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Effect of *in vivo* administered hexachlorobenzene on epidermal growth factor receptor levels, protein tyrosine kinase activity, and phosphotyrosine content in rat liver

Andrea S. Randi[a], Horacio A. Sancovich[b], Ana M. Ferramola de Sancovich[b], Andrea Loaiza[b], Rodolfo A. Kölliker Frers[a], Fernanda Spinelli[b], Diana L. Kleiman de Pisarev[a,*]

[a]*Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 5to piso, Buenos Aires, CP 1121, Argentina*
[b]*Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

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Abstract

In the present study, the effects of hexachlorobenzene (HCB) on epidermal growth factor receptor (EGFR) content of liver microsomes and plasma membrane, and on EGFR-tyrosine kinase activity in the microsomal fraction were investigated. In addition, we studied the parameters of the tyrosine kinase signalling pathway such as protein tyrosine kinase (PTK) activity and phosphotyrosine content in microsomal and cytosolic protein. To determine whether the observed alterations were correlated with a manifestation of overt toxicity, a single very low dose of HCB (1 mg/kg body wt) and two much higher doses (100 and 1000 mg/kg body wt), the highest being toxicologically significant in that it reduced serum thyroxine (T_4) and inhibited uroporphyrinogen decarboxylase (URO-D) (EC 4.1.1.37) activity, were tested. Our results demonstrated that liver microsomes of rats treated with HCB had higher levels of EGFR than untreated rats; treated rats also had less EGFR present in hepatocyte plasma membrane fractions than did untreated rats. HCB altered the phosphotyrosine content and protein phosphorylation of some microsomal and cytosolic proteins in a biphasic dose–response relationship. At the low dose, phosphorylation and phosphotyrosine content of several microsomal proteins were increased; however, these effects were diminished or reversed at the higher doses. Our results suggest that chronic HCB treatment produces a down-regulation of the EGFR and a dose-dependent increase in EGFR-tyrosine kinase activity in the microsomal fraction. This effect may contribute to the alteration of membrane and cytosolic protein tyrosine phosphorylation. The level of sensitivity encountered in our studies is extraordinary, occurring at 1/10 to 1/1000 the doses of HCB known to cause other toxicological lesions.

Keywords: Hexachlorobenzene; Rat liver; EGFR kinase activity; Phosphotyrosine content; Protein tyrosine kinase; EGFR levels

1. Introduction

HBC is a lipophilic chemical compound that is widely distributed in the environment. Although it is no longer used as a fungicide, it is a by-product in several industrial processes [1]. This compound is biodegraded slowly [2], and is extremely persistent in body fat [3]. Chronic administration of HCB to experimental animals produces a number of effects, including increased synthesis of liver microsomal enzymes [4], triggering of hepatic porphyria [5–7], hypothyroxinemia [8,9], and thyroid adenomas [10]. HCB is also known to be carcinogenic in animals [11,12]. URO-D (EC 4.1.1.37) inhibition has been proposed as a primary causative event of HCB-induced porphyria [13]. This cytosolic enzyme catalyzes the conversion of uroporphyrinogen to coproporphyrinogen.

HCB is a ‘‘dioxin-type’’ chemical and a weak agonist of the Ahr [14], a ligand-activated transcription factor. It has also been proposed that the binding of ‘‘dioxin-type’’

* Corresponding author. Tel.: +54-11-4508-3672, Ext. 33; fax: +54-11-4508-3672.
E-mail address: dkleiman@fmed.uba.ar (D.L. Kleiman de Pisarev).

Abbreviations: Ahr, aryl hydrocarbon receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HCB, hexachlorobenzene; PMSF, phenylmethylsulfonyl fluoride; PTK, protein tyrosine kinase; T_4, thyroxine; T_3, triiodothyronine; TCA, trichloroacetic acid; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; URO-D, uroporphyrinogen decarboxylase; XRE, xenobiotic response element.

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chemicals to the Ahr complex could activate the phosphorylation of important proteins in the cytosol, plasma membrane, and other intracellular organelles, eliciting changes in signal transducing and gene expression activities [15]. It has been demonstrated that non-coplanar polychlorinated biphenyls, which do not bind with high affinity to the Ahr [16], alter calcium regulation and relevant signal transduction systems [17]. Nevertheless, it has not yet been determined whether some or all of the effects of HCB are mediated by its binding to the Ahr.

We previously reported that *in vivo* exposure to HCB (1000 mg/kg body wt) for 10 days caused profound changes in some biochemical parameters of rat liver membranes, i.e. reductions in [^{125}I]EGF binding to its receptor, microsomal protein phosphorylation, PTK, and Na^+/K^+ ATPase activities [18]. The EGFR is a member of a family of plasma membrane receptors that, upon binding of the ligand, transduce signals by tyrosine kinase activity, even after the internalization of the liganded receptor into components of the endosomal apparatus [19,20]. An early event in the activation of the EGFR is the stimulation of an intrinsic tyrosine kinase resulting in the autophosphorylation of specific tyrosine residues [21], and the phosphorylation of other cellular proteins [22].

Other chemicals such as TCDD, the most toxic congener of dioxin-type compounds, cause alterations in EGFR levels, PTK activity, and protein phosphorylation [15,23]. Experimental results have confirmed that TCDD causes *in vivo* [24] and *in vitro* [25] down-regulation of EGFR in a variety of tissues and cell types, and that this phenomenon is clearly Ahr-mediated.

In the present study, we evaluated the internalization of the EGFR from the plasma membrane to the microsomal compartment following the administration of HCB *in vivo*, and assessed EGFR tyrosine kinase activity in the microsomal immunoprecipitated fraction. Moreover, we studied parameters of the tyrosine kinase signalling pathway such as protein phosphorylation, PTK activity, and phosphotyrosine content in microsomal and cytosolic proteins. To determine whether the observed alterations were correlated with a manifestation of overt toxicity, a single very low dose of HCB (1 mg/kg body wt) and two much higher doses (100 and 1000 mg/kg body wt), the highest being toxicologically significant in that it reduced serum thyroxine (T_4) and inhibited URO-D activity, were tested.

2. Materials and methods

2.1. Chemicals

HCB (>99% purity, commercial grade) was a gift from Máximo Paz S.A. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from NEN. A mouse monoclonal antibody against the human EGFR (Cat. No. 324862, 200 μg/mL) and nitrocellulose membranes were obtained from Calbiochem. Affinity purified rabbit polyclonal antiphosphotyrosine antibody (No. P11230, 250 μg/mL) was obtained from Transduction Laboratories. Polyclonal goat anti-EGFR-antibody [EGFR (1005)-G 200 μg/mL] was purchased from Santa Cruz Biotechnology. Bellows S.R.L. was the source for the μicroclar filters (0.45 μm). BCIP/NBT was obtained from the Sigma-Aldrich Co. PAGE reagents were from Bio-Rad. All of the other chemicals were ACS grade and were purchased from the Sigma Chemical Co.

