In Vivo Production of Different Chloroform Metabolites: Effect of Phenobarbital and Buthionine Sulfoximine Pretreatment

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The regioselective attack on microsomal phospholipid (PL) polar heads (PH) and fatty acyl chains (FC) demonstrated in vitro has been exploited for the selective quantitation in vivo of the biochemical damages produced by the oxidation and reduction products of CHCl₃ metabolism. Five hours after CHCl₃ injection (60 mg/kg body weight, ip) to control Sprague-Dawley rats, most of the label covalently bound in the liver was associated to PH, indicating a predominant production of COCl₂. The levels of radioactivity bound to both PL moieties increased proportionally when 180 mg/kg body weight ¹⁴CHCl₃ was administered. Buthionine sulfoximine (BSO) pretreatment resulted in a further increase of binding either to PH or FC. The pretreatment of rats with phenobarbital (PB) reduced the PH/FC binding ratio to 3.4, still indicating the predominance of the oxidative metabolism, but giving some indication of the simultaneous presence of CHCl₃ reduction. When reduced glutathione (GSH) was depleted by BSO in PB-induced animals prior to ¹⁴CHCl₃ administration, only the level of radioactivity associated with oxidative intermediates was increased six times. The present results confirmed that GSH is able to exert an efficient protection mainly toward ¹⁴CHCl₃ oxidation intermediates. Furthermore, they indicate that in the liver of the Sprague-Dawley rat the major pathway of CHCl₃ biotransformation is its oxidation and that pretreatment of rats with a GSH-depleting agent (such as BSO) is more relevant than PB induction in enhancing the biochemical damages produced by CHCl₃.

Key words: in vivo metabolism, chloroform, PB induction, BSO pretreatment, reactive intermediates, covalent binding. Sprague-Dawley rat

Introduction

It is now widely accepted that chloroform can be both oxidized to phenol (1,2) and reductively biotransformed in vitro and in vivo to dichloromethyl radical (3,4). Either type of reactive intermediates can react in vitro with cellular structures to form covalent adducts (5–7). Although the production of COCl₂ has been associated with chloroform-induced acute toxicity (8–10), also the reductively produced metabolites can be involved in CHCl₃ toxicity. In fact, CHCl₃ can stimulate lipid peroxidation in PB-pretreated Sprague-Dawley microsomal phospholipids rats (11–13), which are more susceptible to chloroform-induced hepatotoxicity than control rats (14).

It has been shown in vitro that PL are the major site of CHCl₃-induced damage, in experimental conditions resembling the physiological status of the liver (6,7). Moreover, our recent results, obtained in vitro by means of chemical transmethylation or of enzymatic hydrolysis with phospholipase C (15), indicated that the different reactive intermediates (namely, COCl₂ and -CHCl₂) show a typical regioselectivity in the attack to PL. Indeed, while COCl₂ binds preferentially to PL polar heads, PL fatty acyl chains are the main target for dichloromethyl radicals.

This feature has been exploited in this preliminary study for the selective in vivo quantitation of the binding due to each type of intermediates to assess if the two metabolic pathways are simultaneously active in the liver of Sprague-Dawley rats.

Furthermore, we investigated different effects of PB, a well-known enhancer of CHCl₃ toxicity (16,17), and of BSO, which is able to deplete GSH in vivo (18), on the two metabolic pathways of CHCl₃.

Materials and Methods

Chemicals

(¹⁴C)-Chloroform (=3.0 mCi/mnmole, radiochemical purity 99%) was obtained from New England Nuclear (Boston, MA). Unlabeled chloroform (IR purity) was from Merck (Darmstadt, Germany).

BSO was supplied by Sigma (St. Louis, MO), sodium-PB by Fluka (Buchs, Switzerland) and Tanax by Hoechst A.G. (Frankfurt am Main, Germany). Liquid scintillation cocktails Aqualuma and Lipoluma were purchased from Lumac System A.G. (Basel, Switzerland). All other reagents were of the highest purity commercially available.

Animals

Male Sprague-Dawley rats (180–200 g) were from Nossan (Italy). They were maintained on a 12-hr light cycle and provided food and water ad libitum for 1 week before the start of the experiments.

When required, animals were pretreated with 0.1% (w/v) PB sodium salt in drinking water for 1 week; BSO, dissolved in distilled H₂O was injected (2 nmole/kg body weight, ip) 2 hr before ¹⁴CHCl₃ treatment.

Animals were treated with ¹⁴CHCl₃ in corn oil (60–180 mg/kg body weight, ip) 5 hr before sacrifice, obtained by injecting (ip) a lethal dose of Tanax. ¹⁴CHCl₃-specific radioactivity (0.15–0.49 mCi/mnmole) was adjusted in order to inject each animal with the same amount of radioactivity per kg body weight.

