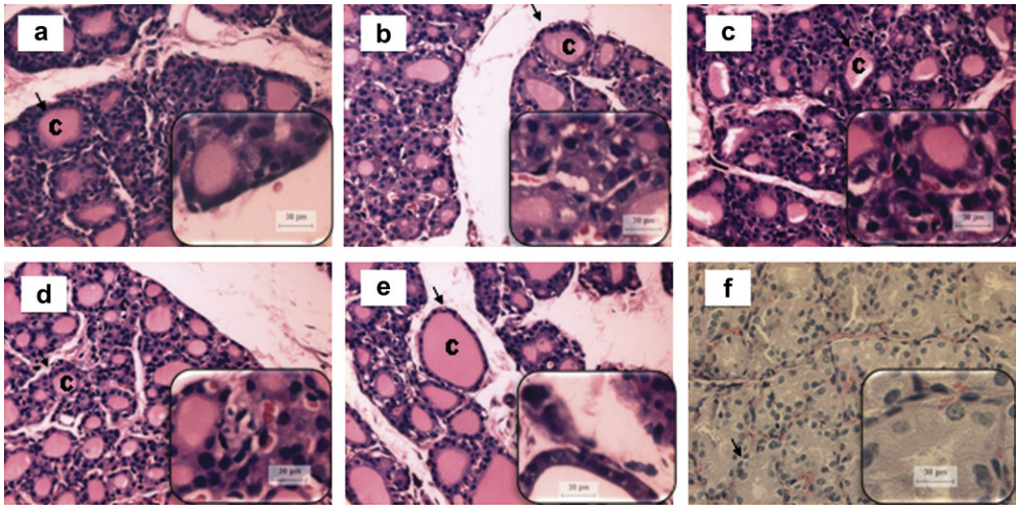

FIG. 1. Thyroid gland morphology. (A) Photomicrographs of representative sections identified in rat thyroids at the end of 30 days of treatment with HCB. (a) Control; (b) HCB 1 mg/kg body weight; (c) 10 mg/kg body weight; (d) 100 mg/kg body weight; (e) 500 mg/kg body weight, and (f) MMI. Colloid in the follicular lumen is denoted by ''C.'' The arrow indicates the thyroid epithelium. Bar = 30 μm. Magnification ×400. (B) Quantification of changes in thyroid gland by measuring the colloid area and the heights of the follicular epithelium. Values are mean ± SEM of 200 follicles from three rats per group. ** and ***Significantly different from control rats ($p < 0.01$ and $p < 0.001$). Statistical comparisons were made by analysis of variance (one-way ANOVA), with a 95% confidence interval followed by Tukey *post hoc* test to identify significant differences between mean values and indicated controls. The experiment was repeated twice with similar results.

