METABOLISM OF CHLOROFORM IN THE HUMAN LIVER AND IDENTIFICATION OF THE COMPETENT P450s

SIMONETTA GEMMA, LUCIANO VITTOZZI, AND EMANUELA TESTAI

Biochemical Toxicology Unit, Comparative Toxicology and Ecotoxicology Laboratory, Istituto Superiore di Sanità, Rome, Italy

(Received July 23, 2002; accepted November 15, 2002)

ABSTRACT:

The oxidative and reductive cytochrome P450 (P450)-mediated chloroform bioactivation has been investigated in human liver microsomes (HLM), and the role of human P450s have been defined by integrating results from several experimental approaches: cDNA-expressed P450s, selective chemical inhibitors and specific antibodies, correlation studies in a panel of phenotyped HLM. HLM bioactivated CHCl₃ both oxidatively and reductively. Oxidative reaction was characterized by two components, suggesting multiple P450 involvement. The high affinity process was catalyzed by CYP2E1, as clearly indicated by kinetic studies, correlation with chlorozoxazone 6-hydroxylation \( r = 0.837; p < 0.001 \), and inhibition by monoclonal antihuman CYP2E1 and 4-methylpyrazole. The low affinity phase of oxidative metabolism was essentially catalyzed by CYP2A6. This conclusion was supported by the correlation with coumarin 7-hydroxylase \( r = 0.777; p < 0.01 \), inhibition by coumarin and by the specific antibody, in addition to results with heterologously expressed P450s. Chloroform oxidation was poorly dependent on \( pO_2 \), whereas the reductive metabolism was highly inhibited by \( O_2 \). The production of dichloromethyl radical was significant only at CHCl₃ concentration \( \geq 1 \) mM, increasing linearly with substrate concentration. CYP2E1 was the primary enzyme involved in the reductive reaction, as univocally indicated by all the different approaches. The reductive pathway seems to be scarcely relevant in the human liver, since it is active only at high substrate concentrations, and in strictly anaerobic conditions. The role of human CYP2E1 in CHCl₃ metabolism at low levels, typical of actual human exposure, provides insight into the molecular basis for eventual difference in susceptibility to chloroform-induced effects due to either genetic, pathophysiological, or environmental factors.

Chloroform is a ubiquitous atmospheric and water contaminant. Beside its extensive use as a solvent in industrial processes, it is formed as a by-product during the chlorination of water intended for human use and paper bleaching. Due to its volatility, chloroform can be easily released from waste or chlorinated waters into the atmosphere or in the ambient air. Therefore, a large part of the human population may be chronically exposed to chloroform from different sources, although drinking water has been considered the main one. Recently, routes of exposure other than oral consumption of chlorinated drinking water (Gallagher et al., 1998; Waller et al., 1998). Moreover, some adverse reproductive outcomes have been recently reported in humans exposed to chloroform via drinking water (Gallagher et al., 1998; Waller et al., 1998). However, the poor assessment of exposure and the concomitant presence of many water contaminants, including other trihalomethanes and disinfection by-products, makes it difficult to establish a causal link between chloroform itself and adverse effects in humans.

The required step for CHCl₃-induced toxicity is the cytochrome P450 (P450\textsuperscript{1})-mediated bioactivation to reactive metabolites (ICPS, 1994; Constan et al., 1999) (Fig. 1). Extensive in vitro and in vivo studies on rodents have demonstrated that chloroform may be metabolized oxidatively to trichloromethanol, which spontaneously decomposes to the electrophilic phosgene (Mansuy et al., 1977; Pohl et al., 1977). COCl₂ is highly reactive and binds covalently to cell components containing nucleophilic groups, including proteins, phospholipid (PL) polar heads, and reduced glutathione (Pohl et al., 1981; 1)