2.2. Animals and treatment

Female Wistar rats (160–180 g at the start of the experiment) were purchased from the National Atomic Energy Commission (CNEA). The rats were fed Purine 3 rat chow (Cabeca S.C.A.) and water *ad lib*. Environmental conditions consisted of a 12-hr light/12-hr dark cycle, temperature 20–24 ± 3°, and 45–75% humidity. Following a 7-day acclimation period, HCB was administered daily by gavage for 5 days of each week over a 4-week period, at doses of 1, 100, or 1000 mg/kg body wt, as indicated in the text. This fungicide was administered as a suspension of 0.04 to 40 mg/mL in water, containing Tween 20 (0.5 mL/100 mL). Control animals received equal volumes of the appropriate solvent by the same route. The maximum dose of HCB employed was chosen based on previous studies from our laboratory and those of others [4,5,7,9], demonstrating that this dose elicited clear manifestations of hepatic porphyria and thyroid hormone metabolism alterations in experiments involving up to 30 days of intoxication.

The general health of the animals was not affected by the doses of HCB employed, as evaluated by the behaviour and appearance of the rats, including examination of their coats, mucous membranes, and body weights, and their food and water consumption.

2.3. Hepatic plasma membrane and microsomal preparations

Following an overnight fast, animals were decapitated, and their livers were perfused with 0.154 M NaCl and used immediately for the isolation of plasma membranes, microsomes, and cytosol. Subcellular fractionation was performed using established procedures. Hepatic plasma membranes were isolated from 6 to 8 g of fresh liver by differential centrifugation, as indicated below, followed by a sucrose gradient purification technique [26]. Essentially, the liver was homogenized in 0.25 M STM (0.25 M sucrose, 5 mM Tris–HCl, pH 7.2–7.6, 1 mM $MgCl_2$), supplemented with the following protease inhibitors: 10 μg/mL of bacitracin, 2 μg/mL of aprotinin, 10 μg/mL of pepstatin A, 10 μg/mL of chymostatin, and 2 μM PMSF, by ten strokes with a loose-fitting pestle in a Dounce-type glass homogenizer. The homogenate was adjusted to 20%

(liver wet weight to total volume) with 0.25 M STM, and filtered through four layers of moistened gauze. The filtrate was centrifuged at 280 *g* for 5 min at 4°, and the sediment was discarded. The supernatant fluid was then centrifuged at 1500 *g* for 10 min at 4°, and the pellet was resuspended by three strokes of the Dounce homogenizer in 1.2 vol. of 0.25 M STM/g liver. STM (2.0 M) was added to obtain a final density of 1.18 g/mL, and sufficient 1.42 M STM (δ: 1.18 g/mL) was added to bring the volume to approximately twice that of the original homogenate. Aliquots of 35 mL were added to centrifuge tubes, and overloaded with 2–4 mL of 0.25 M sucrose. After centrifugation at 82,000 *g* (no brake) for 60 min at 4°, the pellet at the interface was collected and resuspended in 0.25 M sucrose to obtain a final density of 1.05 g/mL. This suspension was centrifuged at 1500 *g* for 10 min at 4°, and the final pellet was resuspended in 0.25 M sucrose and stored at −20°. Microsomes were isolated from a crude homogenate in 25 mM Tris–HEPES (pH 7.4), supplemented with 250 mM sucrose. The homogenizing medium contained the following protease inhibitors: 10 μg/mL of bacitracin, 2 μg/mL of aprotinin, 10 μg/mL of pepstatin A, 10 μg/mL of chymostatin, and 2 μM PMSF. The crude homogenate was centrifuged at 12,000 *g* for 20 min at 4° to remove the unbroken cells, nuclear fraction, and mitochondria. The resulting supernatant fluid was then centrifuged at 100,000 *g* for 60 min at 4° to obtain the microsomal and cytosolic fractions. The homogenates for antiphosphotyrosine immunoblot studies were prepared in the presence of phosphatase inhibitors, 2 mM Na_3VO_4, and 100 mM NaF.

The purity of the plasma membrane and microsomal fractions was characterized by electron microscopy. The membrane fractions were characterized by the distribution of 5′-nucleotidase activity, a marker enzyme of the plasma membrane. 5′-Nucleotidase activity was measured as previously described [27].

2.4. In vitro phosphorylation

Membrane and cytosolic proteins were phosphorylated by a modification of the procedure of Rubin *et al.* [28]. The phosphorylation reaction was performed at 30°, in a total volume of 100 μL containing 100 μg membrane protein, 50 mM PIPES (pH 7.0), 30 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.32 mM EGTA, in the presence or absence of 200 μM Na_3VO_4. Following a 15-min preincubation at 0°, the reaction was started by the addition of 20 μCi of [γ-^{32}P]ATP (2 μM), and stopped after 10 min using 50 μL of 9% sodium dodecylsulphate/6% 2-mercaptoethanol/30 mM Tris (pH 7.8). Tubes were corked and heated to 100° for 5 min. The protein content of isolated fractions was determined by the method of Bradford [29]. Equivalent amounts of total protein for each sample (25–40 μg/lane) were electrophoresed on SDS–polyacrylamide slab gels, according to the method of Laemmli [30]. Gels were treated with 10% (v/v) TCA for 1 hr, and then were stained with Coomassie brilliant blue, destained, dried, and autoradiographed on Kodak XAR-5 film. Exposure time varied from 7 to 15 days. Equal loading of each lane was controlled by Coomassie brilliant blue staining of the gels.

2.5. SDS–PAGE

Liver cytosol and microsomal proteins were separated by SDS–PAGE, according to Laemmli [30], with either a 7.5 or 10% resolving gel and a 4.0% stacking gel, in a Mini-Protean II electrophoresis cell (Bio-Rad). The 0.75-mm slab gels were electrophoresed for 45–60 min at a constant voltage setting of 150 V, at 4°. The running buffer (5×) used was 0.12 M Tris base, 1 M glycine, and 10% SDS (pH 8.3). The molecular weight of the protein bands was approximated from molecular weight standards (Sigma).

2.6. EGFR immunoprecipitation

Microsomal fractions (0.25 mg protein) in immunoprecipitation buffer [200 mM Tris (pH 7.4), 1 M NaCl, 100 mM EDTA, 1% IGEPAL [(octylphenoxy)polyethoxyethanol], 2% Triton X-100] with 1 mM leupeptin, 200 mM PMSF, 1 mg/mL of aprotinin, 2 mM Na_3VO_4, and 1 M NaF were incubated with 4 μg of an anti-EGFR antibody [Santa Cruz; goat polyclonal IgG, EGFR (1005)-G 200 μg/mL] and 30 μL of A/G plus agarose (Santa Cruz), in a final volume of 0.5 mL, for 16–24 hr at 4° under constant shaking. Precipitates were washed four times with immunoprecipitation buffer, centrifuged at 12,000 *g* for 5 min at 4°, and denatured in Laemmli buffer at 95° for 5 min. The immunoprecipitated proteins were subjected to a protein kinase assay or SDS–PAGE for immunoblotting.

