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Hexachlorobenzene-induced alterations on neutral and acidic sphingomyelinases and serine palmitoyltransferase activities. A time course study in two strains of rats

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Abstract

Hexachlorobenzene (HCB) induces porphyria both in humans and rodents, and hepatocarcinoma in rodents. In a previous work we observed that HCB produces a continuous decrease in hepatic sphingomyelin (SM) content in Wistar rats. A distinguishing characteristic of sphingolipids breakdown products is their participation in anti-proliferative and apoptotic processes and in the suppression of oncogenesis. As a first step to elucidate the role of SM decrease in the hepatotoxicity induced by HCB, the present study evaluates the metabolic causes of the continuous decrease in hepatic SM content observed in Wistar rats with HCB intoxication, and its relation with porphyria development. For this purpose, the time-course (3, 7, 15, 21 and 28 days) of the effects of HCB on hepatic SM levels and on some of the enzymes of SM synthesis (serine palmitoyltransferase, SPT) and catabolism (sphingomyelinases, SMases) was followed, using two strains of rats differing in their susceptibility to acquire porphyria: Chbb THOM (low) and Wistar (high). HCB (1 g kg^{-1} b.w. per day) was administered by gastric intubation as an aqueous suspension. After 5 days of HCB treatment, animals were allowed a 2-day recovery period without HCB administration. Two phases in the HCB-induced damages to sphingolipid metabolism were observed. The first stage (7 days of treatment), common to both strains of rats, was characterized by a decrease in hepatic SM levels (17–25%) and in SPT activity (50–43%), while strain differences were found for the later stage. In Chbb THOM rats, hepatic SM content was restored to normal values concomitantly with an increase in SPT activity (44%, at day 28), and without any increase in SM catabolism. In addition, the level of the other phospholipids was not altered. In Wistar rats, hepatic SM levels decreased continuously throughout the experiment, accompanied by increases in SPT, acidic

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sphingomyelinase (A-SMase) and neutral sphingomyelinase (N-SMase) activities (86, 28.5 and 78% increase, respectively). A role for glutathione (GSH) in the interstrain differences or a direct effect of HCB on SM metabolism was not found. The present study: (a) demonstrates that N-SMase, A-SMase, and SPT are some of the enzymes that play a role in the HCB-induced decrease of hepatic SM content; (b) finds that HCB-induced alterations of SM metabolism do not correlate with HCB-induced accumulation of hepatic porphyrins; and (c) proposes a link between HCB-induced alterations in phospholipid pattern and in SM metabolism. The increased SM hydrolysis produced as a consequence of SMases induction could be regarded as a cellular response to liver injury elicited by HCB, perhaps acting through the activation of SM signal transduction pathway delaying the proliferative processes observed after long-term treatment with HCB in some rodent species. However, such protective mechanism appears to be strain-dependent. © 2149 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Hexachlorobenzene; Sphingomyelin; Neutral-sphingomyelinase; Acidic-sphingomyelinase; Serine palmitoyltransferase; Porphyrins

1. Introduction

Hexachlorobenzene (HCB), a highly lipophylic, chlorinated hydrocarbon, is produced as a by-product or impurity in the manufacture of chlorinated compounds and of several registered pesticides (Courtney, 1979; ATSDR, 1994). It is among the most persistent environmental pollutants (Fisher, 1999), and bioaccumulates in the environment, animals and humans (Courtney, 1979; ATSDR, 1994).

During the period 1955–1959, ~4000 people in the southeast of Turkey developed porphyria due to the accidental ingestion of HCB. Some of the clinical symptoms reported were porphyrinuria, hyperpigmentation, hypertrichosis, enlarged thyroid, painless arthritis and enlarged liver. Many of the breast-fed children under the age of 2 whose mothers had ingested HCB-treated grain died, and the only cause reported was secondary pulmonary infections (Courtney, 1979; Gocmen et al., 1989; ATSDR, 1994). In a follow-up study carried out after 20–30 years of exposure, many patients still showed dermatological, neurological, and orthopedic symptoms and signs (Gocmen et al., 1989).

Several studies performed in animals have demonstrated the ability of HCB to induce a porphyria which resembles human porphyria cutanea tarda (Cam and Nigogosyan, 1963; San Martín de Viale et al., 1970). This disease is characterized by a remarkable hepatic accumulation and excretion of highly carboxylated porphyrins as a consequence of the decrease of uroporphyrinogen decarboxylase that induces the deregulation of the heme biosynthetic pathway (San Martín de Viale et al., 1977; Wainstok de Calmanovici et al., 1984). HCB-induced porphyria in rats has been observed to be strain-dependent, i.e. the Chbb THOM strain shows a much lower susceptibility to acquire HCB-induced porphyria than Wistar animals (Wainstok de Calmanovici et al., 1989). In addition, it has been shown that after a long-term exposure, HCB produces hepatomas, haemangio-hepatomas and hepatocellular carcinomas in several rodent species (Smith and Cabral, 1980; Ertürk et al., 1986; ATSDR, 1994).