\textsuperscript{1} Abbreviations used are: P450, cytochrome P450; PL, phospholipid; HLM, human liver microsomes; Abs, antibodies; G6P, glucose-6-phosphate; 4MPYR, 4-methylpyrazole; ORP, orphenadrine; COU, coumarin; TAO, troleandomycin; SFN, sulfaphenazole; PL-PHOS, PL-adduct with phosgene; PL-RAD, PL-adduct with dichloromethyl radical; ChOH, chlorzoxazone 6-hydroxylation; Cou7OH, coumarin 7-hydroxylation.
Testai et al., 1990; De Biasi et al., 1992; Gemma et al., 1996; Vittozzi et al., 2000). Alternatively, phosgene may be hydrolyzed by reacting with water, yielding carbon dioxide and hydrochloric acid (Fig. 1). In anoxic or hypoxic conditions, chloroform may be reduced to dichloromethyl radical (Tomasi et al., 1985; Testai et al., 1995), which is able to bind to PL-fatty acyl chains (De Biasi et al., 1992; Gemma et al., 1996; Vittozzi et al., 2000) or to abstract a hydrogen atom from the biological environment, leading to dichloromethane (Testai et al., 1995) (Fig. 1). The relative ratio between the two pathways depends on the oxygen partial pressure, on chloroform concentration and is specie-, organ- and gender-specific (Ade et al., 1994; Gemma et al., 1996; Vittozzi et al., 2000).

In rodents, it has been evidenced that at low concentration chloroform is oxidized by CYP2E1 (Testai et al., 1996; Constan et al., 1999). At higher CHCl₃ concentration, in the presence of oxygen, phosgene formation is catalyzed by CYP2B1/2, while in anoxic conditions dichloromethyl radical production seemed to be mediated by constitutive P450s (Testai et al., 1996). These direct observations supported a number of studies, showing the potentiation effect of a variety of CYP2E1 and 2B1/2 inducers on chloroform-induced toxicity (Sato et al., 1980; Stevens and Anders, 1981; Branchflower et al., 1983).

Only rare data on chloroform metabolism in human tissues have been published (Fry et al., 1972; Testai et al., 1991). No direct information on the human P450(s) involved in the reaction is available at present, although CYP2E1 is suspected as the major catalyst of chloroform metabolism. The knowledge of the P450(s) responsible for CHCl₃ bioactivation can provide useful information to identify the population group characterized by higher susceptibility to chloroform toxicity. Moreover, it would be possible to evaluate the environmental influence on susceptibility to chloroform-induced effects, through the study of metabolic interaction due to the combined exposure with other xenobiotics, affecting the rate of chloroform metabolism. Therefore, we have undertaken this study to characterize oxidative and reductive chloroform metabolism in human liver microsomes (HLM). In addition, we have identified the human P450(s) responsible for COCl₂ and CHCl₂ formation, by using different experimental approaches, including cDNA-expressed enzymes, correlation studies in a panel of phenotyped HLM, and inhibition by either chemical-selective inhibitors or anti-human P450 antibodies (Abs).

Materials and Methods

Products. [¹⁴C]Chloroform (3.1 mCi/mmol, radiochemical purity 99%) was obtained from PerkinElmer Life Sciences (Boston, MA); [¹³C]CHCl₃ was diluted with unlabeled chloroform (infrared purity) from Merck (Darmstadt, Germany) to achieve the specific radioactivity required. Liquid scintillation cocktails Lipoluma and Optiphase Hisafe were purchased from Lumac Systems A.G. (Basel, Switzerland) and PerkinElmer Wallac (Turku, Finland), respectively. NADP, glucose-6-phosphate (G6P), G6P-dehydrogenase, glucose oxidase, catalase were from Roche Ltd. (Basel, Switzerland). The P450 inhibitors, 4-methylpyrazole (4MPYR), orphenadrine (ORP), coumarin (COU), and troleandomycin (TAO) were purchased from Sigma-Aldrich (St. Louis, MO), whereas sulfaphenazole (SFN) was from Ultrafine (Manchester, UK). Monoclonal anti-human CYP2A6, CYP2B6, CYP2C8, CYP2E1, CYP3A4, and polyclonal anti-human CYP2C for immunoinhibition studies.
were purchased from BD Gentest Corp. (Woburn, MA). All other analytical grade chemicals were obtained from available commercial sources.

**Recombinant Human P450s and Human Liver Samples.** cDNA-derived human P450s (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) coexpressed with NADPH-cytochrome P450 reductase from human B-lymphoblastoid cell line AHLL-TK +/− and control microsomes from untransfected cells were purchased from BD Gentest Corp. HLM were prepared from a single liver sample, kindly gifted by the University of Washington (Seattle, WA) (Kharasch and Thummel, 1993). Protein concentration (23.2 mg/ml) and P450 content ranged between 0.14 and 0.82 nmol/mg of protein. Each sample was characterized by the supplier for single P450 activities (Table 1). In aerobic conditions, chloroform was injected: the lowest concentration obtainable was 15 mM. Further details of incubation procedure have been previously described (Testai et al., 1990).