2.7. Western blot for EGFR

Rat liver plasma membrane and microsomal fractions adjusted to the same protein concentration were treated with SDS sample buffer and boiled for 5 min. Equivalent amounts of total protein for each sample (500 μg/lane) were electrophoresed in a 7.5% SDS–polyacrylamide gel. The gel and nitrocellulose were then equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 10 min. The gel was electroblotted overnight using a Miniprotean electroblot apparatus at 30 V, with agitation and chamber cooling at 4°. Upon completion, the membrane was blocked for 1 hr in TBS (10 mM Tris, 150 mM NaCl, pH 7.6) with 3.0% BSA. The nitrocellulose was incubated for 2 hr with the monoclonal anti-EGFR antibody (1:500) (Calbiochem, 200 μg/mL) in TBSTB (TBS with 0.1% BSA and 0.1% Tween 20), at 37°. The immunoblot was then washed four times in TBSTB, and the bound

antigen–antibody complexes on the blot were detected by incubation for 1 hr with an alkaline phosphatase-conjugated goat antibody to mouse immunoglobulin G (1:1000) in TBSTB, followed by four washes in the same buffer. The colorimetric substrate BCIP/NBT (100 mM Tris-base, pH 9.5, 150 μg/mL of 5-bromo-4-chloro-3-indolyl phosphate, 300 μg/mL of nitroblue tetrazolium, 5 mM $MgCl_2$) was added to the membrane to localize antibody binding, indicated by an insoluble blue color.

Immunoprecipitated EGFR was transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad) and probed with the polyclonal anti-EGFR antibody (1:400) [goat polyclonal anti-EGFR (1005)-G, 200 μg/mL]. The second antibody used was a peroxidase-conjugated IgG (1:2000) (monoclonal anti-goat/sheep IgG, clone GT-34). The immune complexes were visualized by enzyme-linked enhanced chemiluminescence (ECL, Amersham Biosciences, Inc.) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

2.8. EGFR-immune complex kinase assay

The microsomal fraction (0.5 mg protein) was incubated with 8 μg of an anti-EGFR antibody [Santa Cruz, goat polyclonal IgG, EGFR (1005)-G 200 μg/mL] and 60 μL of A/G plus agarose for 16–24 hr at 4° under constant shaking. The immune complexes were washed twice with RIPA buffer (1× PBS, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS with 100 μg/mL of PMSF, 50 μg/mL of aprotinin, and 1 mM Na_3VO_4) and twice with kinase buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 0.1% Triton X-100, and 10% glycerol), as described by Countaway *et al.* [31]. The phosphorylation of 20 μg of acid-denaturated rabbit muscle enolase (Sigma) as exogenous substrate, prepared according to Fresno Vara *et al.* [32], was carried out in a final volume of 100 μL containing 5 mM $MgCl_2$, 20 mM $MnCl_2$. The reaction was started by the addition of 120 μM ATP and 5 μCi of [γ-^{32}P]ATP (New England Nuclear, 6000 Ci/mmol). After 15 min at 30°, 25 μL of 4× Laemmli sample buffer was added, and the samples were heated at 95° for 5 min. Proteins were resolved by reducing 10% SDS–PAGE. Marker proteins were visualized by staining with Coomassie brillant blue. After staining, destaining, and drying, the gels were exposed to X-ray film for autoradiography. Exposure time varied from 7 to 15 days. The ^{32}P-labelled proteins were visualized by autoradiography.

2.9. Western blot for phosphotyrosine

The procedures for obtaining microsomal and cytosolic fractions from the homogenate in the presence of phosphatase inhibitors, Na_3VO_4, and NaF, and to determine phosphotyrosine content were performed according to the same protocol used for the analysis of EGFR levels. The nitrocellulose was incubated with the primary polyclonal antiphosphotyrosine antibody (250 μg/mL) (1:1000) in TBSTB at 37° for 2 hr, followed by 1 hr with alkaline phosphatase-conjugated goat antibody to rabbit immunoglobulin G (1:1000), using BCIP/NBT as the colorimetric assay.

2.10. PTK assay

PTK activity in rat liver cytosolic and plasma membranes was assayed with the synthetic peptide poly-[Glu^{80} Na, Tyr^{20}] (poly-GT), as described by Tremblay and Beliveau [33]. Hepatic plasma membranes were diluted to 16 mg protein/mL in a solution of 50 mM sucrose, 5 mM HEPES–Tris (pH 7.4), and preincubated for 1 hr at 4° in the presence of 0.05% (v/v) Triton X-100 containing protease inhibitors (10 μg/mL of bacitracin, 2 μg/mL of aprotinin, 10 μg/mL of pepstatin A, 10 μg/mL of chymostatin, and 2 mM PMSF). Phosphorylation of the synthetic peptide, poly-GT, was carried out in a reaction mixture containing 80 μg/100 μL of particulate proteins, 500 μg/100 μL of poly-GT, 8 μCi of [γ-^{32}P]ATP (40 μM), 10 mM $MnCl_2$, 20 mM $MgCl_2$, 0.2 mM Na_3VO_4, and 25 mM HEPES–Tris (pH 7.0), for 30 min at 30°. The protein phosphorylation reaction was stopped by the addition of a solution containing 10% (v/v) TCA, 10 mM NaH_2PO_4, and 10 mM $Na_4P_2O_7$. The mixture was then incubated for 30 min on ice and filtered through a μicroclar Bellows filter (0.45 μm), which was then washed three times with stop solution. The radioactivity associated with the filter was measured by liquid scintillation counting in water. Assays in which poly-GT was omitted were used as blanks. Phosphorylation assays were linear for up to 30 min.

2.11. Determination of URO-D activity

Liver homogenates and enzyme preparations were obtained as described by Sopena de Kracoff *et al.* [6]. Homogenates were prepared with 0.154 M KCl (1:5), and centrifuged at 12,000 *g* for 20 min at 4°. Supernatant fractions from livers were then filtered through a Sephadex G-25 column with 0.134 M potassium phosphate buffer, pH 6.8. Eluates with or without traces of fluorescence were pooled and used as the enzyme preparation. The URO-D incubation mixture contained 67 mM potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1.66 mM dithiothreitol (DTT), uroporphyrinogen III (6 μM) as substrate, and the enzyme preparation, in a final volume of 500 μL. Incubations were performed for different times, in the dark at 37°, under N_2, and with mechanical shaking. The reaction was stopped by the addition of concentrated HCl [5% (w/v) final] and DMSO [21% (v/v) final], and was centrifuged at 12,000 *g* for 10 min at 4°. The porphyrins formed were analyzed by HPLC in a 125 × 4 mm column, containing Lichrospher 100 RP-18.5 μm (Merck) in a solvent system consisting of a 15-min linear gradient from 10% (v/v)

Table 1
Effects of HCB administration on body weight, liver weight, liver relative weight, an serum T_4 and T_3 levels

HCB dose (mg/kg body wt)	Body weight (g)	Liver weight (g)	Liver relative weight[a]	T_4 (μg/dL)	T_3 (ng/dL)
0	211.00 ± 10.00	6.74 ± 0.48	3.18 ± 0.12	3.2 ± 0.5	77 ± 5
1	201.17 ± 3.91	7.12 ± 0.51	3.53 ± 0.22	3.0 ± 0.9	74 ± 6
100	200.33 ± 3.59	7.02 ± 0.41	3.49 ± 0.15	2.8 ± 0.7	70 ± 7
1000	194.00 ± 4.59	8.97 ± 0.26*	4.64 ± 0.18**	1.2 ± 0.5*	60 ± 9

The effects of HCB administration (1, 100, and 1000 mg/kg body wt) on body weight, liver weight, liver relative weight, and serum T_4 and T_3 levels were measured at 30 days. T_4 and T_3 levels were determined by RIA, with a double antibody-PEG technique. Data (means ± SEM) are from six rats per group at each time point.

