Biochemical Pharmacology, Vol. 46, No. 6, pp. 1076–1080, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

Chlorotrifluoroethylene trimer and tetramer are inducers of the CYP4A subfamily

(*Received* 1 *April* 1993; *accepted* 10 *June* 1993)

Abstract—Male Wistar albino rats were treated for a 7 day period with equimolar doses of the trimer and tetramer oligomers of chlorotrifluoroethylene (CTFE), resulting in significant hepatomegaly for both compounds. In addition, both trimer and tetramer significantly induced the peroxisomal β-oxidation of fatty acids as assessed by increases in palmitoyl-coenzyme A (CoA) oxidation, thus confirming these oligomers as peroxisome proliferators. Consistent with these conclusions, both trimer and tetramer increased the hydroxylation of lauric acid indicating that the CTFEs were inducers of the CYP4A subfamily, a conclusion further supported by substantial increases in the steady-state levels of the cognate CYP4A1 mRNA as determined by northern blotting. The liver appeared to be more susceptible to induction than the kidney and the CTFE tetramer was more potent than the trimer. These results are discussed with respect to both the differential hepatotoxicity, and biotransformation/disposition of the two polyhalogenated oligomers.

The trimer and tetramer oligomers of chlorotrifluoroethylene (CTFE*) are major components of 3.1 oil used as lubricants, hydraulic fluids and greases, particularly in commercial aircraft and U.S. Air Force aircraft and advanced weapons systems. Concern has been expressed about their safety in use, as inhalation exposure and oral gavage experiments in rats with the trimer and tetramer have indicated that these compounds are hepatotoxic [1, 2], with the tetramer being more toxic than the trimer [2]. The hepatotoxicity and chemical structure of the CTFE oligomers are similar to the perfluoro fatty acids such as perfluoro-*n*-decanoic and perfluoro-*n*-octanoic acid and similar liver changes such as hepatomegaly and induction of the peroxisomal fatty acid β-oxidation system are seen with both groups of compounds [1–4].

The above liver changes elicited by CTFE trimer and tetramer, and their structural similarity to the perfluoro fatty acids would classify these oligomers as peroxisome proliferators, a structurally diverse group of compounds producing characteristic responses in the liver, including peroxisome proliferation [5, 6]. A further characteristic liver response to peroxisome proliferators in rodents is induction of the 12-hydroxylation of lauric acid, a reaction catalysed by members of the cytochrome P4504A subfamily [7, 8]. Accordingly, it is the objective of this study to determine if the trimer and tetramer oligomers of CTFE are inducers of the CYP4A subfamily.

Materials and Methods

Chemicals. The trimer and tetramer CTFE oligomers were given to the U.S. Air Force by Halocarbon Products Corporation (Hackersack, NJ, U.S.A.) and were greater than 99% pure as assessed by gas chromatography/mass spectrometry [2].

Animals, animal dosing and tissue processing. Male Wistar rats, of initial body weight approx. 140 g, were obtained from the University of Surrey Experimental Biology Unit and divided into three groups with six animals in each group. The control group received no treatment and the trimer and tetramer CTFE groups each received seven daily doses of the oligomer by oral gavage (2.3 mmol/kg body weight). Individual body weight gains and food consumption were noted for all animals and the animals killed by cervical dislocation on the eighth day, i.e. 24 hr after the seventh and final dose.

The livers and kidneys were exposed, removed, blotted dry and weighed. Samples of liver and kidney were rapidly removed and snap frozen in liquid N_2, prior to storage at $-70°$ for subsequent RNA analysis. The livers were perfused with 0.9% (w/v) sodium chloride prior to homogenization in 0.25 M sucrose (25%, w/v homogenate). Aliquots of whole liver homogenate were reserved for subsequent peroxisomal and mitochondrial enzyme determinations. Liver microsomal samples were prepared by differential centrifugation [9] and the microsomal pellets resuspended in ice-cold 50 mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol to stabilize cytochrome P450s and stored at $-70°$. Kidneys were decapsulated and subcellular fractions prepared as for liver and stored in aliquots at $-70°$.

Enzyme assays and biochemical determinations. Microsomal cytochromes P450 and b_5 were determined by spectrophotometric methods [10] as was NADPH-cytochrome *c* (P450) reductase [9]. Pentoxy and ethoxyresorufin *O*-dealkylase activities were determined by a fluorometric method [11] and lauric acid 12-(ω) and 11-($\omega - 1$) hydroxylase activities were determined by a radioisotope method after metabolite separation on reverse phase, gradient HPLC [9]. Cyanide-insensitive palmitoyl-coenzyme A (CoA) oxidation was determined by the rate of reduction of NAD^+ at 340 nm by the method of Bronfman *et al.* [12] and carnitine acetyltransferase was determined spectrophotometrically by a previously published method [13].