After 20 min of incubation with [14C]chloroform at 37°C, the reaction was stopped, and microsomal PL were extracted according to the method of Folch et al. (1957). When required, different incubation times (10, 20, 40, 60 min) were applied. The amount of PL extracted was determined by the phosphorous assays as described by Rouser et al. (1966). By means of acid-catalyzed transmethylation of PL extracts, polar heads and fatty-acyl chains were obtained, and covalent binding of [14C]chloroform-derived metabolites to microsomal PL moieties was measured by the method of De Biasi et al. (1992). The levels of PL-adducts due to phosgene (PL-PHOS) and to dichloromethyl radical (PL-RAD) were then calculated as previously described (Gemma et al., 1996).

**Correlation Studies.** PL-PHOS and PL-RAD levels were determined in standard incubations with 12 individual HLM, fully characterized by the supplier for single P450 activities (Table 1). In aerobic conditions, chloroform was tested both at low (0.1 mM) and high (5 mM) concentrations, chosen on the basis of K_m values obtained for the two components of chloroform oxidative metabolism; at 0% pO_2, only 5 mM CHCl_3 was used. Correlation coefficients were calculated by plotting the rate of PL-PHOS and PL-RAD formation versus the marker activity of each isoform.

**Chemical Inhibition Assays.** Isoform-selective P450 inhibitors were added to the standard incubation mixtures carried out with four different individual HLM at the following final concentrations: 50 μM COU, 10 μM ORP, 100 μM 4MPYR, 5 μM SFN, and 100 μM TAO. Chemical inhibitors were diluted in the incubation buffer and added to the mixture, except for COU, SFN, and TAO, which were added as methanolic solution (final methanol concentration 1%); in these latter cases, control incubations (set as 100% metabolic activity) contained the same amount of methanol. The presence of methanol caused <5% decrease with respect to activity measured in the absence of the solvent. Competitive inhibitors such as COU, 4MPYR, and SFN were added to the standard incubation mixture just before [14C]chloroform injection, then the reaction was started by the addition of NADPH. Incubations containing mechanism-based inhibitors such as TAO and ORP were first preincubated with NADPH-generating system for 15 min at 37°C under aerobic conditions. Then [14C]chloroform was added to start the reaction for chloroform metabolism at 20% pO_2. Alternatively, to test the reductive metabolism in anoxic conditions, the mixture was placed on ice, the oxygen-scavenging system was added, the

---

**TABLE 1**

**P450 activities in human liver microsomes**

<table>
<thead>
<tr>
<th>Substrate Concentration</th>
<th>Activity[^a]</th>
<th>Correlation Coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>PL-PHOS formation[^b]</td>
<td>0.1</td>
<td>0.46–3.63</td>
</tr>
<tr>
<td>PL-PHOS formation[^b]</td>
<td>5</td>
<td>0.41–23.93</td>
</tr>
<tr>
<td>PL-RAD formation[^b]</td>
<td>5</td>
<td>3.00–28.83</td>
</tr>
<tr>
<td>Caffeine N3-demethylation (1A2)</td>
<td>5–60</td>
<td>27.73</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation (2A6)</td>
<td>57–4,111</td>
<td>1183.18</td>
</tr>
<tr>
<td>S-Mephenytoin-N-demethylation (2B6)</td>
<td>50–306</td>
<td>132.09</td>
</tr>
<tr>
<td>Tolbutamide methyl hydroxylation (2C9)</td>
<td>70–736</td>
<td>310.27</td>
</tr>
<tr>
<td>S-mephenytoin 4'-hydroxylation (2C19)</td>
<td>4–464</td>
<td>110.00</td>
</tr>
<tr>
<td>Dextromethorphan O-demethylation (2D6)</td>
<td>29–550</td>
<td>227.75</td>
</tr>
<tr>
<td>Chlorozoxazone 6-hydroxylation (2E1)</td>
<td>25–2,977</td>
<td>1916.17</td>
</tr>
<tr>
<td>Dextromethorphan N-demethylation (3A4)</td>
<td>19–205</td>
<td>89.45</td>
</tr>
<tr>
<td>Midazolam 1'-hydroxylation (3A4/5)</td>
<td>504–12,057</td>
<td>5287.45</td>
</tr>
</tbody>
</table>