Disrupted phospholipid metabolism has been reported in female rats following HCB administration (Cantoni et al., 1987; Billi de Catabbi et al., 1997; Cochón et al., 1999). It has been observed that HCB increased phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol hepatic levels during 1–4 weeks of HCB treatment, with a subsequent decrease after 8 weeks of intoxication (Billi de Catabbi et al., 1997). The early increase of these phospholipids was ascribed to the proliferation of microsomal membranes occurred as a consequence of the important and rapid induction of drug-metabolizing enzymes in response to the foreign drug. At a later stage, the oxidative environment elicited by HCB favors porphyrinogens oxidation and lipid peroxidation. The turning point between both phases in phospholipid metabolism alterations was the moment at which the level of hepatic porphyrins rose, thus empha-

sizing a possible relationship between the alterations induced by HCB in both metabolisms.

In contrast, the continuous decrease in hepatic sphingomyelin (SM) content observed in Wistar rats, following eight weeks of HCB treatment (Billi de Catabbi et al., 1997), is of particular interest. SM is usually a significant component of the phospholipid bilayer, and occurs in especially high concentrations in the plasma membrane. Sphingolipid breakdown products (Fig. 1) appear to be involved in signal transduction, cell regulation, and cell viability (Hannun, 1996). Thus, sphingolipids signaling pathway may play critical roles in monitoring the health of cells and tissues, and in preventing premature or undesirable growth of cells in response to internal or external injury by activating SM cycle (Hannun and Linardic, 1993). Although the precise mechanism by which sphingolipid alterations are involved in toxicological processes is not clear, it was postulated (Merrill et al., 1997) that carcinogens and toxins may be acting via initiation, or inhibition of sphingolipids signaling pathway. In addition, disruption of sphingolipid metabolism by food and feed contaminants has been implicated in several animal diseases and possible human cancer, as well as dietary sphingolipids may play a protective role against diseases (Merrill et al., 1997).

The first step of de novo SM biosynthesis is the condensation of L-serine and palmitoyl-CoA by serine palmitoyltransferase (SPT) (3-ketosphinganine synthase; palmitoyl-CoA:L-serine C-palmitoyltransferase (decarboxylating); EC 2.3.1.50) in

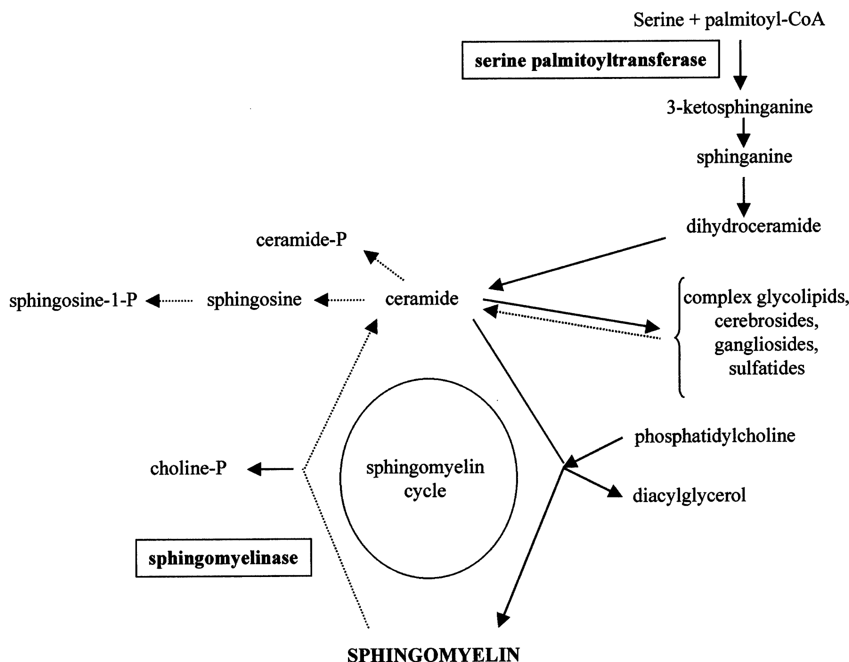

Fig. 1. Sphingolipids metabolic pathway. De novo SM biosynthesis begins in the endoplasmic reticulum with the condensation of serine and palmitoyl-CoA by SPT. 3-Ketosphinganine is reduced to sphinganine, which is acylated to dihydroceramides by ceramide synthase. The dihydroceramide is desaturated to ceramide and incorporated into SM (by SM synthase), and into other complex sphingolipids. Sphingolipids are assembled within the endoplasmic reticulum and Golgi apparatus prior to delivery to the cell surface, and so they are also found intracellularly. Turnover occurs in the plasma membrane, lysosomes, endosomes, cytosol and extracellular media to liberate ceramide, sphingosine and sphingosine-1-P. Only the enzymes analyzed in the present study are shown. Solid arrows: sphingolipid biosynthesis. Dashed arrows: sphingolipid catabolism.

the endoplasmic reticulum (Fig. 1). It has been reported that its activity correlates with the relative level of sphingolipid synthesis by tissues (Merrill and Wang, 1992).

