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METABOLISM OF L-CYSTEINE *S*-CONJUGATES AND *N*-(TRIDEUTEROACETYL)-L-CYSTEINE *S*-CONJUGATES OF FOUR FLUOROETHYLENES IN THE RAT

ROLE OF BALANCE OF DEACETYLATION AND ACETYLATION IN RELATION TO THE NEPHROTOXICITY OF MERCAPTURIC ACIDS

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Abstract—The relationship between the relative nephrotoxicity of the mercapturic acids (NAc) of the fluorinated ethylenes tetrafluoroethylene (TFE), chlorotrifluoroethylene (CTFE), 1,1-dichloro-2,2-difluoroethylene (DCDFE) and 1,1-dibromo-2,2-difluoroethylene (DBDFE), and the biotransformation by activating (*N*-deacetylase and β-lyase) and inactivating (*N*-acetyltransferase) enzymes was studied in the rat. After intraperitoneal (i.p.) administration of 50 μmol/kg of *N*-(trideuteroacetyl)-labeled mercapturic acids of DCDFE and DBDFE to rats, significant amounts of the dose were excreted unchanged: with DCDFE-NAc, 17% of the dose, and DBDFE-NAc, 31% of the dose. In contrast, the corresponding deuterium-labeled mercapturic acids of TFE and CTFE were excreted unchanged at less than 1% of the dose. With DCDFE-NAc and DBDFE-NAc, also high amounts of unlabeled mercapturic acids were excreted, respectively 48% and 28% of the dose, indicating extensive N-deacetylation followed by reacetylation *in vivo*. Only small amounts (<2%) of unlabeled mercapturic acids were excreted with TFE-NAc and CTFE-NAc. After administration of the cysteine *S*-conjugates DCDFE-Cys and DBDFE-Cys to rats, high amounts of the corresponding mercapturic acids were detected in urine, respectively 57% and 45% of the dose. After administration of TFE-Cys and CTFE-Cys, however, only small amounts were excreted as the corresponding mercapturic acid, approximately 4% of the dose. The strongly different amounts of mercapturic acids in urine may be attributed to the strong differences in N-deacetylation activities which were found in rat renal fractions. The threshold dose of the mercapturic acids to cause nephrotoxicity in male Wistar rats increased in the order: CTFE-NAc (25 μmol/kg) < TFE-NAc (50 μmol/kg) < DCDFE-NAc (75 μmol/kg) <DBDFE-NAc (100 μmol/kg). A higher ratio of N-deacetylation and N-acetylation activities, resulting in a higher availability of cysteine *S*-conjugate, in addition to a higher specific activity of cysteine *S*-conjugate β-lyase, probably explains the higher nephrotoxicity of TFE-NAc and CTFE-NAc when compared to DCDFE-NAc and DBDFE-NAc. The much lower activities of N-deacetylation and β-lyase which are observed in hepatic fractions may explain the lack of hepatotoxicity of the mercapturic acids studied.

Halogenated alkenes, such as tetrafluoroethylene (TFE), chlorotrifluoroelthylene (CTFE), 1,1-dichloro-2,2-difluoroethylene (DCDFE), hexafluoropropene and hexachloro-1,3-butadiene (HCBD), have been shown to produce severe proximal tubular toxicity in kidneys of rodents both after acute exposure and after chronic exposure to low levels [1–3]. The bioactivation mechanism of halogenated alkenes is generally believed to involve initial conjugation to glutathione (GSH) in the liver [4–6]. The rate of GSH-conjugation, therefore, may be an important determinant in the relative nephrotoxicity of fluorinated ethylenes. GSH-conjugation to CTFE by hepatic fractions indeed proceeded at a 10–12 times higher rate than the less potent nephrotoxin TFE [3, 7]. To cause nephrotoxicity, however, GSH-conjugates of halogenated alkenes have to be processed by enzymes present in several tissues, Fig. 1. GSH-conjugates formed in the liver are primarily excreted via the bile in the small intestine [8]. The relatively hydrophilic GSH-conjugates are rapidly degraded by biliary and intestinal γ-glutamyl transpeptidases and dipeptidases to the more lipophilic cysteine *S*-conjugates which may be reabsorbed again from the small intestine. GSH-conjugates released in the general circulation are hydrolysed to the corresponding cysteine *S*-conjugates predominantly by renal peptidases [9]. The cysteine *S*-conjugates are ultimately bioactivated by renal cysteine *S*-conjugate β-lyase (β-lyase) to sulfur containing reactive intermediates which have been shown to bind covalently to DNA and proteins [10, 11], thus being responsible for the toxic effects to the kidney.