[^a]: PL-PHOS-marker activities are expressed as nmol · mg protein −1 · min −1.
[^b]: PL-PHOS and PL-RAD formation are expressed as nmol [14C] · mg PL −1.
[^*]: p < 0.05; **: p < 0.01; ***: p < 0.001.
reaction tubes were sealed and flushed with nitrogen for 20 min, warmed at 37°C for 10 min, and injected with [14C]chloroform to start the reaction.

**Immunoinhibition Assays.** In immunoinhibition experiments, monoclonal Abs (both single and mixtures) or antisera were preincubated at 4°C for 20 min and at room temperature for 30 min, respectively, with 1.2 mg of microsomal protein from HLM pooled from five donors. Following the protocol indicated by the supplier, the amounts of Abs or antisera used were expected to produce maximal inhibition (>80%) of the specific enzymatic activity: 3 μl/100 μg of protein anti-CYP1A2 Ab and anti-CYP2B6 Ab; 5 μl/100 μg of protein anti-CYP2A6 Ab, anti-CYP2C8 Ab, and anti-CYP2E1 Ab; 10 μl/100 μg of protein anti-CYP3A4 Ab; 25 μl/100 μg of protein anti-CYP2C2. In control incubations, the presence of monoclonal Abs or antisera was replaced by Tris buffer or normal goat serum, respectively, and was set as 100% metabolic activity. Immunoinhibited microsomes were then added to the incubation mixture to test the in vitro bioactivation of [14C]chloroform following the standard operating procedure.

**Calculation.** The kinetic parameters \(K_{\text{app}}\) and \(V_{\text{maxapp}}\) were obtained from the Eadie-Hofstee plot. Linear regression was carried out by using the least-squares method to obtain the best fitting of single experimental data. In correlation studies, P450 activities measured in 12 HLM tested toward both chloroform and model substrates, exceeding \(>2\) S.D. of the mean, have been considered outliers and were not included in the calculation of the Pearson’s regression coefficient (r). Student’s t test was used to assess the significance of results of inhibition studies; the cut-off of statistical significance was set at \(p < 0.05\).

**Results**

**In Vitro Chloroform Metabolism by HLM. Substrate and time dependence.** In vitro characterization of chloroform metabolism was carried out by using three individual HLM, characterized by different relative P450 content. The ratio of CYP2E1-to-CYP2A6 was 14.4, 0.33, and 0.15 in HLM1, HLM2, and HLM3, respectively, the latter HLM being characterized also by high levels of CYP1A2- and 2B6-related activities. The marker activities corresponding to the other P450s were in the average range expressed within the panel of individual HLM tested during the study. Results showed that all HLM samples can bioactivate [14C]CHCl3, both oxidatively and reductively, as illustrated in Fig. 2 with HLM3. The Eadie-Hofstee plots clearly identified two components, characterized by different affinity for chloroform, suggesting multiple P450 involvement (Fig. 2A inset; Table 2). The \(K_{\text{app}}\) values ranged between 20 and 650 μM, being the lowest value obtained in HLM3, characterized by a high level of CYP2E1 and low levels of CYP2A6. In HLM2, which is characterized by an extremely low content of CYP2A6, the low affinity phase was less defined; indeed, both \(K_{\text{app}}\) and \(V_{\text{maxapp}}\) were one order of magnitude lower than kinetic parameters obtained with the other two samples. Although the HLM2 and HLM3 differed for their content in CYP1A2 and 2B6, their \(K_{\text{app}}\) and \(V_{\text{maxapp}}\) values resulted very similar, in line with their almost equal levels of CYP2A6.

When 5 mM chloroform was tested, the oxidative bioactivation of chloroform was linear with time up to 20 min (Fig. 2B). No formation of PL-RAD was measured in aerobic conditions at any substrate concentration or incubation time (data not shown). In anoxic conditions, substantial levels of PL-RAD adducts were detectable at 5 mM CHCl3 (26.1 ± 2.8, 19.4 ± 3.3, and 13.8 ± 2.2 nmol · mg PL-1 · nmol P450-1 in HLM1, HLM2, and HLM3, respectively); on decreasing chloroform concentration at 1 mM, reductive metabolism was drastically reduced by about 80 to 90%, attaining negligible levels of PL-RAD at 0.1 mM chloroform. PL-RAD production appeared to be linear up to 60 min of incubation time (\(R^2 = 0.986\)) (Fig. 2B), whereas PL-PHOS adducts were not detected (data not shown).