[a] Liver relative weight: [liver weight (g)/body weight (g) × 100].

* $P < 0.005$; ** $P < 0.0005$.

acetonitrile to 30% acetonitrile in 1 M ammonium acetate buffer (pH 5.2), followed by isocratic elution at 30% acetonitrile for another 10 min. The flow rate was 1 mL/min, and the fluorescence detector was set at excitation and emission wavelengths of 404 and 618 nm, respectively, as described in Lim *et al.* [34].

2.12. Total serum T_4 and T_3

Total serum T_4 and T_3 concentrations were determined by radioimmunoassay (RIA), with a double antibody-PEG technique (Diagnostic Products Corp.). Each sample was assayed in duplicate.

2.13. Statistical analysis

Data from dose effects were analyzed by a one- or two-way ANOVA, followed by Tukey's test. Other statistical analyses were performed using Student's *t*-test, as indicated in the text. Differences between control and treated animals were considered significant when *P* values were <0.05.

3. Results

In the present study, several biochemical parameters presumably linked to the EGFR signal transduction pathway were investigated. Very low doses of HCB (1 mg/kg body wt) were assayed, to detect sensitive biochemical parameters modified by HCB intoxication. To determine whether the observed alterations were correlated with the manifestation of overt toxicity, two much higher doses of HCB (100 and 1000 mg/kg body wt), the highest being toxicologically significant in terms of reducing serum T_4 and inhibiting URO-D activity, were tested [5,9,35].

3.1. Effects of HCB administration on body and liver weights, the liver/body weight ratio, and serum T_4 and T_3 levels

To determine the dose-related effects on general parameters of HCB intoxication, the liver/body weight ratio and serum T_4 and T_3 levels were evaluated. Only the highest HCB dose (1000 mg/kg body wt) resulted in a decrease in total T_4 serum levels, and a significant increase in liver weight and liver relative weight [(liver weight/body weight) × 100] (Table 1).

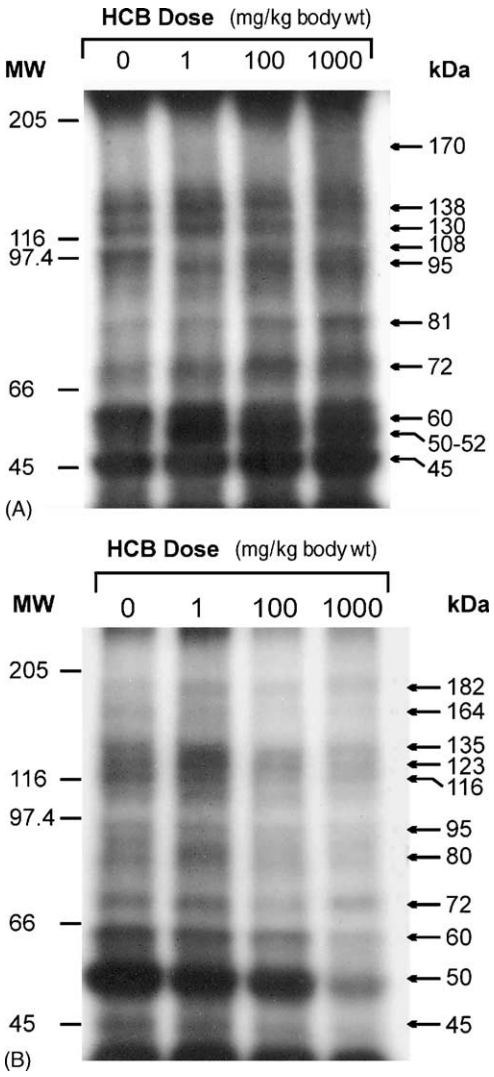

Fig. 1. *In vivo* effects of HCB on the phosphorylation of rat liver endogenous proteins, as determined by autoradiography. Rats were treated with HCB or vehicle at the doses indicated on the figure. The proteins were phosphorylated and analyzed by 7.5 or 10% SDS–PAGE, as indicated under Section 2. (A) Microsomal protein phosphorylation. (B) Cytosolic protein phosphorylation. Each lane was loaded with 30 μg of protein; MW: molecular weight markers. This experiment was repeated three times with similar results.

3.2. Hepatic protein phosphorylation

The *in vivo* effect of HCB administration on hepatic protein phosphorylation in various membrane and cytosolic preparations was studied. Plasma membrane preparations, highly enriched in 5′-nucleotidase activity and with minimal cross-contamination with other subcellular fractions, were used. The microsomal fractions were isolated using established procedures and contained the endosomes, but were free of nuclei and mitochondria.

Microsomal protein phosphorylation showed a biphasic dose–response relationship. At the lowest dose, phosphorylation of some microsomal proteins (52–50 and 138–130 kDa) was enhanced. This effect was reversed at higher doses. On the other hand, the phosphorylation of the 95, 81, and 72 kDa proteins was enhanced with increasing HCB doses (Fig. 1A).

In a similar fashion, phosphorylation of several cytosolic proteins (135–116, 80, and 72 kDa) was increased at the lowest HCB dose, and this effect was diminished or

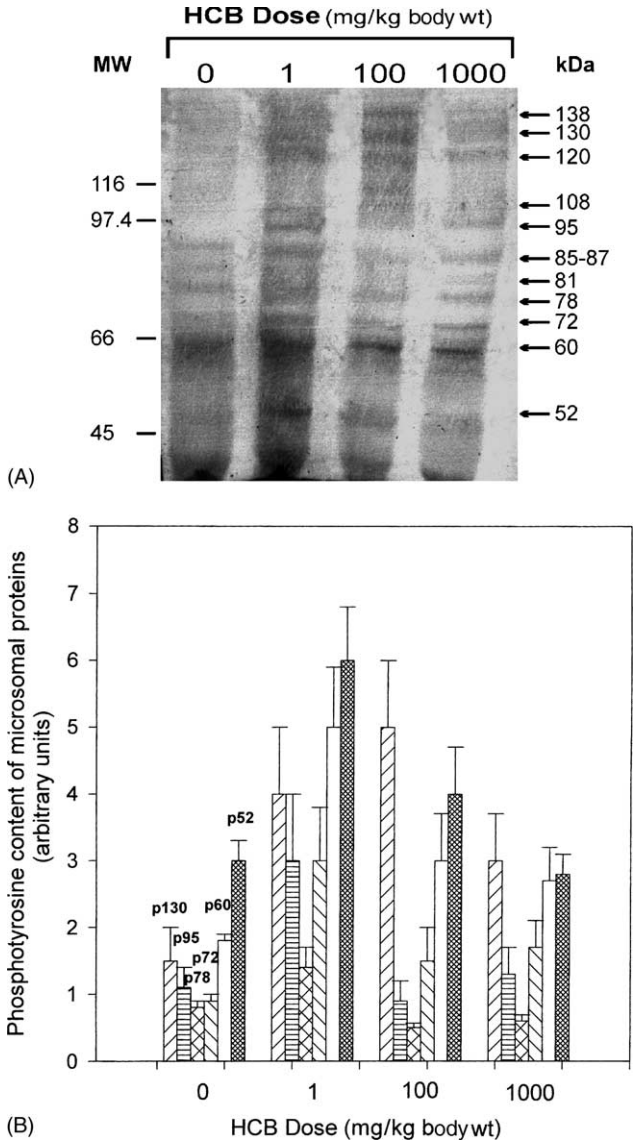

Fig. 2. (A) Immunochemical detection of the phosphotyrosine content of rat liver microsomal proteins from HCB-treated animals. The corresponding HCB dose (1, 100, and 1000 mg/kg body wt) or vehicle was administered as indicated in Section 2. Proteins were resolved on a 7.5% SDS–polyacrylamide gel, and phosphotyrosine content was detected by western blotting using a polyclonal antibody. A goat anti-rabbit IgG antibody conjugated with alkaline phosphatase was used to detect the primary antibody binding. MW: molecular weight markers. (B) Quantification of phosphotyrosine content of several proteins by densitometry scanning of three independent experiments. Values are means ± SD.