Cysteine *S*-conjugates may also be N-acetylated by microsomal cysteine *S*-conjugate *N*-acetyltransferases to mercapturic acids (*N*-acetylcysteine *S*-conjugates; NAc). Many GSH-conjugates are excreted in urine as mercapturic acids [12]. Analysis of mercapturic acids in urine is considered to be a powerful tool in determining exposure to alkylating agents [13]. The relatively high amount of mercapturic acid excreted in urine of rats treated

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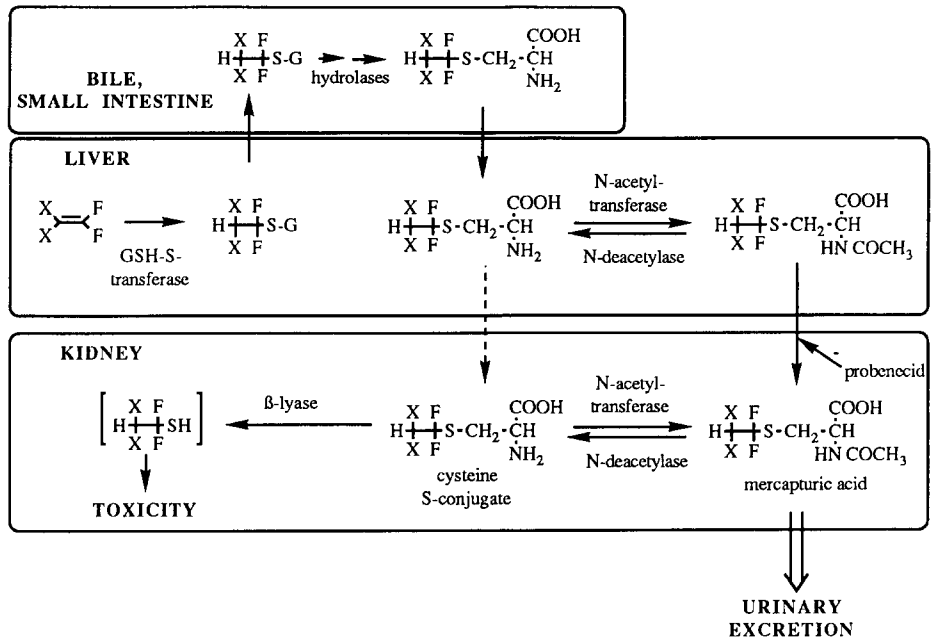

Fig. 1. Interorgan distribution of enzymes involved in the bioactivation mechanism responsible for the nephrotoxicity caused by 1,1-difluoroethylenes (X,X = F, F; Cl, F; Cl, Cl; Br, Br).