**Oxygen concentration dependence.** When the dependence on \(pO_2\) of 5 mM CHCl3 metabolism was studied, PL-PHOS production was detected at similar high levels both in aerobic and in hypoxic conditions (1–5% \(pO_2\)), whereas it was negligible at 0% \(pO_2\) (Fig. 3). On the other hand, PL-RAD formation was very efficiently inhibited by oxygen, attaining at 1 and 5% \(pO_2\) only 14 and 2%, respectively, the level measured in anoxic conditions (Fig. 3).

**Interindividual variability.** A quantitative analysis of our data, obtained in a panel of 12 individual HLM showed that PL-PHOS levels varied by 8- and 58-fold at 0.1 and 5 mM CHCl3, respectively (Table 1). Among the tested samples PL-RAD amounts, which were quantitatively higher than PL-PHOS ones, showed a level of variation by about 10-fold (Table 1). The average production of the two types of PL-adducts was very similar when measured at 5 mM chloroform in the oxygenation conditions favoring their respective formation (Table 1).

**Correlation Studies.** To correlate PL-PHOS and PL-RAD levels with the activity of each single isoform measured with marker substrates, CHCl3 metabolism by microsomes from a panel of 12 phenotyped individual human livers was measured. Different chloroform concentrations were chosen to investigate the possible contribution of high affinity (0.1 mM) and low affinity component (5 mM) of the reaction. A strong positive correlation between PL-PHOS formation and Ch6OH (\(r = 0.837; p < 0.001\)) was found at 0.1 mM CHCl3, in aerobic conditions (Table 1). A correlation with a weaker level of significance (\(p < 0.05\)) was evidenced also with CYP1A2- and 2C9-marker activities; however, this result seemed to be misleading, since the content of these two P450s paralleled that one of CYP2E1, showing a Pearson’s coefficient \(r = 0.68\) and 0.70, respectively (\(p < 0.01\)). On increasing substrate concentration up to 5 mM, PL-PHOS significantly correlated (\(r = 0.777; p < 0.01\)) with Cou7OH (Table 1).

No significant correlation was observed between PL-PHOS formation and all of the other tested activities at any CHCl3 concentration tested (Table 1). In anoxic conditions, a strong positive correlation was found between PL-RAD production at 5 mM chloroform and Ch6OH (\(r = 0.744; p < 0.01\)). Due to the involvement of CYP2E1 in PL-RAD formation, results were affected by the same misleading correlation of PL-RAD with CYP1A2 and 2C9 activities, previously evidenced in aerobic incubations at low CHCl3 concentration. All other isoform marker activities did not significantly correlate with CHCl3 reductive biotransformation (Table 1).

**In Vitro Chloroform Metabolism by cDNA-Expressed Human P450s.** Microsomes from human B-lymphoblastoid cell lines expressing single P450s were tested to characterize their abilities to metabolize [14C]CHCl3 both oxidatively and reductively. At 5 mM CHCl3, in aerobic conditions, CYP2A6, CYP2B6, and to a lesser extent CYP2C19 were found to catalyze CHCl3 oxidation (Fig. 4A). At lower CHCl3 concentrations (1 mM) only CYP2A6 showed a significant activity in the oxidative reaction, which was no more significant at 0.1 mM. The levels of PL-PHOS produced by CYP2B6 and 2C19 were negligible at 1 mM chloroform yet (Fig. 4A). CYP2E1, tested only at 15 mM CHCl3, due to its sensitivity to solvent inhibition, resulted as a poor catalyst for PL-PHOS production in these experimental conditions (Fig. 4A). When the oxygen concentration was lowered down to 5% \(pO_2\), the activity of CYP2A6 and 2B6 in forming PL-PHOS resulted in 82 and 52% of their production measured in aerobic conditions. CHCl3 reductive metabolism was associated with all the tested isoforms but CYP1A2, CYP2B6, and CYP3A4 (Fig. 4B). Levels of PL-RAD produced by CYP2C9 and CYP2C19 (about 20 nmol · mg PL-1 · nmol P450-1) doubled those measured with the other P450s. The PL-RAD levels produced by CYP2E1 (tested at 15 mM CHCl3) were 2 to 3 times higher than those ones produced by other isoforms (at 5 mM). At lower CHCl3 concentrations, no reductive metabolism by any cDNA-expressed P450 could be detected (data not shown).
Moreover, the reductive metabolism was highly inhibited by oxygen; indeed, at 1% pO$_2$ the PL-RAD formation catalyzed by CYP2C9 was less than 50% the one measured in anoxic conditions, while 2C19 was no more active.