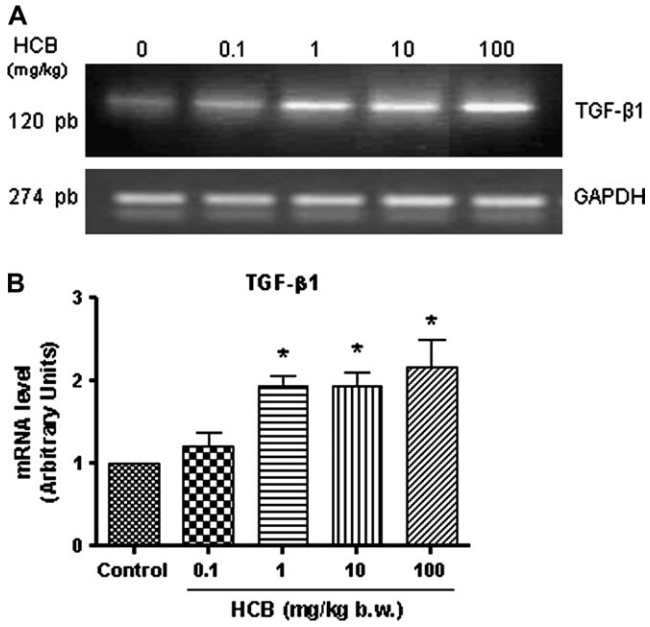

FIG. 2. Analysis of TGF-β1 expression in thyroid glands from HCB-treated rats. Rats were treated with HCB (0.1, 1, 10, and 100 mg/kg body weight) for 30 days. (A) Representative pattern of RT-PCR amplification of both control cDNA and HCB (0.1, 1, 10, and 100 mg/kg body weight) cDNA, synthesized from 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. The values are mean ± SEM of two independent experiments of three rats per group. *Significantly different from control rats ($p < 0.05$). Statistical comparisons were made by analysis of variance (one-way ANOVA), with a 95% confidence interval followed by Tukey *post hoc* test to identify significant differences between mean values and indicated controls.

stained apoptotic thyrocytes. The mean percentage of apoptotic thyrocytes increased (450, 375, and 200%) in HCB (1, 10, and 100 mg/kg body weight) treated rats respectively compared with control thyroids (Fig. 4B). It must be underlined that the apoptotic process did not involve one entire follicle at once, but only some cells. No difference was observed in the wet weight of thyroid glands between TUNEL-positive and -negative groups.

HCB-Initiated Mitochondrial Events

In order to investigate if the increase in the apoptotic nuclei occurs downstream of a possible damage to mitochondria, we analyzed the release of cytochrome *c* after 30 days of treatment with different concentrations of HCB (0.1, 1, 10, and 100 mg/kg body weight). As shown in Figure 5, HCB induced a dose-dependent increase of cytochrome *c* in the cytosol. This increase was enhanced by 88, 213, and 239% after HCB (1, 10, and 100 mg/kg body weight), respectively.

HCB-Enhanced Cleavage of Procaspase-9

To explore if caspase-9 is activated as a consequence of cytochrome *c* release into the cytosol, in response to HCB treatment, we documented caspase-9 proteolytic processing of full-length protein. When apoptosis was induced at HCB (1, 10, and 100), caspase-9 was processed in a dose-dependent manner to yield fragments of ~17 kDa (p17) corresponding to the active form. The antibody does not recognize full-length caspase-9 of 51 kDa. As shown in Figure 6, active caspase-9 was increased by 192, 270, and 296% after HCB treatment (1, 10, and 100 mg/kg body weight), respectively.

HCB Did Not Enhance Cleavage of Procaspase-8

As caspase-8 has been known to be able to release cytochrome *c* from mitochondria, we examined if the proteolitic processing of caspase-8 was activated in thyroids of HCB (0.1, 1, 10, and 100)–treated rats. Our results show that procaspase-8 was present primarily as a 53- to 55-kDa protein, and the active form as a 18-kDa protein. By immunoblotting, we documented that HCB did not affect the processing of caspase-8 at any dose (Fig. 7).

DISCUSSION

The daily rates of exposure of the vast majority of the population for HCB, are lower than the WHO tolerable daily intake, for noncancer effects and for neoplastic effects in humans, and are well below the doses used in most experimental studies (Falcó *et al*., 2008). In the present study, we found that serum HCB concentration in the highest dose group (500 mg/kg) was 600 ng/ml (results not shown). Analysis of serum samples from 100 subjects of a general population highly exposed to airborne HCB, revealed HCB concentrations ranging between 1.1 and 953 ng/ml (To-Figueras *et al*, 1997). These observations suggest that the doses used in the present study, are relevant to human exposures in highly contaminated populations.

TSH is a well-known growth stimulator of benign goiter and thyroid cancer, however it possibly does not play any particular role in organochlorinated-related thyroid growth in human adults, as results from no increase of TSH level with increasing polychlorinated biphenyls (PCBs) and HCB levels (Rádiková *et al*., 2008; Sala *et al*., 2001).