The enzyme sphingomyelinase (SMase) (sphingomyelin phosphodiesterase; sphingomyelin choline phosphohydrolase; EC 3.1.4.12), catalyzes the hydrolysis of SM to ceramide and choline phosphate (Fig. 1). To date, at least five types of sphingomyelinases have been identified, including an acidic SMase (A-SMase) associated with lysosomes, that has also been detected in soluble form in cytosol and extracellular media, and a neutral pH-optimum, magnesium-dependent SMase (N-SMase), associated to the plasma membrane (Liu and Hannun, 1997).

The present study evaluates the metabolic causes of the continuous decrease in hepatic SM content observed in Wistar rats with HCB intoxication, and its relation with porphyria development. For this purpose, the time-course of the effects of HCB on hepatic SM levels and on some of the enzymes of SM synthesis (SPT) and catabolism (sphingomyelinases, SMases) was followed, using two strains of rats differing in their susceptibility to acquire porphyria: Chbb THOM (low) and Wistar (high).

2. Materials and methods

2.1. Chemicals

HCB (commercial grade) was generously provided by Compañía Química (Argentina). Serine L-[^{3}H(G)] 27.5 Ci mmol^{-1}, was purchased from Du Pont NEN. Palmitoyl-CoA, HEPES, 2-(*N*-hexadecanoyl-amino)-4-nitrophenyl phosphocholine (HDA-PC), 2-(*N*-hexadecanoyl-amino)-4-nitrophenyl (HDA), serine, dithiotreitol, reduced glutathione (GSH), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), were purchased from Sigma Chemical Co., St. Louis, MO. Precoated thin-layer chromatography (TLC) plates (particle size 2–25 μm, layer thickness 200 μm, on an aluminum support) were supplied by Aldrich. All other chemicals were of the highest available grade.

2.2. Animals and treatment

Female Wistar and Chbb THOM rats of the same age, weighing 160–180 g at the start of the experiment, were used. Procedures involving animals (care and use) were conducted in conformity with international guidelines (Guide for the Care and Use of Laboratory Animals, National Research Council, USA, 1996, and the Council of the European Comunities Directive, 86/609/ECC).

Rats were maintained on a 12 h-light/dark cycle, and fed Purina 3 diet and water ad libitum. Animals of each strain were randomly assigned to two groups: (1) controls; and (2) HCB-treated. HCB was administered 5 days/week by stomach tube. Each day the animals received a single dose of HCB (1 g kg^{-1} body weight per day) suspended in water (40 mg ml^{-1}) containing Tween 20 (0.5%). Animals from the control group received the vehicle alone.

Evidences demonstrate that HCB administration in oil shows a high absorption (80%) (Koss and Koransky, 1975). However, because this was a study on HCB effect on lipid metabolism, we chose to administer HCB as an aqueous suspension in order to avoid the addition of extra dietary lipids. The HCB dose used was chosen based on previous studies demonstrating that it elicits clear manifestations of hepatic porphyria in Wistar rats, with no deaths or external alterations (San Martín de Viale et al., 1970, 1975, 1977; Wainstok de Calmanovici et al., 1989; Billi de Catabbi et al., 1997; Randi et al., 1998; Cochón et al., 1999). Taking into account that when HCB is administered in water it is very poorly absorbed ($\sim$5% of the dose is absorbed) (Courtney, 1979; Koss and Koransky, 1975), the calculated dose used in the present work would be 50 mg kg^{-1} b.w. per day.

Rats were sacrificed by decapitation between 08:00 and 09:00 h, following 3, 7, 14, 21 and 28 days of treatment.

2.3. Porphyrin content

Porphyrin content was determined in 5% (w/v) HCl, as described by San Martín de Viale et al. (1977).

2.4. Analysis of phospholipid levels

Twenty percent liver homogenates in 0.154 M KCl were prepared, and total lipids were extracted according to Bligh and Dyer (1959). Phospholipid levels were measured as described previously (Billi de Catabbi et al., 1997). Briefly, the lipids were separated by one-dimensional, two-solvent, TLC, using chloroform–methanol–acetic acid–water (40:10:10:1, v/v) followed by a second step with chloroform–methanol–acetic acid–water (120:46:19:3, v/v), as developing solvent systems. Lipid fractions were detected with I_2 vapor, and those spots migrating with phospholipid standards were scraped off and quantified by a phosphate assay.