with DCDFE, 45–60% of the dose, points to very efficient GSH-conjugation of this highly nephrotoxic fluoroalkene [14]. Since the liver is the major organ to metabolize compounds reabsorbed from the intestine and since the liver has a high *N*-acetyltransferase activity, reabsorbed cysteine *S*-conjugates will be N-acetylated predominantly in this organ [15–18]. The resulting mercapturic acids are transferred to the kidney where they are concentrated by a probenecid-sensitive organic anion transporter [19]. Because mercapturic acids are not substrate for β-lyase, N-acetylation can be regarded as a detoxification pathway. Nevertheless, the mercapturic acids of HCBD [19] and the fluorinated ethylenes TFE, CTFE, DCDFE and 1,1-dibromo-2,2-difluoroethylene (DBDFE) [14, 20] caused severe nephrotoxicity similar to that of the parent compounds. This has been explained by enzymic deacetylation of the mercapturic acids to cysteine *S*-conjugates and subsequent bioactivation by β-lyase [21–23]. Because the nephrotoxicity of hexachloro-1,3-butadiene [19] and *S*-(1,2-dichlorovinyl)-L-cysteine [24] was inhibited *in vivo* by probenecid, which blocks renal uptake of mercapturic acids but not of cysteine *S*-conjugates, Fig. 1, it was suggested that the mercapturic acids rather than the cysteine *S*-conjugate may be the primary agents seen by the kidney *in vivo*.

The involvement of at least five enzymes in the bioactivation mechanism of halogenated alkenes, Fig. 1, implicates that the relative toxicity of the parent fluorinated ethylenes can be determined by differences in metabolism and transport mechanisms at numerous levels. However, by studying metabolism and nephrotoxicity of mercapturic acids as model compounds, variations in GSH-transferase and peptide activities can be eliminated from the extremely complex structure toxicity relationship. Previously, we showed that the relative nephrotoxicity of the mercapturic acids of TFE, DCDFE and DBDFE decreased significantly in the order *N*-acetyl-*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFE-NAc) > *N*-acetyl-*S*-(2,2-dichloro-1,1-difluoroethyl)-L-cysteine (DCDFE-NAc) > *N*-acetyl-*S*-(2,2-dibromo-1,1-difluoroethyl)-L-cysteine (DBDFE-NAc) [20]. The aim of the present study was to investigate the relationship between metabolism and nephrotoxicity of mercapturic acids *in vivo* by analysis of urinary metabolites. To distinguish between unchanged mercapturic acids and mercapturic acids which result from N-deacetylation–N-acetylation cycling, the metabolism of *N*-(trideuteroacetyl)-labeled mercapturic acids was studied. Because the relative nephrotoxicity of CFTE-NAc was not investigated previously, the dose toxicity relationship of this mercapturic acid was also studied *in vivo*. To investigate the role of different enzyme activities in determining the relative toxicity and the organ selectivity of toxicity of these compounds, the activities of *N*-deacetylase, β-lyase and *N*-acetyltransferase were studied *in vitro* using renal and hepatic fractions.

MATERIALS AND METHODS

Chemicals. Acetyl coenzyme A (acetyl CoA) was obtained from Boehringer Mannheim. Acetic anhydride-d_6 was obtained from Aldrich (Brussels, Belgium).

TFE-Cys, CTFE-Cys, DCDFE-Cys and DBDFE-Cys were prepared by the methods described

previously [20, 22]. *N*-(Trideuteroacetyl)-derivates of the mercapturic acids were prepared by acetylating the corresponding cysteine-conjugates with (acetic anhydride)-d6. The cysteine *S*-conjugates (about 250 mg) were dissolved in 5 mL ice-cold 2 N sodium hydroxide and 50 μL portions of (acetic anhydride)-d_6 were added repeatedly while vortexing until the pH of the reaction mixtures decreased to pH < 7. Then the mixtures were allowed to stand at room temperature for 10 min. After acidification of the mixtures to pH 2, the trideuterated mercapturic acids were extracted with ethyl acetate and evaporated to dryness. After crystallization from diethylether/pentane the products were analysed by ^{1}H- and ^{19}F-NMR. The methyl esters formed by treatment with ethereal diazomethane were analysed by GC/MS. The analytical data were consistent with the *N*-trideuteroacetyl-L-cysteine *S*-conjugates: (a) the ^{19}F-NMR spectra were identical to those of the unlabeled mercapturic acids [20, 22]; (b) the ^{1}H-NMR spectra were identical to those of the unlabeled mercapturic acids [20, 22] except for the lack of the CH_3-group of the *N*-acetyl-moiety; (c) the fragmentation pattern of the methyl ester upon electron impact mass spectrometry, was similar to that of the unlabeled derivates [20, 22] except that characteristic key ions for mercapturic acids [25] were shifted due to the presence of a deuterium-label: *m/z* 176, 144 and 88 are replaced by *m/z* 179, 147 and 89.