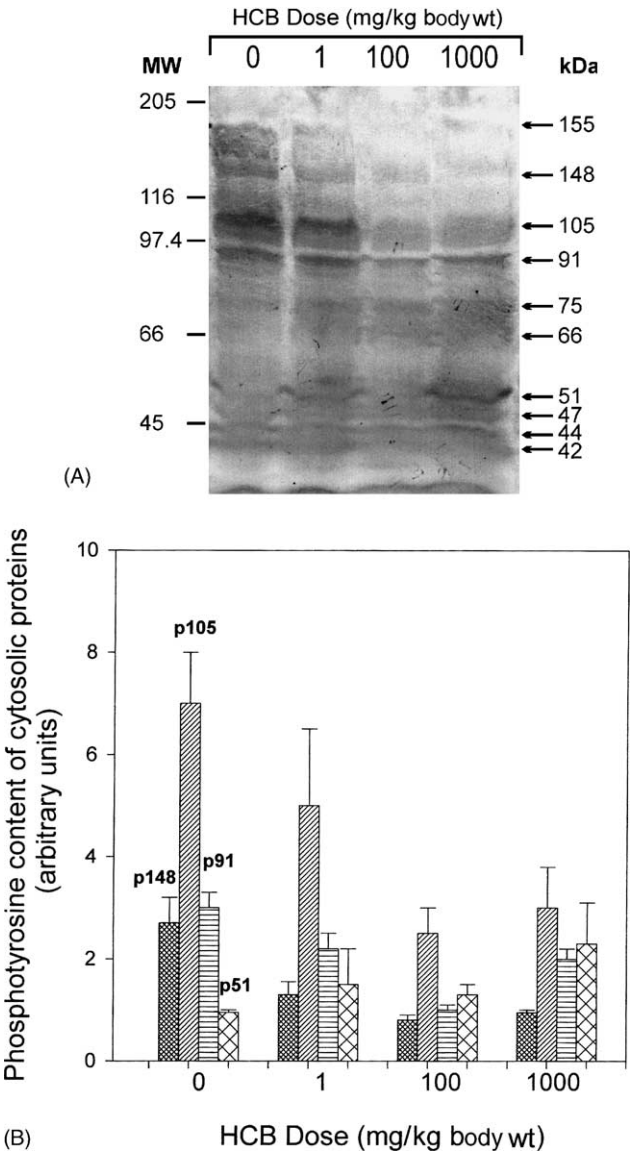

Fig. 3. (A) Immunochemical detection of the phosphotyrosine content of rat liver cytosolic proteins from HCB-treated animals. The corresponding HCB dose (1, 100, and 1000 mg/kg body wt) or vehicle was administered as indicated before. Proteins were resolved on a 7.5% SDS–polyacrylamide gel, and phosphotyrosine content was detected by western blotting as indicated in Section 2. MW: molecular weight markers. (B) Quantification of phosphotyrosine content of several proteins by densitometry scanning. Values are means ± SD of three independent experiments.

reversed to levels lower than those in untreated animals at the higher doses. The phosphorylation of other cytosolic proteins was also diminished at the higher doses. The most conspicuous band, 50 kDa, was noticeably inhibited at 1000 mg/kg body wt (Fig. 1B). This band was also found in murine hepatic cytosol [36].

3.3. Western blot analysis of phosphotyrosine content

Western blot analysis was performed to study the phosphotyrosine content of microsomal and cytosolic proteins of *in vivo* HCB-treated rats. In the case of microsomal phosphotyrosine content, a biphasic effect was observed with a maximum increase at 1 mg HCB/kg body wt, except for several bands between 108 and 138 kDa proteins, which increased at HCB doses of 100 mg/kg body wt (Fig. 2). There was a remarkable tyrosyl phosphorylation of the 60 and 52 kDa bands, which may correspond to two proteins involved in a tyrosine kinase-signalling pathway: the tyrosine-phosphorylated SRC-type protein, which undergoes an increase in phosphorylation after TCDD treatment [37], and the 50–52 kDa protein, which corresponds in molecular weight to an isoform of SHC, a SH2-containing adapter protein, that is a well-characterized substrate for the EGFR [38].

In the cytosolic fraction, the phosphotyrosine content of the 148 kDa protein decreased with increasing doses of HCB when compared to the control, while the phosphotyrosine content of other proteins, such as the one of 105 kDa, decreased only at the higher doses. In contrast, tyrosyl phosphorylation of the 51 kDa protein was augmented only at 1000 mg HCB/kg body wt (Fig. 3).

3.4. Western blot analysis of the EGFR

We have demonstrated previously that HCB (1000 mg/kg body wt) decreased EGF binding to its membrane receptor and microsomal PTK activity [18]. To evaluate the cellular compartmentalization of the EGFR, hepatocyte plasma membrane and microsomal EGFR levels were determined by western blotting. Our results showed that treated rats had lower levels of the EGFR in the plasma membrane fractions than did untreated rats. On the other hand, liver microsomes from rats treated with HCB had much higher levels of the EGFR than untreated rats, with a maximum effect at the lowest dose (Fig. 4). In some