**Chemical Inhibition Studies.** The effect of isoform-selective chemical inhibitors on oxidative and reductive chloroform metabolism has been studied in a panel of four individual HLM, characterized by quite different single P450 content, to take into account the

---

**Fig. 2.** Bioactivation of chloroform in individual human liver microsomes illustrated with HLM: dependence on substrate concentration (A) and incubation time (B).

PL-PHOS (squares) and PL-RAD (circles) formation were measured in aerobic and anoxic conditions, respectively; they represent the mean ± S.D. of three independent determinations and are expressed as nmol [${}^{14}$C] · mg PL$^{-1}$ · nmol P450$^{-1}$. The inset in panel A shows the Eadie-Hoffstee plot of PL-PHOS formation in aerobic conditions. When substrate concentration dependence was studied, incubation time was 20 min. Time dependence of chloroform oxidation and reduction was tested at 5 mM chloroform. Details are described under Materials and Methods. CYP, cytochrome P450.
individual variability in the response (Table 3). The addition of 4MPYR to aerobic incubations containing 0.1 mM CHCl₃ caused the higher inhibitory effect in all the tested samples. Depending on CYP2E1 content, the level of inhibition in the presence of 4MPYR varied between 98 to 61% in HLM showing the highest and the lowest Ch6OH activity, respectively. TAO, COU, and ORP were also able to decrease to a lesser extent the production of PL-PHOS. Although the four HLM tested were characterized by very different Cou7OH, 3A4-, and 2B6-related activities, the inhibition of PL-PHOS formation due to COU, TAO, and ORP was not proportionally variable and did not parallel the related P450 content. Therefore the inhibition by TAO, COU, and ORP can be considered unspecific, very likely due to the inhibitor concentrations used. SFN addition did not affect the high affinity phase of chloroform oxidative metabolism (Table 3). At 5 mM chloroform and 20% pO2, COU was the most efficient inhibitor: indeed, inhibition of chloroform oxidation ranged between 94 and 45%, in HLM showing the highest and the lowest CYP2A6 content, respectively. 4MPYR inhibition of PL-PHOS production attained about 30% in all the tested samples, independently on the CYP2E1 content (Table 3). The presence of ORP and TAO evidenced some inhibition on PL-PHOS production, totally unrelated to the corresponding P450 level, whereas SFN was completely ineffective (Table 3).

The reductive metabolism of 5 mM chloroform was significantly reduced in all the anoxic incubations containing 4MPYR (Table 3), with 90 and 60% inhibition of PL-RAD production measured in samples with high and low CYP2E1 content, respectively. The addition of all the other chemical inhibitors caused only marginal decrease in chloroform reductive metabolism (Table 3).

**Immunoinhibition Studies.** The effects of human-selective Abs were studied in a combinatorial immunoinhibition assay with HLM pooled from five donors. Data showed that at low CHCl₃ concentration (0.1 mM), PL-PHOS production was not affected until anti-CYP2E1 was added to the Abs mixture, when 90% inhibition was measured (Fig. 5A). Anti-CYP3A4 tested singularly did not produce any decrease in chloroform oxidation (data not shown). At 5 mM CHCl₃, the presence of anti-CYP2A6 as the first Ab in the mixture caused a strong decrease of PL-PHOS levels (84%); all further addition did not cause any change (Fig. 5B). To pick up other significant inhibition, anti-CYP2E1 and anti-CYP2B6 Ab were singularly added to the incubation mixture, producing a decrease in PL-PHOS formation as low as 20.2% and 15.1%, respectively. In anoxic conditions and 5 mM CHCl₃, PL-RAD levels decreased by 93% only in the presence of anti-CYP2E1 in the Ab mixture (Fig. 5C).