The current study demonstrates that subchronic exposure to HCB (500 mg/kg) causes reductions in circulating T_4 levels, without a compensatory increase in plasma TSH, after 30 days of treatment, indicating that thyroid homeostasis was significantly altered at this dose. Interestingly, other authors reported that TSH concentrations were increased three-fold at week 2, declining to near control values at week 19, in a study on promotion of thyroid tumors in rats by Pregnenolone-16 -carbonitrile (Vansell *et al*, 2004). We do not think that in the present study, TSH levels would have increased earlier, returning to its control level at 30 days, because we have previously demonstrated that shorter times of HCB (1000 mg/kg) administration did not modify TSH levels (Kleiman de Pisarev

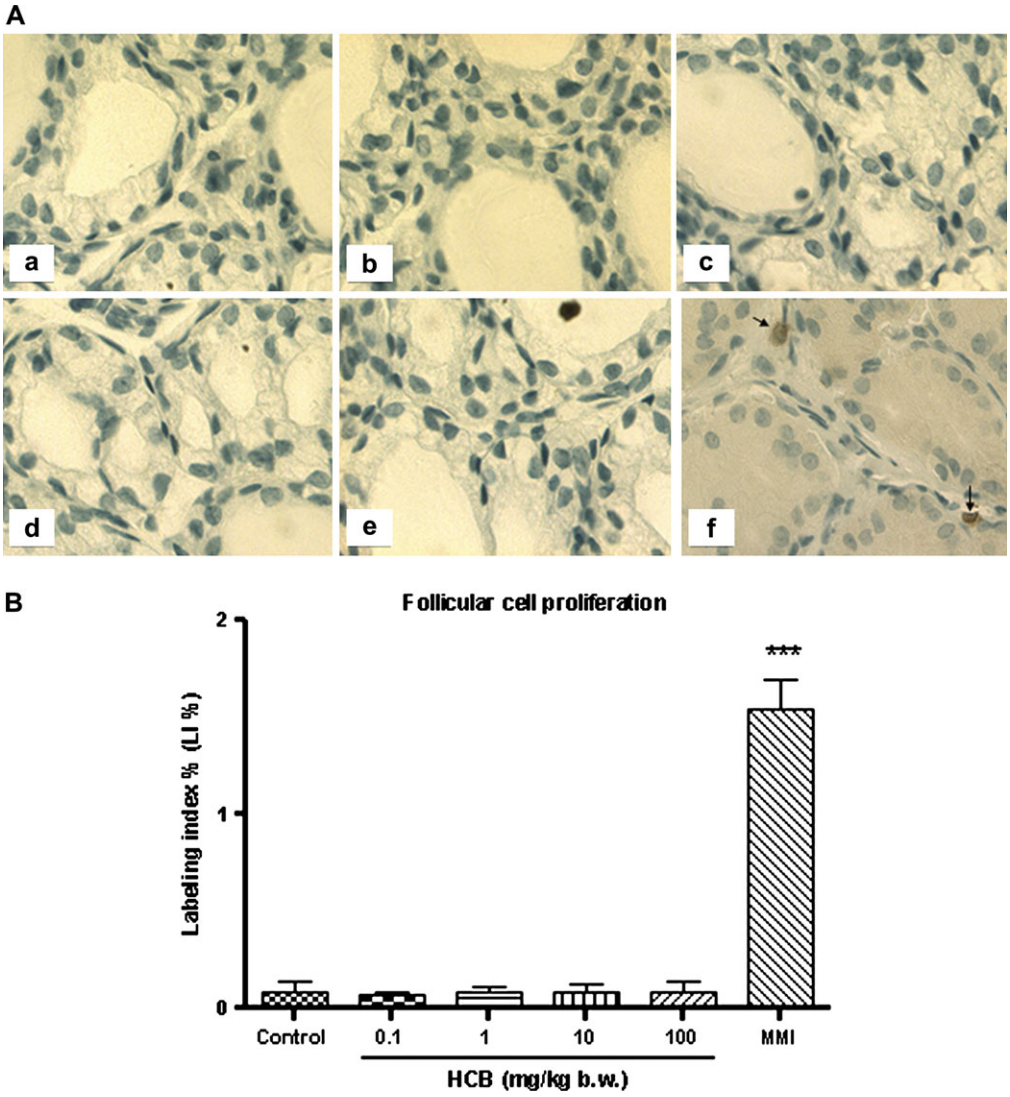

FIG. 3. Effect of HCB on thyroid-follicular cell proliferation. Rats were treated with HCB (0.1, 1, 10, and 100 mg/kg body weight) for 30 days. Cell proliferation was evaluated by BrdU incorporation into DNA of replicative thyroid-follicular cells. (A) Thyroid-follicular cell nuclei labeled with BrdU are indicated with an arrow. Magnification ×400. (B) LI, number of labeled cells with BrdU over the total number of cells. Each value represents the mean ± SEM ($n = 1000$ nuclei per rat) from three rats per group. ***Significantly different from control rats ($p < 0.001$). Statistical comparisons were made by analysis of variance (one-way ANOVA), with a 95% confidence interval followed by Tukey *post hoc* test to identify significant differences between mean values and indicated controls. The experiment was repeated twice with similar results.