During treatment, rats were maintained in all glass metabolism cages (Metabowls...
MK III, Jencons Scientific Ltd.), provided with water and with an air flow of 500 ml/min.

Covalent Binding of 14C-Chloroform Metabolites

Microsomal preparations from rat livers were obtained as previously described (5). Microsomal lipid extracts were obtained from an aliquot of microsomal suspension (250 μl) corresponding to about 0.5 g of wet liver tissue, according to the method of Folch (19), and repeatedly dried to eliminate the residues of free substrate. Then covalent binding of 14CHCl3 metabolites to PL polar heads (PH) and fatty acyl chains (FC) was determined after the acid-catalyzed transmethylation of liver microsomal PL, as described in detail by De Biasi et al. (15). Briefly, the lipid extract was dissolved in 1 ml of anhydrous methanol:benzene:H2SO4 (75:25:1) and vigorously shaken for 1 hr at 70°C. The reaction mixture was cooled in an ice bath, then 2 ml petroleum ether (BP 40°-70°) and 1 ml 0.26 M K2HPO4 were added. After 10 min vigorous shaking, the mixture was centrifuged (3,000 rpm, 10 min). The lower aqueous layer, containing the free hydrophobic PH, was transferred into a plastic vial containing 17 ml Aqualuma. The upper organic phase, containing the PL fatty acid methyl esters, was washed twice and then a 2-ml aliquot was transferred into a plastic vial containing 10 ml Lipoluma. Data were compared by means of the Student’s t-test.

Results

After the ip administration of 60 mg/kg body weight 14CHCl3 to control male Sprague-Dawley rats, most of the label covalently bound in the liver was associated to PL hydrophilic PH (Figure 1). Indeed the binding was about 6-fold higher than the label bound to FC.

This amount of radioactivity bound either to PH or FC increased proportionally when a 3-fold higher dose (180 mg/kg body weight) was administered (Figure 1).

When control rats were pretreated with BSO prior to 14CHCl3 administration, the effect of GSH depletion on covalent binding could be observed. The total amount of radioactivity was 7-fold higher (Figure 2). The binding associated to both microsomal PL moieties resulted similarly affected (no significant changes in the ratio PH/FC-associated label).

The pretreatment of rats with PB nearly doubled the total PL covalent binding due to 14CHCl3 metabolites (Figure 3). The radioactivity bound to FC increased slightly but significantly more than that one associated to PH.

When BSO was administered to PB-induced animals prior to 14CHCl3 injection, the level of total radioactivity bound to PL was 6.5 fold increased with respect to that measured in PB-induced rat liver (Figure 3). The variation was due only to the increase of radioactivity associated to PH, since the level of covalent binding to FC was not significantly changed.

Discussion

Injection of a single subtoxic dose of 14CHCl3 to variously pretreated Sprague-Dawley rats resulted in a predominant binding of the 14C label to the PH of hepatic microsomal PL, indicating that COCl3 is the major product of CHCl3 bioactivation. Indeed, the ratio of radioactivity associated with PH with respect to that of FC (PH/FC) was about six and remained almost constant at the two dosage levels tested of 14CHCl3. The observed binding to FC may not be attributed with certainty to the radicals derived from CHCl3 reduction, since the regioselectivity of binding to PL shown by the two CHCl3 metabolites is not absolute (15).

This finding is in line with previous in vitro results (7,15), suggesting that in hepatic microsomes from control Sprague-Dawley rats in experimental conditions mimicking the physiologic oxygenation of the liver, reductive metabolism is not significantly expressed or requires very low PO2 values. Pretreatment of Sprague-Dawley rats with PB prior to 14CHCl3 caused the binding to FC to increase significantly more than the binding to PH. The PH/FC binding ratio decreased to 3.4.
These data indicate the predominance of CHCl₃ oxidative metabolism, and suggest that some contribution by the reductive pathway is also present.

The marked increase of total PL binding (together with the increase of PH/PC binding ratio up to 21.8) in liver microsomes of rats pretreated with BSO indicated that GSH exerts in vivo a highly efficient scavenging action toward the CHCl₃ intermediate produced through the oxidative pathway. The greater susceptibility to CHCl₃-induced hepatotoxicity observed in GSH-depleted rats (16) may be associated mainly to a decreased protection against COCl₂, the major product of CHCl₃ oxidation (1,2,8).

These preliminary results suggest that in the liver of either control or PB-induced Sprague-Dawley rats the predominant bio-transformation pathway of CHCl₃ is its oxidation and that the damages exerted by CHCl₃ on the microsomal PL are potentiated by GSH-depleting agents such as BSO, more than PB-like inducers of CHCl₃ metabolism.

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