The in vitro effect of HCB on SM content was determined in hepatic slices from control animals. Tissue slices (0.5 mm thick) were obtained with a Stadie–Riggs microtome, and maintained in a solution of 10 mM Tris pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$ (Tris electrolyte buffer), and 5 mM glucose. The incubation mixture contained 10^{-4} M of HCB in dimethylsulfoxide, tissue slices (20 mg), and 0.2 ml of Tris electrolyte buffer containing 5 mM glucose. Incubations were carried out at 37°C for 2, 4 and 6 h. Controls were performed with dimethylsulfoxide. Total lipids were extracted and treated as described above.

2.5. SPT Activity assay

To determine SPT activity, microsomes were prepared as described by Williams et al. (1984), and were assayed within two days of freezing at −18°C.

Enzyme activity was assayed by following the incorporation of radiolabel from [^{3}H] serine into chloroform-soluble products, as described by Merrill and Wang (1992). Briefly, each 100 µl assay contained 0.1 M HEPES (pH: 8.3 at 25°C), 5 mM DTT, 5 mM EDTA, 1 mM [^{3}H] serine 60 mCi $mmol^{-1}$, 50 µM pyridoxal 5′-phosphate, enzyme (150 µg of microsomal protein), and 0.2 mM palmitoyl-CoA. The reaction was initiated by the addition of palmitoyl-CoA. After 10 min at 37°C, the reaction was terminated by adding 0.2 ml of 0.5 N NH_4OH. The labeled lipid product was extracted, and the organic phase radioactivity was measured by scintillation counting using Econofluor. Control tubes contained all of the added components except palmitoyl-CoA.

Enzymatic activity was expressed as pmol of 3-ketosphinganine formed per min. per mg protein after subtracting the background radioactivity. Assays were conducted in duplicate and the data expressed as mean ± S.E.M.

2.6. Neutral and acidic SMase activity assay

A-SMase activity was assayed with minor modifications as described by Levade et al. (1983). Liver homogenate (1:6 w/v) was prepared in 0.1% Triton-X 100. Each reaction mixture contained 14 mM HDA-PC, 0.1 M sodium acetate buffer (pH: 5.5 at 25°C), and enzyme (1.5–2 mg of protein). Endogenous SM represented 0.6% of the added analog substrate. After incubation at 37°C for 60 min, the reaction was stopped by addition of 10 N NaOH (0.6 ml) and 2.5 ml of ethyl acetate: 2-propanol (5:1 v/v). The upper phase was then extracted and measured at 417 nm. Control assays were performed without incubation.

The same procedure was adjusted to measure N-SMase activity. The incubation mixture contained 14 mM HDA-PC, 0.25 M Tris HCl buffer (pH:7.4 at 25°C) and 2.5 mM $MgCl_2$, and enzyme (1.5–2 mg of protein). Under these conditions, N-SMase activity was found to be magnesium-dependent, and, EDTA- and GSH-inhibited.

A molar absorption coefficient ε of 27×10^{-9} M^{-1} cm^{-1} was obtained from a calibration curve performed with HDA, submitted to the same extraction procedure as the incubation products.

To assay the in vitro effects of HCB on the enzyme activity, HCB dissolved in DMSO was preincubated with the enzyme for 15 min at 37°C, before substrate addition. Control assays contained DMSO.

Enzymatic activity was expressed as nmol of HDA formed in 1 h per mg of protein. The assays were conducted in duplicate and the data expressed as mean ± S.E.M.

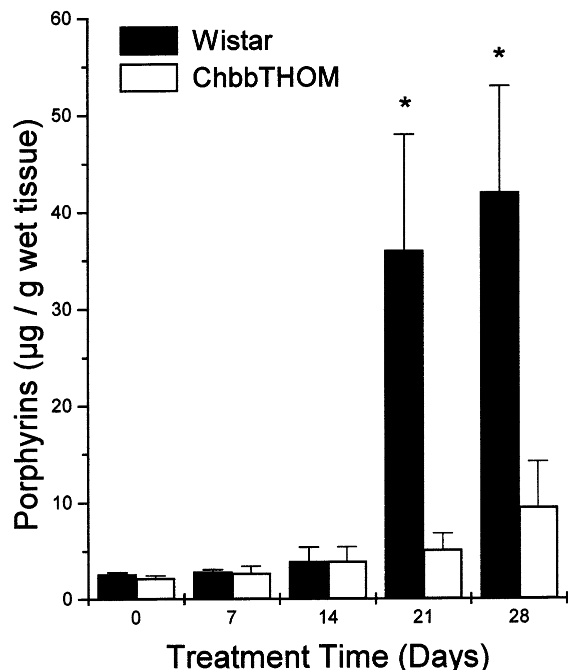

Fig. 2. Effects of HCB on hepatic porphyrin accumulation in Wistar and Chbb THOM rats. Animals were treated with HCB for 7, 14, 21 and 28 days. Controls received vehicle alone. Porphyrins were spectrophotometrically quantified. Each value represents the mean ± S.E.M. of five animals. (*) Significantly different from control rats ($P < 0.05$). Control values were not altered by vehicle administration up to 28 days.