et al., 1989). On this respect we have reported that there is a discrepancy between the ability of HCB to reduce circulating levels of T_4 and its ability to produce a functional shift in liver thyroid status (Kleiman de Pisarev *et al.*, 1995). It is well known that increased rate of intrapituitary T_4 to T_3 conversion by deiodinase type II results in inhibition of TSH release, although, vice versa, TSH release is stimulated by decreased rate of such conversion. Thus, it is possible that type II deiodinase activity is upregulated in the pituitary, in response to HCB 500 mg/kg induced hypothyroxinemia.

Morphometric measurements may be helpful in characterizing early and/or transient responses to toxicants that have multiple disrupting effects. Our results show that frank alterations in the gland histomorphology and reductions in circulating T_4 are only apparent in animals exposed to HCB (500 mg/kg) dose, suggesting a hypfunctioning gland.

In the present study we have shown that HCB doses that do not disrupt thyroid hormone economy or thyroid morphology, did not alter thyroid-follicular cell proliferation evaluated by BrdU incorporation and PCNA-labeled nuclei. On this respect it has been reported that microsomal enzyme inducers that do not increase serum TSH, that is, 3-methylcholantrene and Aroclor 1254 (PCB), do not increase thyroid-follicular cell proliferation (Klaassen and Hood, 2001).