### TABLE 2

<table>
<thead>
<tr>
<th>HLM</th>
<th>( K_m^{app} ) (a)</th>
<th>( V_{max}^{app} ) (a)</th>
<th>( K_m^{app} ) (b)</th>
<th>( V_{max}^{app} ) (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM₁</td>
<td>18 ± 9</td>
<td>71 ± 28</td>
<td>302 ± 165</td>
<td>93 ± 21</td>
</tr>
<tr>
<td>HLM₂</td>
<td>92 ± 60</td>
<td>112 ± 42</td>
<td>3122 ± 999</td>
<td>928 ± 188</td>
</tr>
<tr>
<td>HLM₃</td>
<td>655 ± 233</td>
<td>238 ± 61</td>
<td>4592 ± 1902</td>
<td>901 ± 171</td>
</tr>
</tbody>
</table>

(a) M, (b) nmol PL-PHOS · mg PL⁻¹ · nmol P450⁻¹ · min⁻¹.

**5 mM CHCl₃**

**Fig. 3.** Oxidative and reductive bioactivation of chloroform in human liver microsomes: dependence on oxygen partial pressure.

Formation of PL-PHOS (dotted bars) and PL-RAD (hatched bars) was measured at any pO₂ after 20 min standard incubations; they represent the mean ± S.D. of three independent determinations and are expressed as nmol [¹⁴C] · mg PL⁻¹ · nmol CYP⁻¹. CYP, cytochrome P450.
PL-PHOS (A) and PL-RAD (B) were measured, respectively, after 20-min aerobic and anoxic standard incubations, as described under Materials and Methods. Incubations contained a single heterologously expressed P450 and 0.1, 1, 5, or 15 mM chloroform, as indicated. Results are expressed as nmol [14C] · mg PL−1 · nmol P450−1, and represent the mean ± S.D. of three independent determinations, n.d., not detectable. CYP, cytochrome P450.

**TABLE 3**

Effect of P450-specific inhibitors on CHCl₃ metabolism in human liver microsomes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>TAO (CYP3A4)</td>
<td>48.8 ± 8.8*</td>
</tr>
<tr>
<td>COU (CYP2A6)</td>
<td>58.6 ± 13.1*</td>
</tr>
<tr>
<td>4-MPYR (CYP2E1)</td>
<td>80.1 ± 6.3**</td>
</tr>
<tr>
<td>SFN (CYP2C9)</td>
<td>11.6 ± 16.4</td>
</tr>
<tr>
<td>ORP (CYP2B6)</td>
<td>37.0 ± 10.0*</td>
</tr>
</tbody>
</table>

* Inhibition (%) was calculated with respect to activity measured in control incubations; values are means of triplicate determinations.

**Discussion**

The characterization of the P450-dependent oxidative and reductive metabolism of chloroform in HLM is reported for the first time. The study has been carried out with a multifaceted experimental strategy: the comparison and integration of the obtained results have allowed the identification of P450s involved in chloroform bioactivation in the human liver.

It has been clearly demonstrated that, depending on substrate and oxygen concentration, HLM are able to bioactivate chloroform to its oxidative and reductive metabolites, COCl₂ and dichloromethyl rad-
as CYP2E1: Ch6OH strongly correlated with PL-RAD formation, which was decreased only by 4MPYR and anti-CYP2E1 Ab. In line with this result, CYP2E1 has been indicated as the primary isoform responsible for CCL4 reduction in the human liver (Zangar et al., 2000). On the contrary, although the quite similar chemical structure, halothane reductive metabolism has been reported to be catalyzed by two different P450s, namely 2A6 and 3A4 (Spracklin et al., 1996).

The amount of chloroform-derived adducts produced by HLM in strictly anaerobic or in room air-equilibrated incubations were similar and even higher, when compared with those obtained in the same experimental conditions with rodent hepatic microsomes (Gemma et al., 1996). A strong similarity was found also between rodents and human hepatic P450s involved in low dose-chloroform metabolism, which is exclusively oxidative. Indeed, similar to the human data, CYP2E1 has been reported as the major catalyst of chloroform oxidation in vitro by rat liver microsomes (Testai et al., 1996) and in vivo in mice (Constan et al., 1999). Therefore, the commonly used rodent species appear to make reasonable models for studying metabolism and toxicity of low levels of CHCl3, very likely the more appropriate to extrapolate data to the actual human exposure condition.