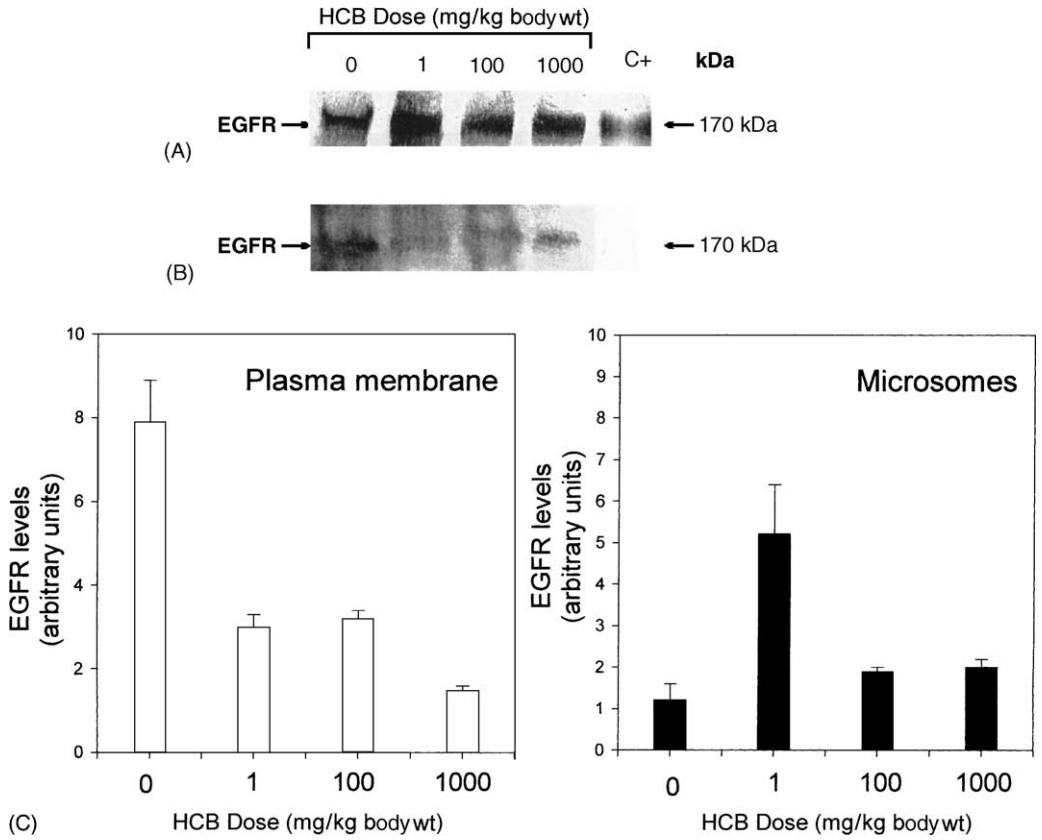

Fig. 4. Dose-dependent effects of HCB treatment on microsomal and plasma membrane EGFR levels. The corresponding HCB dose (1, 100, and 1000 mg/kg body wt) or vehicle was administered as indicated before. Proteins were resolved on a 7.5% SDS–polyacrylamide gel, and the EGFR was detected by western blotting using a monoclonal antibody. A goat anti-mouse IgG antibody conjugated with alkaline phosphatase was used to detect primary antibody binding, as described in Section 2. Immunochemical detection of EGFR levels in (A) microsomal proteins and (B) plasma membrane proteins. C+: positive control of cell lysate A-431 that overexpresses EGFR. (C) Quantification of plasma membrane and microsomal EGFR content by densitometry scanning of immunoblots. Values are means ± SD of three independent experiments.

experiments, EGFR protein was immunoprecipitated from liver microsomes using a polyclonal anti-EGFR antibody, and the immunoprecipitates were electrophoresed and transferred to PDVF membranes for detection using a polyclonal anti-EGFR antibody. Our results showed that the data were not affected significantly by the method of measurement, although in some experiments the amount of EGFR was higher at 100 mg/kg body wt of HCB than at 1 mg/kg body wt, as shown in Fig. 5.

3.5. PTK activity

Taking into account that *in vivo* HCB administration caused a decrease in rat liver plasma membrane EGFR levels, the PTK activity in this subcellular fraction was examined, using an artificial substrate specific for PTK, poly [Glu^{80} Na, Tyr^{20}]. The results obtained showed that PTK activity was increased significantly at 1 mg HCB/kg body wt, returning to control levels at higher doses (Fig. 6). The effect of EGF (100 ng/100 μL) on plasma membrane PTK activity was assayed in control and HCB-treated rats. Our results showed that PTK activity was stimulated significantly over their corresponding controls only in untreated animals (Fig. 6). A non-significant stimulation effect was observed at the higher doses of HCB. These results suggest that HCB could induce an internalization of receptors, with a negative correlation between EGFR internalization and EGF-stimulated PTK activity. An alternative interpretation of the results is that HCB potentially alters other tyrosine-kinase receptor levels, such as insulin receptors (IR) and insulin-like growth factor-I receptors (IGF-IR), in which case the observed changes in PTK activity would not be EGFR-mediated. PTK activity assayed in the cytosolic fraction was increased significantly at HCB doses of 1 and 100 mg/kg body wt, while at 1000 mg/kg body wt, the activity returned to control values (Fig. 7).

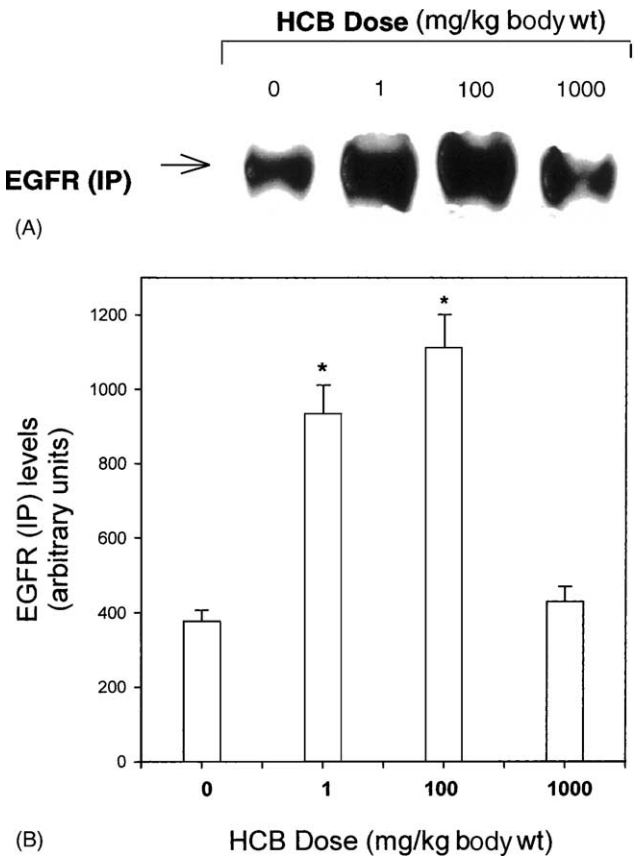

Fig. 5. Dose-dependent effects of HCB treatment on microsomal immunoprecipitated EGFR levels. The corresponding HCB dose (1, 100, and 1000 mg/kg body wt) or vehicle was administered as indicated before. The EGFR was immunoprecipitated from microsomes by a specific antibody. The immunocomplex was resolved on a 7.5% SDS–polyacrylamide gel, and the EGFR was detected by western blotting using a specific antibody. A goat anti-mouse IgG antibody conjugated with peroxidase was used to detect the primary antibody binding, as described in Section 2. (A) Immunochemical detection of EGFR levels. (B) Quantification of microsomal EGFR contents by densitometry scanning of the immunoblots of three independent experiments. Values are means ± SD. Key: (*) significantly different from control values at $P < 0.05$.

3.6. HCB induction of EGFR kinase activity

Our results suggested that HCB administration induced EGFR internalization; therefore, we examined the ability of HCB to stimulate EGFR kinase activity. EGFR protein was immunoprecipitated from liver microsomal fractions of rats, control and treated with HCB (1, 100, and 1000 mg/kg body wt), using a specific antibody. EGFR kinase activity was measured in the immune complex using acid-denatured enolase as an exogenous substrate. Our results showed that the amount of phosphorylated enolase was elevated markedly in HCB-treated animals, with a higher effect at 100 and 1000 mg HCB/kg body wt (Fig. 8A).