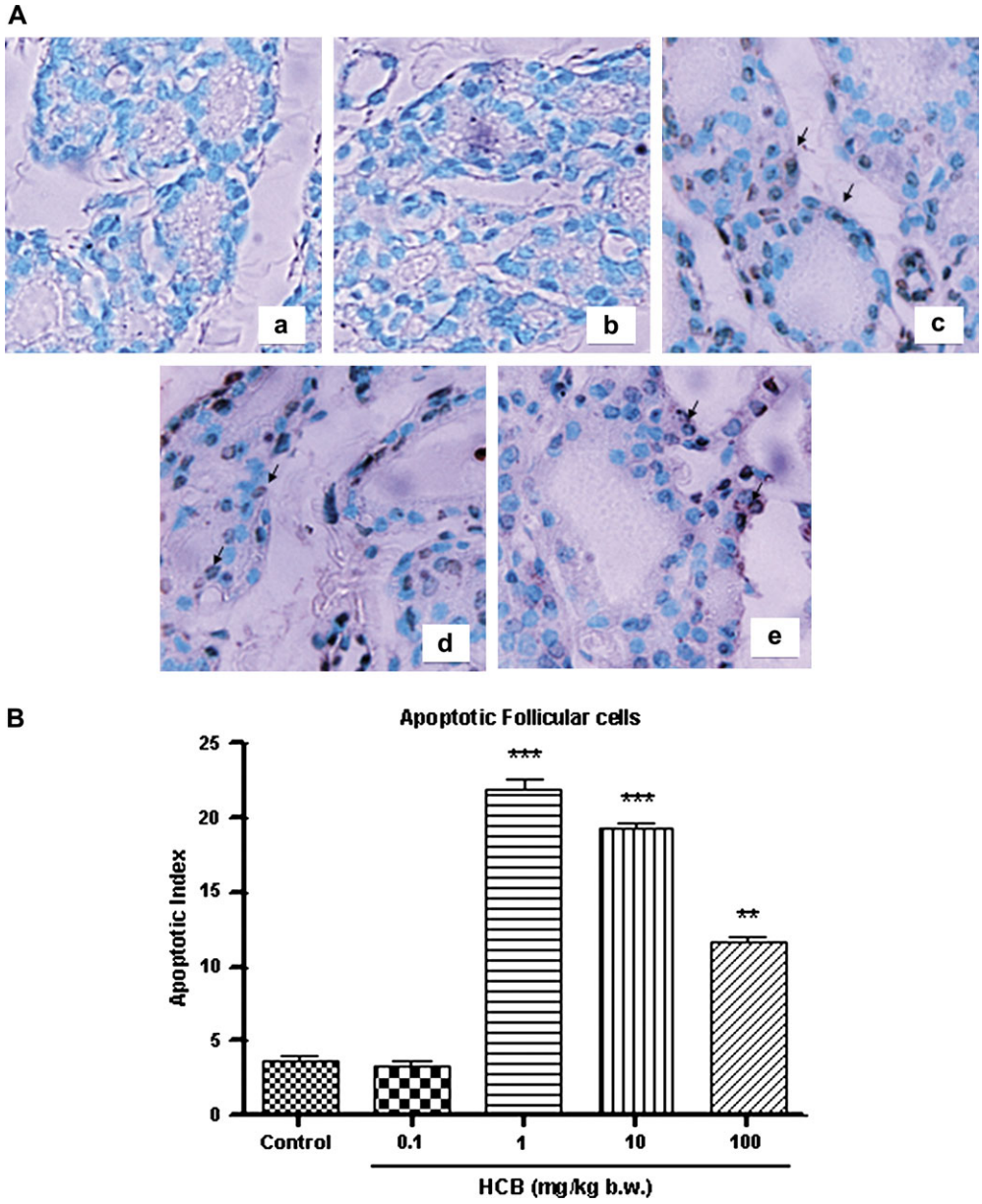

FIG. 4. Detection of *in situ* DNA breaks by TUNEL staining. (A) TUNEL staining of thyroid-follicular cells from control and HCB-treated rats. (a) Control, (b) 0.1, (c) 1, (d) 10, and (e) 100 mg/kg body weight. Magnification ×600. Arrows point to brown-stained apoptotic cells. (B) Values represent the mean ± SEM of TUNEL-positive cells ($n = 1000$ cells per rat) from two independent experiments of three rats per group. ** and ***Significantly different from control rats ($p < 0.01$ and $p < 0.001$). Statistical comparisons were made by analysis of variance (one-way ANOVA), with a 95% confidence interval followed by Tukey *post hoc* test to identify significant differences between mean values and indicated controls.

Iodine and TGF-β are the only known negative regulators of thyroid cell number. To control epithelial cell number, TGF-β acts through two independent pathways. First, it inhibits proliferation through Smad-dependent c-myc repression, depending on the cell type. Second, in some cell types TGF-β has been shown to induce apoptosis (Chen *et al.*, 2002).