2.7. Determination of hepatic GSH concentration

Twenty percent liver homogenates in 5% trichloroacetic acid in 1 mM NaEDTA, were prepared and centrifuged 5 min at $2000 \times g$. GSH concentration was measured in the supernatant fraction by the method of Benke et al. (1974), using DTNB to measure total non protein thiols.

2.8. Proteins

Proteins were determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.9. Statistical analysis

Results were expressed as means ± S.E.M., and were submitted to a one-way ANOVA. For each analysis, the F test was used to determine statistical significance. In all cases, 0.05 was used as the level of significance.

3. Results

3.1. Hepatic porphyrin content

Taking into account that hepatic porphyrin content is usually considered a biomarker of HCB exposure, the time course accumulation of liver porphyrins following 7, 14, 21 and 28 days of HCB treatment was studied in both Wistar and Chbb THOM rats (Fig. 2).

As was expected, interstrain differences in the susceptibility to acquire HCB-induced porphyria were observed. While Wistar rats showed a significant increase (~ 13-fold) by the 21st day of treatment, Chbb THOM animals showed no significant increases in hepatic porphyrin content after 28 days of treatment. It is interesting to point out that, following 28 days of HCB treatment, all the animals of the Wistar strain exhibited clear signs of porphyria.

3.2. Changes in hepatic phospholipids content

HCB treatment gradually decreased SM levels in Wistar rats, from day 3 of treatment. The greatest change was observed on day 28, when the content decrease reached 49%. In contrast, SM levels in Chbb THOM rats reached a minimum by day 7 (25% of decrease), and returned to normal values by the end of the experiment (Fig. 3).

With respect to the other phospholipids, in Chbb THOM rats, only phosphatidylcholine levels showed a significant increase following 15 days of HCB treatment, then returning to control values (results not shown). This fact is in contrast to the previously observed altered pattern of hepatic phospholipids elicited by HCB in Wistar rats (Billi de Catabbi et al., 1997).

Incubation of liver slices from normal, untreated rats with HCB (10^{-4} M) up to 6 h, showed no changes in SM content (results not shown).

3.3. Enzyme activities

In order to investigate whether the drop in SM levels observed following HCB treatment was due to a decreased biosynthesis and/or increased catabolism of this phospholipid; SPT, A-SMase, and N-SMase activities were measured as a function of HCB-treatment time in both strains.

At the beginning of the treatment, SPT activity was found to be decreased to the same degree in both strains of rats (50 and 43% decrease for Wistar and Chbb THOM rats, respectively) (Fig. 4). In Chbb THOM rats, the activity of this enzyme was restored to normal values by the 14th day, while in Wistar rats it was not until the 21st day that this activity was within normal values. By day 28, SPT activity was significantly increased in both strains, though the effect was more pronounced in Wistar (86 and 44% increase for Wistar and Chbb THOM, respectively).

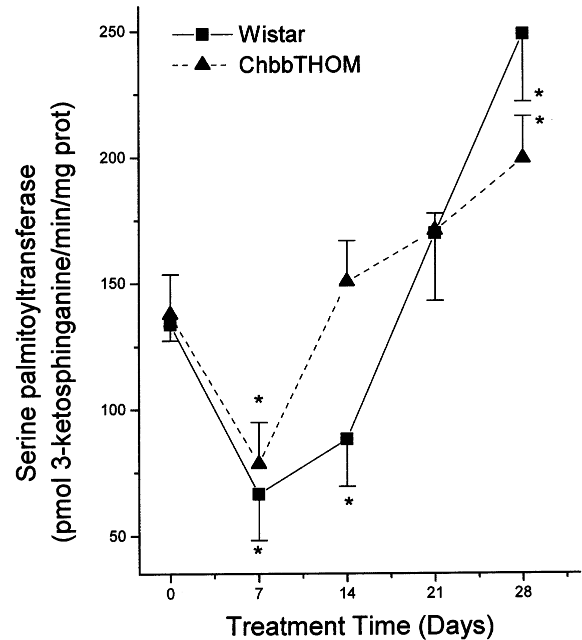

Fig. 4. Time-course of the effects of HCB on SPT activity in Wistar and Chbb THOM rats. Enzyme activity was evaluated through the incorporation of [^{3}H] serine into 3-ketosphinganine in the livers of rats treated with HCB or vehicle for 3, 7, 14, 21 and 28 days. Results are the means ± S.E.M. of five rats. (*) Significantly different from control rats ($P < 0.05$). Control values were not altered by vehicle administration up to 28 days.