Metabolism of the cysteine S-conjugates and mercapturic acids in rats. Male Wistar rats (180–200 g), obtained from Harlan–Sprague–Dawley (Zeist, the Netherlands), were housed in plastic cages in temperature (22°) and humidity (50%) controlled rooms equipped with a 12 hr lighting cycle. Food (Hope Farms) and water were provided *ad lib.*

Groups of three rats were injected intraperitoneally (i.p.) with the deuterium-labeled mercapturic acid (NAc-d_3) or the cysteine *S*-conjugate (Cys) of TFE, CTFE, DCDFE or DBDFE dissolved in saline (2.5 mL/kg) at a dose of 50 μmol/kg. After treatment the rats were individually housed in all-glass metabolism cages, designed for separate collection of urine and faeces. Urine was collected 8, 24 and 48 hr after treatment in cooled (0°) vessels.

To quantify mercapturic acids in urine of rats, 0.1 mg DCDFE-NAc (in case of rats treated with cysteine *S*-conjugates or mercapturic acids of TFE, CTFE and DBDFE) or 0.1 mg DBDFE-NAc (in case of rats treated with cysteine *S*-conjugates or mercapturic acids of DCDFE) was added as an internal standard to 5 mL fractions of urine. These fractions were then acidified to pH 2 and extracted twice with 5 mL ethyl acetate. The ethyl acetate phases were combined, concentrated by evaporation, treated with excess of ethereal diazomethane and examined with GC/MS, as described below. Standard curves were obtained by spiking blank urine with known amounts of deuterium-labeled or unlabeled mercapturic acids.

Cysteine-conjugates in urine were analysed according to the method described previously [23]. In short, this method involves N-acetylation of the cysteine *S*-conjugates with acetic anhydride and subsequent analysis of the mercapturic acids formed, as described above.

Metabolism of cysteine S-conjugates and mercapturic acids by renal and hepatic subcellular fractions. Renal and hepatic microsomal and cytosolic fractions were prepared from male Wistar rats according to Wolf *et al.* [26]. Activities of cysteine *S*-conjugate β-lyase were determined by formation of pyruvic acid in incubations of cysteine *S*-conjugates (4 mM) with renal and hepatic cytosol (1 mg/mL) at 37° in 50 mM potassium borate buffer at pH 8.6 and in the presence of 0.1 mM α-keto-γ-methiolbutyric acid. Pyruvic acid was quantified by reversed phase HPLC with fluorescence detection after derivatization with 1,2-phenylene diamine (OPD) [27]. In short, 50 μL samples of the incubation mixtures were taken after 10 min of incubation and treated with 150 μL of 0.13% (w/v) of OPD in 3 N hydrochloric acid. After heating for 30 min at 80°, 200 μL of methanol were added and the mixture was centrifuged for 10 min at 4000 *g* to precipitate denaturated proteins. For separation of OPD-adducts, a RP Lichrosorb 5RP18 column (150 × 4.6 mm) was used with isocratic elution with 60% methanol at a flow rate of 0.4 mL/min. Fluorimetric detection took place at 376 nm excitation and 407 nm emission wave lengths.

Activities of *N*-deacetylase were determined by formation of cysteine *S*-conjugates in incubations of the corresponding mercapturic acids (4 mM) with renal and hepatic cytosol (4 mg/mL) in 50 mM potassium phosphate buffer at pH 7.4, at 37°, and in the presence of 0.2 mM aminooxyacetic acid. Cysteine *S*-conjugates were determined by reversed phase HPLC after derivization with *o*-phtaldialdehyde, as described previously [22].