In this study we demonstrate for the first time that HCB, a UDPGT inducer, increases TGF-β1 gene expression in the rat thyroid follicular cells, in the absence of an increase of endogenous TSH. Given the relationship between HCB and the increase in TGF-β1 expression, we hypothesized that thyroid apoptosis might also be increased. In the current study we demonstrate that thyroid-follicular cells are sensitive to the apoptotic inducing effect of HCB, at all assayed doses, although the increase is less pronounced at 100 mg/kg. However the apoptotic effect is still remarkable at this dose, as shown by the high percentage of apoptotic cells (200%). In a similar manner, the goitrogenic compound propylthiuracil

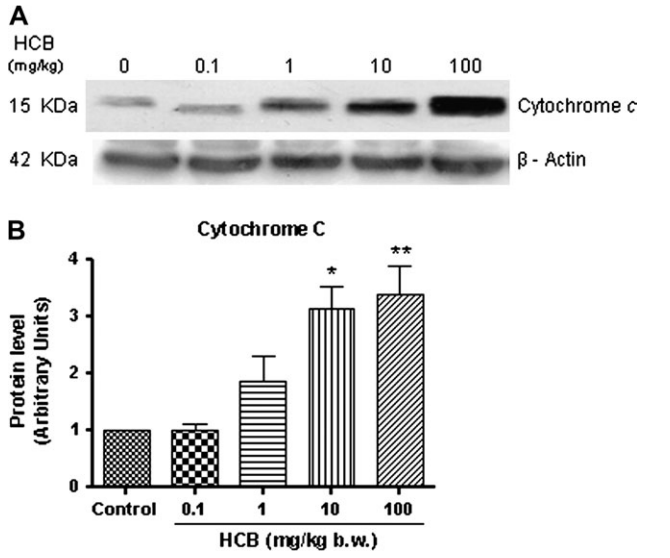

FIG. 5. HCB induces cytochrome *c* release in thyroid-follicular cells. (A) Immunochemical detection of cytochrome *c* levels in the cytosolic fraction of thyroid tissue. Rats were treated with HCB (0.1, 1, 10, and 100 mg/kg body weight) during 30 days. The release of cytochrome *c* into the cytoplasm was detected by Western blotting with anti-cytochrome *c* antibody. The same samples were probed with β-actin antibody as a loading control. (B) Densitometric quantification of cytochrome *c* content by scanning of the immunoblots. The levels of cytochrome *c* are expressed as arbitrary units. Values are mean ± SEM of three independent experiments of three rats per group. * and **Significantly different from control rats ($p < 0.05$ and $p < 0.01$). Statistical comparisons were made by analysis of variance (one-way ANOVA), with a 95% confidence interval followed by Tukey *post hoc* test to identify significant differences between mean values and indicated controls.

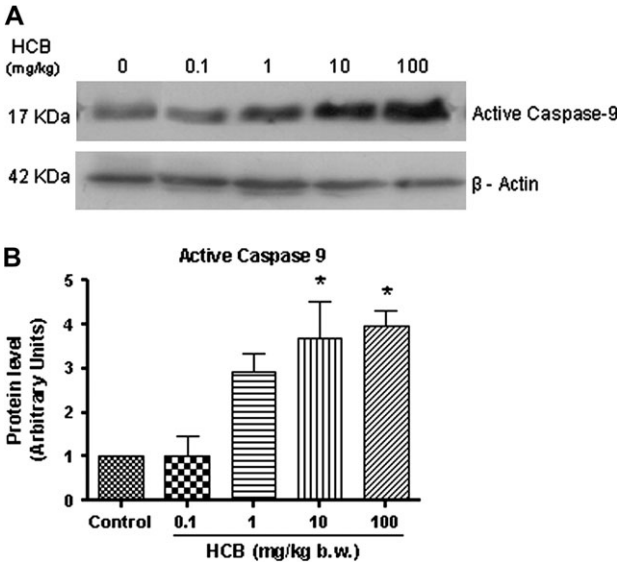

FIG. 6. Enhanced cleavage of procaspase-9 induced by HCB. (A) Active caspase-9 was determined by immunoblotting in the cytosolic fraction of thyroid tissue from HCB (0.1, 1, 10, and 100 mg/kg body weight)–treated rats during 30 days. The same samples were probed with β-actin antibody as a loading control. (B) Quantification of cytosolic active caspase-9 by densitometric scanning of the immunoblots. Values are means ± SEM of three independent experiments of three rats per group. *Significantly different from control rats ($p < 0.05$). Statistical comparisons were made by analysis of variance (one-way ANOVA), with a 95% confidence interval followed by Tukey *post hoc* test to identify significant differences between mean values and indicated controls.