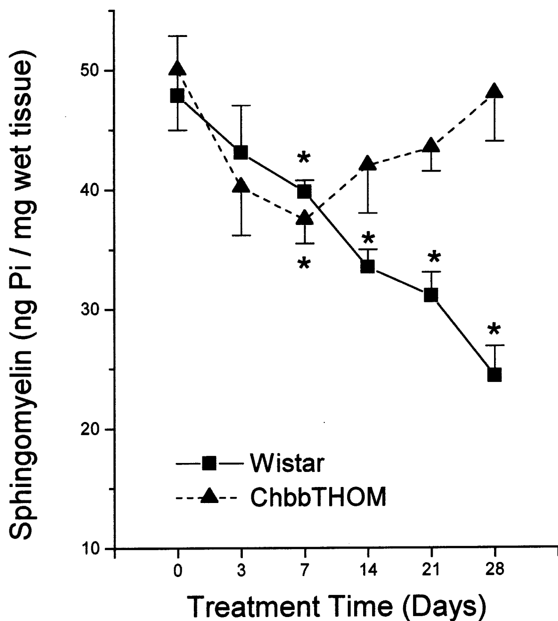

Fig. 3. Time-course of the effects of HCB on SM content in Wistar and Chbb THOM rats. SM was separated by TLC from the livers of rats treated with HCB for 3, 7, 14, 21 and 28 days. Following mineralization, the inorganic phosphate was quantified. Control, age-matched rats received vehicle alone. Results are the means ± S.E.M. of five animals. (*) Significantly different from control rats ($P < 0.05$). Control values were not altered by vehicle administration up to 28 days.

With regard to SM catabolism, by day 3 of HCB treatment A-SMase activity was significantly diminished in rats of both strains (Fig. 5). In Chbb THOM animals, this activity remained below normal levels throughout the experiment. In contrast, Wistar rats showed a significant increase in A-SMase activity from day 21 (28.5 and 18.7% of increase for the 21st and 28th day, respectively).

HCB treatment did not modify N-SMase activity in Chbb THOM rats, while it significantly increased the activity of this enzyme in Wistar animals (Fig. 6). The increase could be noted from day 14 of treatment and reached a maximum by day 21 (78% increase).

Taking into account that the peak increase of SMase activities in Wistar rats coincided with the onset of a marked accumulation of hepatic porphyrins (Figs. 2, 5 and 6), the possibility arises that SMase induction occurs only when hepatic porphyrin accumulation becomes manifest. To test this hypothesis, animals were treated with HCB for up to 56 days (Table 1). In Chbb THOM rats, though the hepatic porphyrin content was significantly increased, SMase activities were decreased. In Wistar rats, on the other hand, these enzyme activities remained elevated.

To determine whether there were any direct effects of HCB on the enzymes of SM catabolism, N-SMase and A-SMase activities were measured after in vitro preincubation with 10^{-6}–10^{-4} M HCB. In neither case could any direct effect of HCB on the enzyme activity be observed (results not shown).

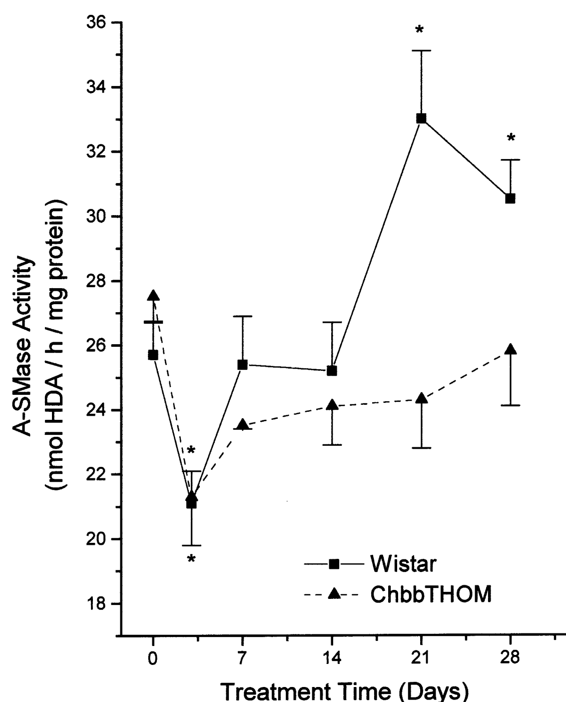

Fig. 5. Time-course of the effects of HCB on A-SMase activity in Wistar and Chbb THOM rats. Enzyme activity was evaluated using a synthetic substrate (2-(*N*-hexadecanoylamino)-4-nitrophenyl) phosphocholine (HDA-PC), at pH 5.5, in the livers of rats treated with HCB or vehicle for 3, 7, 14, 21 and 28 days. The HDA hydrolyzed was spectrophotometrically quantified. Results are the means ± S.E.M. of five rats. (*) Significantly different from control rats ($P < 0.05$). Control values were not altered by vehicle administration up to 28 days.