Isolation of RNA from liver and kidney, labelling of a 2.1 kb CYP4A1 cDNA probe and northern blot analysis for CYP4A1 mRNA were carried out as described previously [14]. The blots were washed with 2× standard saline citrate (SSC), 0.1% (w/v) sodium dodecyl sulphate (SDS) for 2 × 20 min at room temperature, 0.5× SSC, 0.1% (w/v) for 2 × 20 min at 42° and finally 5× SSC for 20 min at room temperature. The exposure time for autoradiography was 48 hr.

Results

Over the 7 day dosing period, the CTFE trimer caused a slight drop in the body weight gain and food intake, whereas the CTFE tetramer was without effect on these parameters (Table 1). More strikingly, there was a marked increase in the absolute liver weight and the liver:body weight ratio for both oligomers (Table 1). In addition, both compounds caused substantial increases in both palmitoyl

* Abbreviations: CoA, coenzyme A; CTFE, chlorotrifluoroethylene; SSC, standard saline citrate; SDS, sodium dodecyl sulphate.

Table 1. Influence of CTFE trimer and tetramer on body weight gain, food intake and liver/kidney weights in male rats*

	Control	Treatment CTFE trimer	CTFE tetramer
Body weight increase (g/7 days)	50.0 ± 1.2	43.9 ± 3.2†	51.1 ± 2.60
Food intake (g/7 days)	140.0 ± 4.8	135.7 ± 3.9†	148.3 ± 4.8
Liver weight (g)	5.8 ± 0.2	10.3 ± 0.3†	10.1 ± 0.2†
Liver:body weight ratio (%)	3.0 ± 0.1	5.5 ± 0.2†	5.3 ± 0.1†
Kidney weight (g)	1.6 ± 0.1	1.7 ± 0.1	1.71 ± 0.1
Kidney:body weight ratio (%)	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1

* Results are presented as the mean ± SEM from six rats.
† Significantly different from the control group (Student's *t*-test) at $P < 0.001$.

Table 2. Influence of CTFE trimer and tetramer on peroxisomal palmitoyl-CoA oxidation and mitochondrial carnitine acetyltransferase in liver and kidney*

	Control	Treatment CTFE trimer	CTFE tetramer
Palmitoyl-CoA oxidation (nmol/min/mg)			
Liver	5.8 ± 0.3	7.3 ± 0.5‡	14.3 ± 1.5†
Kidney	4.9 ± 0.6	6.4 ± 0.4	6.1 ± 0.2
Carnitine acetyltransferase (nmol/min/mg)			
Liver	1.6 ± 0.2	2.4 ± 0.3	7.4 ± 0.8†
Kidney	12.4 ± 0.1	17.0 ± 0.3†	11.6 ± 0.9

* Results are presented as the mean ± SEM from six animals in each group.
† Results are significantly different from control group (Student's *t*-test) at either †$P < 0.001$ or ‡$P < 0.01$.

CoA oxidation and carnitine acetyltransferase activity in the liver and kidney (particularly the former organ) (Table 2), thus providing evidence that the two oligomers were peroxisome proliferators in this species and strain of rat with this particular dosing regimen.

As the majority of peroxisome proliferators modulate the cytochrome P450-dependent mixed function oxidase system [7], it was of interest to examine if the CTFE oligomers had this property. As shown in Table 3, both the trimer and the tetramer approximately doubled the total amount of hepatic cytochrome P450 and its associated flavoprotein reductase, with no effect on the specific content of microsomal cytochrome b_5. However, in spite of the increases in cytochrome P450 and its reductase, the alkoxyresorufin dealkylase activities were substantially inhibited by both CTFE trimer and tetramer treatments (Table 3). In contrast, the 11- and 12-hydroxylations of lauric acid were induced (particularly the latter) indicating induction of the cytochrome P4504A subfamily and it appeared that the CTFE tetramer was more potent in this respect (Table 3). These hepatic effects were largely mirrored in the kidney mixed function oxidase system, although the effects appeared less pronounced (Table 4).

The above fatty acid hydroxylase induction data appeared to indicate that the CTFE trimer and tetramer were inducers of the CYP4A subfamily. To substantiate further this and to explore the mechanism of induction, RNA was extracted from liver and kidney and the steady-state levels of CYP4A mRNA analysed by northern blotting with a CYP4A1 cDNA probe. As shown in Fig. 1, there is a substantial increase in both liver and kidney CYP4A1 mRNA after CTFE tetramer treatment, whereas this response is less marked for the CTFE trimer, consistent with the fatty acid hydroxylase data in Tables 3 and 4.