increased thyroid-follicular cell proliferation, the expression of TGF-β1 and apoptosis in the thyroid (Kolaja *et al*., 1999). It has also been demonstrated that in cultured porcine thyroid-follicular cells, TGF-β1 induces apoptosis (Bechtner *et al*., 1999). The doses of HCB causing significant increases in TGF-β1 expression and apoptosis, suggest that HCB can affect these parameters by direct actions on the gland rather than by producing a disruption in thyroid hormone homeostasis. High levels of TGF-β expression has also been associated to a counter-regulatory mechanism to prevent tumor growth (Derwahl and Studer, 2002). On this respect, it has been demonstrated that in a human medullary thyroid carcinoma cell line, TGF-β1 treatment decreases cell proliferation and increases the rate of cell death (Khosla *et al*., 1994). It has been demonstrated that HCB can induce alveolar adenomas of the thyroid in Syrian male golden hamsters fed 4, 8 or 16 mg/kg body weight per day of HCB (Cabral and Shubik, 1986; Cabral *et al*., 1977). The results of the present study, may be relevant to the molecular events involved in HCB tumor promoter activity, because it has been reported that TGF-β may become a stimulator of proliferation, invasion, angiogenesis and metastasis (Stover *et al*., 2007). It has been proposed that induced TGF-β1 may assist exaggerated proliferation of certain preneoplastic thyrocytes that are more resistant to TGF-β1 induced cell death than surrounding cells (Kolaja *et al*, 1999). Although our results are far from establishing a direct connection between the induction of apoptosis by HCB and human proliferative thyroid diseases, they provide the groundwork for further research in this area.

Among most of the numerous proposed hierarchical models of the caspase cascade, caspase-8, caspase-9, and cytochrome *c* are upstream regulatory elements in apoptotic pathways. Our work demonstrates that HCB induces a significant increase of cytochrome *c* in the cytosol, with HCB 10 and 100 mg/kg, providing evidence that the mitochondrial pathway is involved in HCB-induced apoptosis. Following release from mitochondria, cytochrome *c* binds to Apaf-1 resulting in the activation of caspase-9, which in turn activates the effector caspases responsible for the cleavage of many of the substrates associated with the characteristic biochemical and morphological changes of apoptosis (Cain, 2003). The enhanced dose-response cleavage of procaspase-9, reported in the present study is consistent with the role of caspase-9 in the apoptotic pathway activated by HCB in thyroid-follicular cells. It is interesting that this caspase cascade, with caspase-9 at the apex, is the major pathway initiated in many forms of chemically induced apoptosis (Sun *et al*., 1999). It has been demonstrated that apoptosis induced by TGF-β1 is accompanied