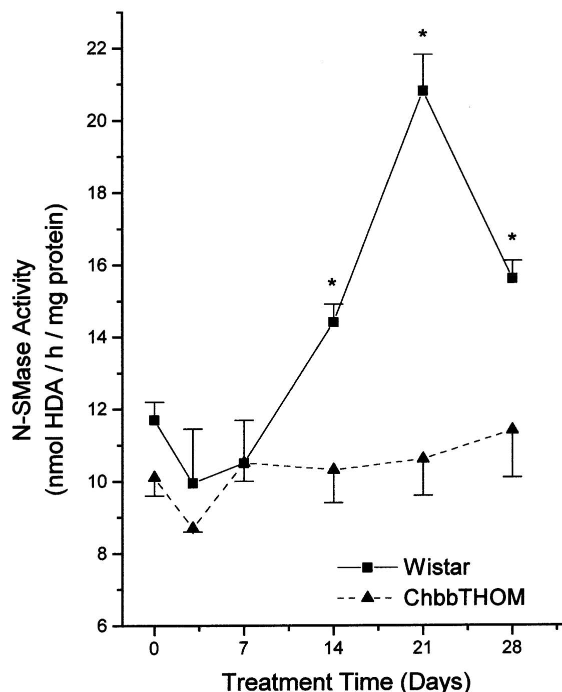

Fig. 6. Time-course of the effects of HCB on N-SMase activity in Wistar and Chbb THOM rats. Enzyme activity was evaluated using a synthetic substrate (2-(*N*-hexadecanoylamino)-4-nitrophenyl) phosphocholine (HDA-PC), at pH 7.4, in the presence of $MgCl_2$ 2.5 mM, in the livers of rats treated with HCB or vehicle for 3, 7, 14, 21 and 28 days. Hydrolyzed HDA was spectrophotometrically quantified. Results are the means ± S.E.M. of five rats. (*) Significantly different from controls ($P < 0.05$). Control values were not altered by vehicle administration up to 28 days.

3.4. Hepatic GSH content

It has been reported that at physiologically relevant concentrations GSH inhibits N-SMase but not A-SMase, and that depletion of cellular GSH results in the hydrolysis of SM (Liu and Hannun, 1997; Liu et al., 1998a).

To investigate whether the effects of HCB treatment on SM content and on N-SMase activity were due to GSH depletion, Wistar and Chbb THOM rats were treated with HCB for 7, 14, 21

and 28 days, and hepatic GSH levels measured. Both strains showed similar basal GSH levels (6.64 ± 0.42 and 6.27 ± 0.35 μmol GSH g^{-1} tissue for Wistar and Chbb THOM rats, respectively). HCB treatment did not modify hepatic GSH levels in either group (results not shown).

4. Discussion

Though previous studies with Wistar and Fisher rats had shown HCB-induced decreases in hepatic SM levels (Cantoni et al., 1987; Billi de Catabbi et al., 1997), so far no attempt had been made to study the reasons for this decrease. In this study, the time-course of the in vivo effects of HCB on enzymes involved in SM metabolism and their relation to the development of porphyria, were studied.

Our studies showed that hepatic SM content in HCB-treated Chbb THOM rats transiently decreased and then returned to normal values, which somewhat paralleled the change in SPT activity. The SMase activities did not seem to be affected. The dynamic equilibrium between rate of synthesis and rate of degradation appears to provide an explanation for SM content in this rat strain.

In Wistar rats SM content declined continuously with time. SPT first declined in parallel, but then increased by about threefold from day 7 to 28. These time-sequential changes, in this enzyme activity, resemble what was observed in Chbb THOM rats. However, an increase in the SMase catabolic enzymes, A-SMase and N-SMase activities was observed, thus contributing to the further decline in SM levels. The fact that SM content remained diminishing without any further increase in SMase activity by day 56 of treatment provides evidence that other steps in sphingolipid metabolism should be affected in Wistar rats. It could be speculated that as natural endogenous substrate (SM) decreases, SMase activities towards the analog HDA-PC would increase. However, increases in SMase activities towards the analog substrate did not parallel the continuous decrease in tissue SM content. Therefore, we discard the hypothesis that the effects found in this work could be ascribed to endogenous substrate depletion favoring analog substrate utilization by the enzymes.