However, some differences exist, when high CHCl3 concentrations have to be considered, starting with the pO2 dependence of chloroform metabolism. The rate of both chloroform oxidation and reduction in the two limit oxygenation conditions (anoxic and aerobic) are comparable both in human and in rodent liver microsomes (Gemma et al., 1996). The differences arise when comparing results obtained in hypoxic conditions (5 and 1% pO2) (Gemma et al., 1996), better representing the physiological situation of hepatic centrilobular region, where most of drug metabolism takes place. In HLM the oxidative pathway strongly prevailed on radical formation; the oxidation/reduction (ox/red) ratio was 34 and 5 at 5 and 1% pO2, respectively. In the same conditions, rat and mouse liver microsomes were characterized by much lower values of the ox/red ratio: 1.5 to 6 at 5% pO2 and 0.2 to 0.5 at 1% pO2 (Gemma et al., 1996), clearly suggesting a more efficient hepatic reductive metabolism. Therefore, in the absence of investigations able to quantitate the actual proportion of each pathway in vivo, these data suggest that, within a broad range of chloroform concentrations, the major toxicologically relevant metabolite produced by the human liver is phosphine.

Secondly, at variance with the human CYP2A6 involvement, the low affinity phase of chloroform oxidation in rat liver microsomes is mediated mainly by CYP2B1/2 (Testai et al., 1996), as supported also by the potentiation of chloroform toxicity due to animal pretreatment with CYP2B inducers (Lavigne and Marchand, 1974; Stevens and Anders, 1981). Regarding reductive metabolism, in the rat constitutive isoforms and CYP2B1/2 at high CHCl3 concentrations were identified as active, and no contribution has been attributed to CYP2E1 (Testai et al., 1996). A similar situation was reported for CCl4: CYP2B has been implicated in its reductive metabolism in rodent (Frank et al., 1982), whereas in HLM the only isoform active in the reaction was CYP2E1 (Zangar et al., 2000).

Concluding, chloroform can undergo both oxidative and reductive metabolism in the human liver, depending on oxygen and substrate concentration. At the low levels, typical of actual human exposure through the use of chlorinated waters, CHCl3 is metabolized primarily to phosphine by CYP2E1, a typical high affinity-low capacity enzyme. When the CYP2E1-mediated reaction is saturated, the predominant role in phosphine production is for CYP2A6, efficient even in highly hypoxic conditions (1% pO2). CYP2E1 is a unique isoform, in that beside its role in chloroform oxidation, it is able to catalyze also dichloromethyl radical formation. This latter pathway seems to be

---

**FIG. 5.** Combinatorial Abs analysis of individual P450 contribution to 0.1 mM (A) and 5 mM (B, C) chloroform bioactivation.

PL-PHOS and PL-RAD were measured after 20-min aerobic (A, B) and anoxic (C) standard incubations, respectively. Incubations were carried out with HLM, pooled from five donors. Details of experimental conditions were described under Materials and Methods. The order of Abs addition is indicated under each bar, representing the mean ± S.D. of duplicate determinations. Results are expressed as nmol [14C] · mg PL⁻¹ · nmol CYP⁻¹. Stars indicate the statistical significance of results (p < 0.001). CYP, cytochrome P450.
scarcely relevant in the human liver, since it is active only at high substrate concentrations and in strictly anaerobic conditions.

The relevance of CYP2E1 in chloroform metabolism in humans provides useful information to identify eventual differences in susceptibility to chloroform-induced adverse effects. Variations in the level of expression of CYP2E1 is not uncommon, being determined either by genetic features, by pathophysiological conditions such as diabetes, or by environmental factors, such as alcoholic beverages consumption or exposure to other xenobiotics known to affect CYP2E1 expression. A peculiar situation may occur in the human fetus, between gestational days 50 and 113, when the fetal brain CYP2E1 mRNA is expressed at relatively high amounts corresponding to a fairly constant level of enzymatic activity of ethanol metabolism (Brzezinski et al., 1999). The eventual effects related to chloroform exposure during this stage of development might be of interest, providing some biological basis for reproductive outcomes in humans exposed to chloroform via drinking water.

References