The specific EGFR activity was calculated by dividing the arbitrary units of the scanned autoradiograph of phosphorylated enolase from the kinase assay by the amount of EGFR present on the immunoblot probed with the anti-EGFR antibody, which was determined by densitometric scanning of the appropriate immunoreactive band (Fig. 5). Our results showed that EGFR kinase activity increased in a dose-dependent manner after HCB administration (Fig. 8B). We observed that although a higher internalization of EGFR was seen at either 1 mg or 100 mg HCB/kg body wt, the highest increase of EGFR kinase activity was achieved at 1000 mg/kg body wt, when toxic effects were observed.

3.7. Effect of HCB on URO-D activity

The activity of the enzyme URO-D, proposed as a primary triggering agent of HCB experimental porphyria, was measured. Our study showed a significant decrease (71%) in URO-D activity at only 1000 mg HCB/kg body wt (Fig. 9).

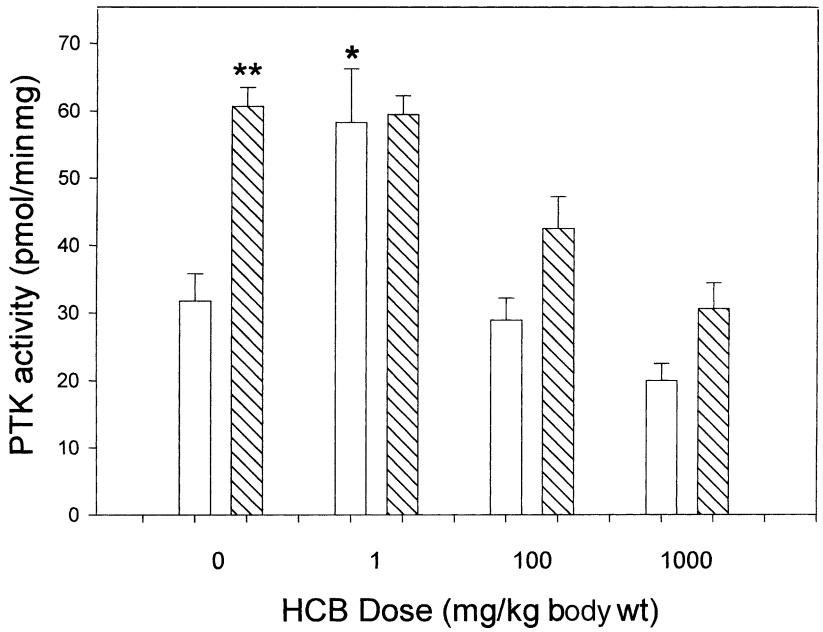

Fig. 6. Dose-dependent HCB-induced changes in plasma membrane PTK activity in the presence or absence of EGF. The corresponding HCB dose (1, 100, and 1000 mg/kg body wt) or vehicle was administered daily as indicated before. Plasma membrane preparations were preincubated for 15 min with EGF (100 ng/100 μL). PTK activity was measured in the absence (open bars) or presence (hatched bars) of EGF. Values are means ± SEM (N = 3). Key: significantly different from control values without EGF at: (*) $P < 0.05$ or (**) $P < 0.005$ (one-way ANOVA).

4. Discussion

We demonstrated earlier that *in vivo* administration of HCB (1000 mg/kg body wt) caused time-dependent alterations in microsomal protein phosphorylation and PTK activity, as well as dose-dependent modifications in the labelling of several bands, by day 10 of poisoning [18]. In the present study, the effects of chronic HCB administration on EGFR content, EGFR kinase activity, and biochemical parameters of the tyrosine kinase signal transduction pathway were investigated. To determine whether the observed alterations were correlated with a manifestation of overt toxicity, a very low dose (1 mg/kg body wt) and two higher doses (100 and 1000 mg/kg body wt) of HCB were tested. Our results showed that HCB increased liver/body weight ratios, and reduced T_4 serum levels, two well-characterized parameters of HCB exposure, only at the maximum dose

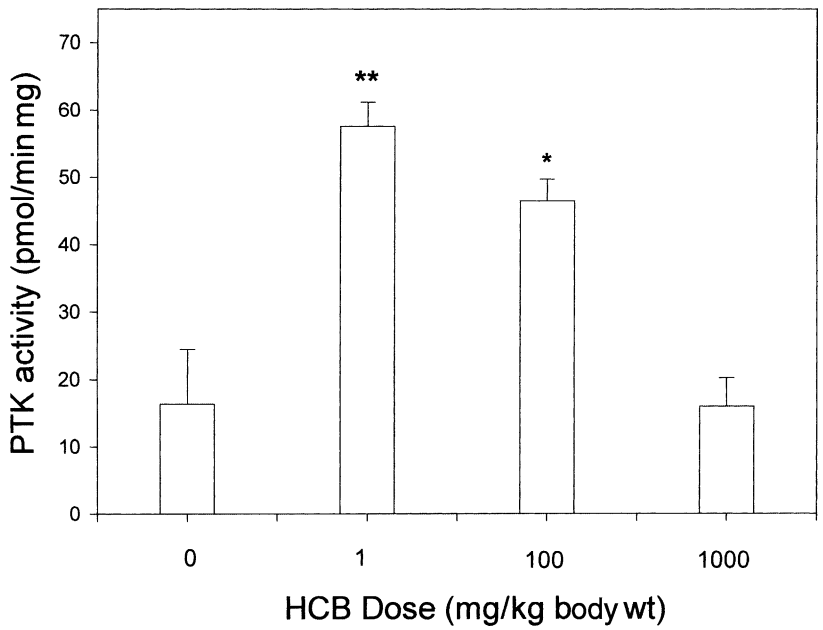

Fig. 7. Dose–response curve for rat liver cytosolic PTK activity. PTK activity was determined using poly Glu-Tyr as substrate. Each sample point represents the mean ± SEM (N = 3). Key: significantly different from control values at: (*) $P < 0.05$ or (**) $P < 0.005$ (one-way ANOVA).