In view of our results, there seem to be at least two phases in the mechanism of HCB-elicited damage of sphingolipids biosynthesis. The first stage is characterized by a decrease in hepatic SM levels in parallel with SPT activity. This stage seems to be common to both strains of rats. At a later stage, other factor(s) appear which determine that, in spite of the low SM levels, SMase activities become induced in Wistar animals. One of these factors could be the disrupted phospholipid metabolism elicited by HCB in Wistar rats, which

Table 1
Effects of long-term HCB intoxication on N-SMase and A-SMase activities and on porphyrin content in Chbb THOM and Wistar rats[a]

Strain	Treatment	Duration of treatment (days)	N-SMase activity (nmol HAD h^{-1} per mg protein)	A-SMase activity (nmol HDA h^{-1} per mg protein.)	Porphyrin content (μg porphyrin g^{-1} liver)
Chbb THOM	Vehicle	42	10.1 ± 2.0	27.5 ± 2.0	2.9 ± 0.6
	HCB	42	7.3 ± 1.0 (72)[b]	22.5 ± 1.6 (82)	21 ± 11*
	Vehicle	56	9.9 ± 2.1	27.0 ± 1.9	2.5 ± 0.8
	HCB	56	8.5 ± 1.1 (86)	26.2 ± 2.7 (97)	95 ± 20*
Wistar	Vehicle	56	11.0 ± 1.8	25.7 ± 1.4	2.7 ± 0.6
	HCB	56	13.2 ± 0.8* (120)	34.2 ± 1.3* (133)	142 ± 46*

[a] HCB was administered as described in Section 2. Results are expressed as the mean ± S.E.M. ($n = 4$).
[b] In parenthesis, percentage of control values.
* Significantly different from control, $P < 0.05$.

in turn could trigger major alterations on SM metabolism in the second stage. At this regard, it is generally accepted that the activity of some membrane-bound enzymes depends on their lipid environment (Sanderman, 1979). More over, phosphatidylserine is a regulator of SM levels in membranes by its inhibitory effect on phosphatidylcholine:ceramide–phosphocholine and phosphatidylethanolamine:ceramide–phosphoethanolamine transferase (Petkova et al., 1991; Nikolova et al., 1992) and its activating effect on N-SMase (Liu et al., 1998b). In addition, it has been reported that in Wistar rats, HCB produces alterations in the activity of two plasma membrane bound enzymes: 5′-nucleotidase and Na^{+}/K^{+} ATPase, as well as changes in membrane protein phosphorylation, (Randi et al., 1998).

GSH, at physiologically relevant concentrations, has been reported to inhibit N-SMase, while depletion of cellular GSH results in the hydrolysis of SM (Liu and Hannun, 1997; Liu et al., 1998a). In addition, differences in hepatic GSH levels have been postulated as a factor that might contribute to interstrain differences in susceptibility to the porphyrinogenic effects of HCB (Debets et al., 1981). Nevertheless, our results exclude a role for GSH in the interstrain differences observed in both porphyrin and SM metabolisms. Direct HCB effect on SM content and on acidic or neutral SMases as judged from the in vitro assays should also be discarded, as well as the fact that the increase in endogenous porphyrin content could be related to the second phase of SM metabolism disruption.

In summary, the present study: (1) identifies for the first time neutral and acidic SMases and SPT as some of the enzymes involved in the HCB-induced decrease in hepatic SM content; (2) proposes a link between HCB-induced alterations in phospholipid pattern and in SM metabolism; (3) finds that the HCB-induced alterations of SM metabolism do not correlate with HCB-induced accumulation of hepatic porphyrins, nor with endogenous GSH levels; and (4) also demonstrates interstrain differences in HCB-induced alterations of phospholipid metabolism. At this respect, the Wistar strain is more susceptible to the effects of HCB on both porphyrin and SM metabolisms than Chbb THOM. Further investigation is required to understand the relationship between the biochemical events that lead to strain-dependent susceptibility to both alterations. It also remains to be elucidated whether other steps of sphingolipids metabolism contribute to the decrease in hepatic SM levels elicited by HCB in Wistar rats.

The relevance of the present findings on the oncogenicity of HCB remains to be determined. Nevertheless, taking into account that SM breakdown products participate in antiproliferative and apoptotic processes and in the suppression of oncogenesis, it could be speculated that the increased SM hydrolysis observed in the present study would be a protective mechanism acting as an early cellular response to the liver injury elicited by HCB. In addition, if the SM cycle do play a role in protecting the hepatocytes from HCB oncogenicity, Chbb THOM strain should be more sensitive to HCB carcinogenic effects.

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