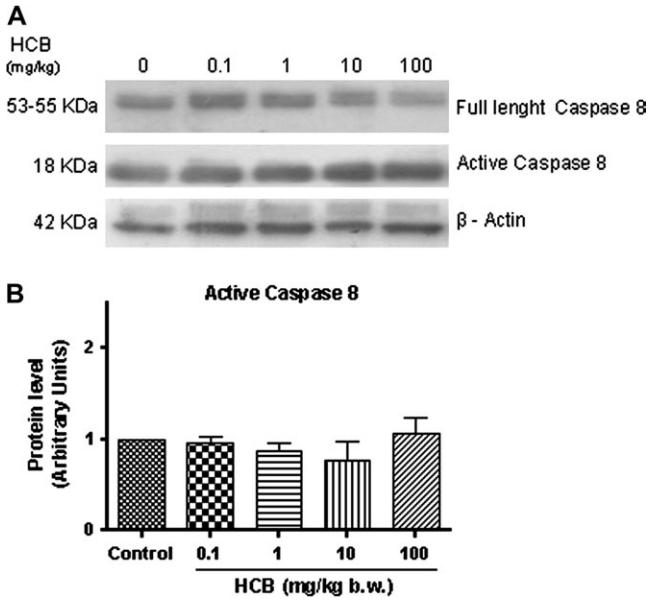

FIG. 7. HCB did not enhance cleavage of procaspase-8. (A) Pro- and active caspase-8 were determined by immunoblotting in the cytosolic fraction of thyroid tissue from HCB (0.1, 1, 10, and 100 mg/kg body weight)–treated rats. (A) Western blot analysis of pro- and active caspase-8 and β-actin. (B) Quantification of cytosolic active caspase-8 by densitometric scanning of the immunoblots. Values are means ± SEM of three independent experiments of three rats per group. Statistical comparisons were made by analysis of variance (one-way ANOVA), with a 95% confidence interval followed by Tukey *post hoc* test to identify significant differences between mean values and indicated controls.

with ROS generation. Thus, we could speculate that HCB induced ROS generation in a dose-dependent manner, eliciting dose-dependent increases in cytochrome *c* and caspase-9 activity (Wang *et al*., 2008).

Bcl-2 family proteins, including Bax, Bak, and Bcl-Xs for proapoptosis and Bcl-2, Bcl-XL, and Mcl-1 for antiapoptosis, have been demonstrated to be involved in the process of mitochondrial induced apoptosis (Jurgensmeier *et al*., 1998). Preliminary studies from our laboratory indicated that the levels of proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins were deregulated by HCB treatment (results not shown). Caspase-8 can be activated by one of several death receptors when bound by the appropriate ligands, including TNF, FasL, and TNF-related apoptosis-inducing ligand. It can also be activated in the absence of a death receptor signal, as has been demonstrated for drug-induced apoptosis (Wesselborg *et al*., 1999) suggesting that other mediators of apoptosis might also be involved. The results from the present study show that although caspase-8 was not activated by HCB, we cannot exclude death receptor-induced apoptosis mediated by the activation of caspase-3 by caspase-10 as has been described by Wang *et al*., (2001), eliciting an increased programmed cell death at HCB 1 and 10 mg/kg.

There is an apparent discrepancy among TGF-β1 mRNA expression, induced apoptosis decreasing at the highest HCB dose (100 mg/kg) and the increasing trend in cytochrome *c* levels and caspase-9 activity. Our results suggest that caspase-dependent and -independent pathways may be operating together in HCB-induced apoptosis in the thyroid gland. The possibility exists that the higher TUNEL-positive nuclei at HCB 1 and 10 mg/kg, when compared with HCB 100 mg/kg, could be related to induction of other caspases (such as caspase 10) that may potentiate the apoptotic effect. We could also speculate that a caspase-independent pathway may be also operating to increase relative apoptotic index at the lower doses. On that respect, HCB (1 and 10 mg/kg) treatment might change mitochondrial membrane permeability leading to release of the apoptotic inducing factor which after entering nucleus results in increased apoptosis, as recently demonstrated in HeLa cell lines (Jayaraj *et al*., 2009). Future experiments would be necessary to clarify those points.

In summary, these studies indicate for the first time that the administration of HCB doses that do not disrupt thyroid economy, induce TGF-β1 expression and apoptosis in the thyroid gland, involving the mitochondrial pathway. As the delicate balance between apoptosis and cell proliferation may play an important role in the control of thyroid gland mass, our observations provide a clue to a potentially important mechanism to the molecular events involved in HCB carcinogenic activity.

FUNDING

National Agency of Scientific and Technological Promotion (PICT 05-25849); National Council of Scientific and Technological Research (PIP6060); and University of Buenos Aires (PID M041).

ACKNOWLEDGMENTS

Many thanks are given to Rodolfo Kölliker-Frers and Victor Hugo Tomassi for their technical assistance. D.L.K de P and A.R. are established researchers of the CONICET. Rodolfo Kölliker-Frers and Victor Hugo Tomassi are Technical Professionals of the CONICET.

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