Discussion

The results of our study support the view that CTFE

Table 3. Influence of CTFE trimer and tetramer treatment on liver mixed function oxidase components*

	Control	Treatment CTFE trimer	CTFE tetramer
Cytochrome P450 (nmol/mg)	0.63 ± 0.03	1.40 ± 0.01†	1.20 ± 0.06†
Cytochrome b_5 (nmol/mg)	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01
NADPH-cytochrome *c* (P450) reductase (nmol/min/mg)	104.8 ± 4.5	251.7 ± 6.0†	251.1 ± 11.7†
Ethoxyresorufin *O*-deethylase (pmol/min/mg)	285 ± 40	180 ± 10§	145 ± 10§
Pentoxyresorufin *O*-depentylase (pmol/min/mg)	86 ± 10	29 ± 10†	29 ± 10†
Lauric acid hydroxylation (nmol/min/mg)			
11-hydroxylase	1.2 ± 0.1	1.7 ± 0.1‡	2.1 ± 0.1†
12-hydroxylase	3.7 ± 0.2	5.9 ± 0.4†	10.1 ± 0.7†

* Values are given as mean ± SEM from six animals in each group and are significantly different from the control value at †$P < 0.001$ or ‡$P < 0.05$ or §$P < 0.01$.

Table 4. Influence of CTFE trimer and tetramer treatment on kidney mixed function oxidase parameters*

	Control	Treatment CTFE trimer	CTFE tetramer
Cytochrome P450 (nmol/mg)	0.11 ± 0.01	0.20 ± 0.02‡	0.09 ± 0.01
Cytochrome b_5 (pmol/mg)	16 ± 1	9 ± 1‡	3 ± 1†
NADPH-cytochrome *c* (P450) reductase (nmol/min/mg)	27.0 ± 3.3	47.4 ± 2.2‡	22.7 ± 0.8
Ethoxyresorufin *O*-deethylase (pmol/min/mg)	61 ± 7	50 ± 5	58 ± 2
Pentoxyresorufin *O*-deethylase (pmol/min/mg)	62 ± 5	84 ± 9	34 ± 3
Lauric acid hydroxylation (nmol/min/mg)			
11-hydroxylase	1.2 ± 0.1	2.1 ± 0.1‡	1.5 ± 0.2
12-hydroxylase	3.4 ± 0.2	7.1 ± 0.3†	4.9 ± 0.4‡

* Results are expressed as mean ± SEM on three determinations on a pool of 12 kidneys in each treatment group and are significantly different from the control group (Student's *t*-test) at †$P < 0.001$ or ‡$P < 0.05$.