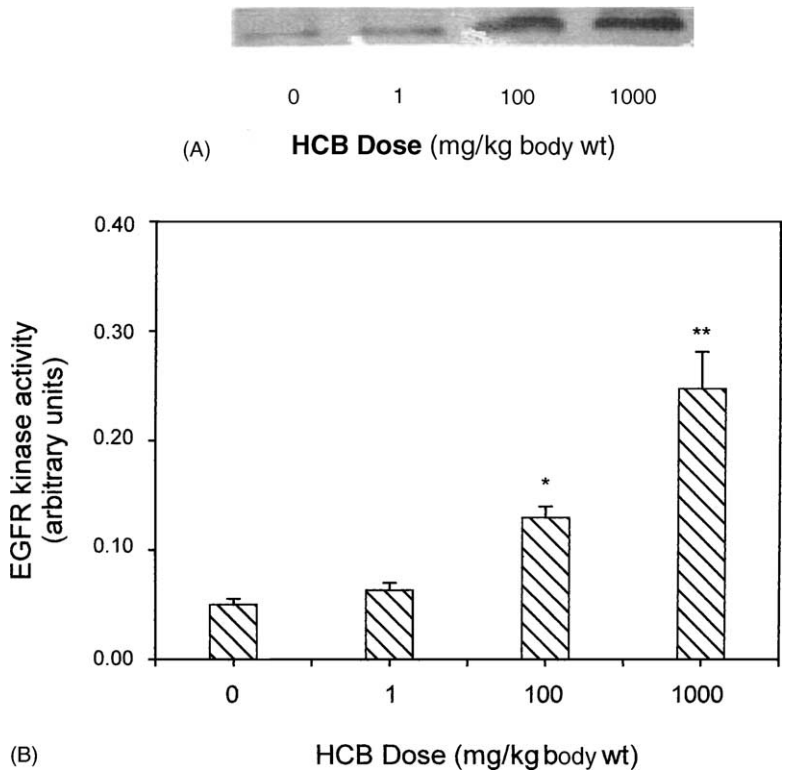

Fig. 8. EGFR-immune complex kinase activity. EGFR was immunoprecipitated from rat liver microsomes of control and HCB-treated rats by a specific antibody. Immunoprecipitates were incubated with [γ-^{32}P]ATP and acid-denatured enolase, a substrate for kinase activity. Enolase phosphorylation was detected following SDS–PAGE and autoradiography (A). Specific EGFR kinase activity was calculated as described in Section 3 (B). Data represent the means ± SEM of three independent experiments. Key: significantly different from control values at: (*) $P < 0.05$ or (**) $P < 0.005$ (one-way ANOVA).

(1000 mg/kg body wt). In a similar way, URO-D activity was inhibited significantly only at the highest dose of HCB. The results confirm previous observations from our laboratory [5].

The current study has demonstrated that HCB administration resulted in dose–response alterations in cytosolic and microsomal protein phosphorylation, and phosphotyrosine content, suggesting that altered protein kinase and

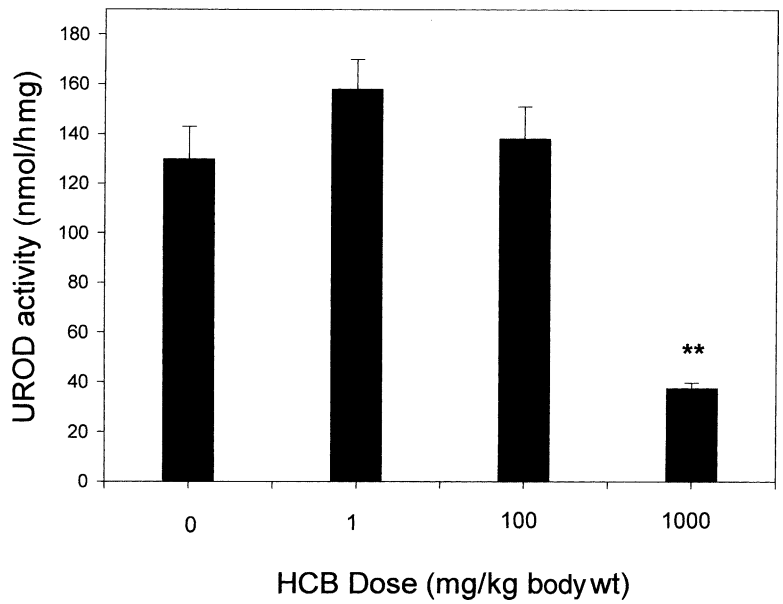

Fig. 9. Dose–response curve for URO-D activity. Rats were treated daily with HCB (1, 100, and 1000 mg/kg body wt) or vehicle. Each sample point represents the mean ± SEM (N = 3). URO-D activity was determined in rat liver cytosol as indicated in Section 2. Key: (**) significantly different from control values at $P < 0.005$ (one-way ANOVA).

phosphatase activities could be involved in the pleiotropic effect of HCB. It is worth mentioning that the steady-state level of phosphoproteins reflects the ratio of phosphorylation and dephosphorylation rates as a result of HCB action on cellular protein kinases and/or phosphatases. In addition, the changes in the profile of microsomal and cytosolic proteins indicate that the alterations occurred even at the lowest dose of HCB (1 mg/kg body wt). In the case of microsomal phosphotyrosine content and protein phosphorylation, a biphasic dose–response relationship was seen for some proteins. At the lower doses (1 and 100 mg/kg body wt), phosphorylation was enhanced, and this effect was reversed at higher doses, reaching values lower than those in untreated animals. The same effect was observed in several cytosolic proteins.

Earlier results have shown that HCB (1000 mg/kg body wt) decreased EGF binding to the high-affinity components of the EGFR, those with tyrosine kinase activity [18,31], suggesting that the EGFR may be involved in the mechanism of action of HCB. The present study is the first demonstration that HCB caused a significant reduction of immunodetectable EGFR in the plasma membrane, accompanied by increases in microsomal EGFR levels at the same concentrations with a greater effect at the lowest dose (1 mg/100 g body wt). These results suggest that HCB enhances plasma membrane EGFR internalization. Other laboratories have also shown that administration of TCDD to female rats decreases EGFR levels in the hepatocyte plasma membrane and also decreases EGFR mRNA [24,39].

HCB can increase rat liver EGFR-tyrosine kinase activity in a dose-dependent manner. That the type of HCB-activated kinase is likely to be a tyrosine kinase has been shown by the fact that enolase has only tyrosine residues as phosphorylatable sites, with no serine or threonine residues. The increase in EGFR kinase activity may be involved in the phosphorylation of other endogenous substrates.

Plasma membrane PTK activity was enhanced at 1 mg HCB/kg body wt in the absence of EGF. Moreover, there is a lack of stimulation by EGF of plasma membrane PTK activity at this concentration. These results suggest that the increased PTK activity at 1 mg HCB/kg body wt may be attributable to the presence of other tyrosine kinase-receptors, such as insulin and/or other growth factor receptors in the plasma membrane preparations. Although our results indicate that the alteration in PTK and phosphotyrosine content at a very low HCB dose (1 mg/kg body wt) is probably not due to an increase in EFGR-specific kinase activity, the biochemical changes observed at 100 and 1000 mg/100 g body wt of HCB could be, in part, EGFR-mediated. These findings are relevant because it has been shown that long-term exposure to very low doses of toxicants can promote preneoplasic foci formation in rat liver, following a medium-term initiation/promotion study design [40].

Our striking finding of a biphasic effect of HCB on the phosphorylation of several proteins (increased effects at low doses and a decline at higher doses) was also reported for other biochemical parameters such as prostaglandin release by the chick embryo heart in response to polyhalogenated aromatic hydrocarbons [41] and stimulation of DNA synthesis in cultured rat hepatocytes upon exposure to TCDD [42].

In conclusion, in this study we have shown, for the first time, that (a) HCB can increase rat liver EGFR-kinase activity in a dose-dependent manner, and (b) the administration of low non-toxic doses of HCB for 30 days alters biochemical parameters of phosphotyrosine signal transduction pathways of membrane and cytosolic proteins. Although at this time we are not able to link any actual or potential toxicity with this treatment, our observations provide a clue to a potentially important mechanism by which HCB can exert pleiotropic effects through EGFR kinase, altering functions of many proteins through a cascade of protein phosphorylation. The level of sensitivity encountered in these changes is extraordinary, occurring at 1/10 to 1/1000 the doses of HCB known to cause other toxic lesions.

Acknowledgments

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