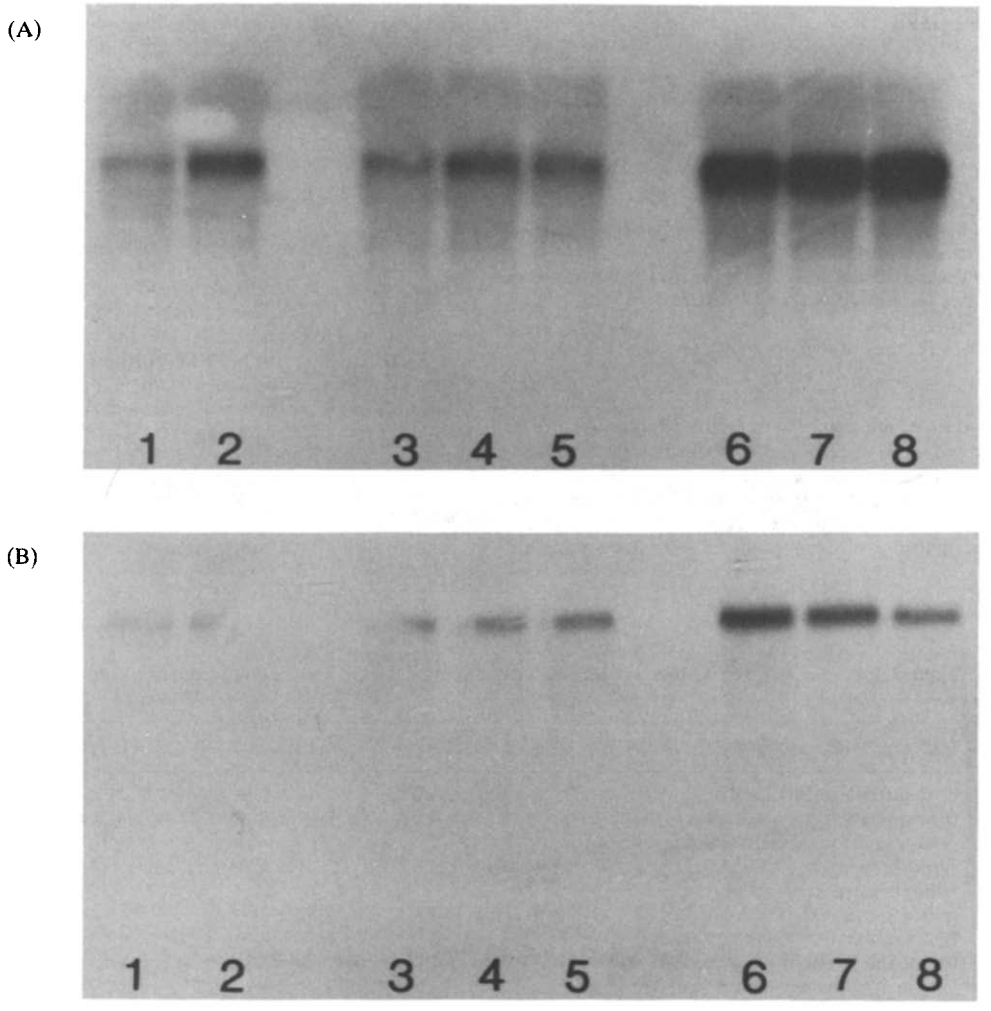

Fig. 1. Northern blot analysis of CYP4A1 mRNA in liver (A) and kidney (B) after CTFE trimer and tetramer treatment. Animals were dosed and northern blot analysis carried out as described in Materials and Methods. Equal amounts of RNA (20 μg) were loaded in each track corresponding to RNA derived from control tissue [1, 2], trimer-treated [3–5] or tetramer-treated [6–8].

oligomers are peroxisome proliferators in that they increase the liver:body weight ratio and cause induction of the peroxisome palmitoyl-CoA oxidation. Furthermore, the fatty acid hydroxylase data (Tables 3 and 4) and the CYP4A1 northern blot data (Fig. 1) provide strong evidence that the CYP4A subfamily is being induced, another characteristic of peroxisome proliferators [7, 15]. In this context it should be noted that the cytochrome P4504A subfamily consists of approximately nine members [16], the majority of which exhibit fatty acid ω-hydroxylase activity (i.e. lauric acid 12-hydroxylase activity). Therefore examination of the catalytic data alone does not allow identification of which specific CYP4A subfamily members are induced by the CTFE oligomers. However, because the CYP4A1 cDNA hybridization experiments were carried out under relatively strict conditions of stringency, we are confident that, at least, the CYP4A1 protein is induced. Whether smaller amounts of structurally related CYP4A mRNAs are additionally induced is unlikely, but cannot be discounted by our experiments. Furthermore, although our data have shown that the CTFE oligomers increase the steady-state level of CYP4A1 mRNA, it is not clear how this induction arises. The obvious possibilities are oligomer-dependent mRNA stabilization or transcriptional gene activation as has been described for CYP4A1 and clofibrate [17]. Further possibilities that are the subject of our future work include the possible role of the peroxisome proliferator activated receptor [18] in the induction process as has been recently described for the CYP4A6 gene [19].

Our data would also indicate that the CTFE tetramer is a more potent inducer of peroxisomal and CYP4A enzyme activities than the trimer as they were both given in equimolar doses and are consistent with their relative hepatotoxicities [2]. However, it must be emphasized that a more definitive analysis of their relative potencies must await more extensive dose–response studies. For the CTFE oligomers, disposition and pharmacokinetic considerations make an important contribution to their relative potencies in that the tetramer is selectively retained in the liver, to approximately double the liver concentrations achieved by the trimer after a 7 day dosing period [2], as a result of their different pharmacokinetic properties [19]. In addition, this selective retention of CTFE oligomers in the liver may rationalize our data described herein, whereby the liver appeared to be a more responsive tissue than the kidney.

Although some exceptions are known, by far the majority of peroxisome proliferators and CYP4A inducers are carboxylic acids or bioisostere equivalents [20], and it is therefore puzzling why polyhalogenated hydrocarbons such as the CTFE oligomers should exhibit similar properties. However, this apparent contradiction has recently been clarified by reports that the CTFE oligomers undergo reductive dehalogenation (at the terminal carbon bearing two chlorine atoms) to the corresponding carboxylic acid metabolites identified both in urine and liver homogenates [21–23]. It would appear that the carboxylic acid metabolites are the active inducing agents, as direct addition of the acids to rat hepatocyte primary cultures results in similar liver enzyme changes as described herein (Del Raso NJ, personal communication). In addition, after 7 days of separate trimer and tetramer dosing, the amount of tetramer carboxylic acid metabolite present in liver was approximately 11-fold that of the trimer metabolite, consistent with the relative inductive potencies of equimolar oligomer doses described herein.

Acknowledgements—This research was supported in part by grants from the European Science Foundation and the U.S. Air Force Office of Scientific Research.

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