To assess cysteine *S*-conjugate *N*-acetyl-transferase activities, cysteine *S*-conjugates (4 mM) were incubated with renal and hepatic microsomes (1 mg protein/mL) at 37° in 100 mM potassium phosphate buffer at pH 7.4 and in the presence of 1 mM acetyl CoA. After 0, 5, 10 and 20 min, 0.5 mL samples were added to 0.5 mL 2 N HCl. To these fractions 0.1 mg CTFE-NAc (in incubations with TFE-Cys, DCDFE-Cys and DBDFE-Cys as substrate) or 0.1 mg DCDFE-NAc (in incubations with CTFE-Cys as substrate) was added as an internal standard. Subsequently, the fractions were extracted twice with ethyl acetate, concentrated by evaporation, treated with excess of diazomethane and ultimately examined with GC/MS, as described below.

Analysis of mercapturic acids and cysteine S-conjugates by GC/MS. GC/MS analyses were carried out on a HP 5890/MSD system. A CP Sil SE 30 capillary column (25 m, 0.22 i.d.) obtained from Chrompack B.V. (Middelburg, The Netherlands) was used. The operation temperatures were 280° (split injector and ion source) and electron impact ionization was performed at an electron energy of 70 eV. The column temperature was programmed from 150° (1 min) to 280° at 20°/min. Retention times of the methyl esters of the mercapturic acids under these conditions were: TFE-NAc 4.8 min, CTFE-NAc 6.0 min, DCDFE-NAc 7.0 min and DBDFE-NAc 8.2 min. Mercapturic acids in urine were analysed by selective ion monitoring of ions

Table 1. Excretion of mercapturic acids in urine of rats i.p. treated with the *N*-trideuteroacetyl-labeled mercapturic acids (NAC-d_3) or cysteine conjugates (Cys) of four structurally related fluoroethylenes

	Urinary mercapturic acid 0–24 hr (% of dose)*			
	Dose (μmol/kg)	d_3-Labeled	Unlabeled	Total
TFE-NAc-d_3	50	0.4 ± 0.2	1.6 ± 0.5	2.0 ± 0.4
CTFE-NAc-d_3	50	0.5 ± 0.2	1.0 ± 0.3	1.5 ± 0.4
DCDFE-NAc-d_3	50	17 ± 3	48 ± 5	65 ± 5
DBDFE-NAc-d_3	50	31 ± 4	28 ± 6	59 ± 7
TFE-Cys	50	0	3 ± 1	3 ± 1
CTFE-Cys	50	0	4 ± 2	4 ± 2
DCDFE-Cys	50	0	57 ± 8	57 ± 8
DBDFE-Cys	50	0	48 ± 10	45 ± 10

* Values represent means ± SD of excretion data obtained from three rats. TFE-, *S*-(1,1,2,2-tetrafluoroethyl)-conjugate; CTFE-, *S*-(2-chloro-1,1,2-trifluoroethyl)-conjugate; DCDFE-, *S*-(2,2-dichloro-1,1,-difluoroethyl)-conjugate; DBDFE-, *S*-(2,2-dibromo-1,1,-difluoroethyl)-conjugate.

characteristic for deuterium-labeled (*m/z* 147 and 179) and unlabeled mercapturic acids (*m/z* 144 and 176). The mass spectra of deuterium-labeled mercapturic acid methyl esters do not contain the fragments *m/z* 144 and 176, while the unlabeled compounds do not contain *m/z* 147 and 179, thus allowing simultaneous detection of both unlabeled and labeled mercapturic acids.

Assessment of liver and kidney toxicity of CTFE-NAc in rats. To determine the relative nephrotoxicity of CTFE-NAc, groups of four rats were injected i.p. with CTFE-NAc, dissolved in saline (2.5 mL/kg), at doses of 10, 25 and 50 μmol/kg. Control animals received vehiculum only. After treatment, the rats were individually housed in all-glass metabolism cages, designed for separate collection of urine and faeces. Urine was collected for 48 hr in portions of 24 hr in cooled (0°) vessels. Blood was collected by heart puncture 48 hr after treatment and centrifuged (4000 *g*, 10 min) subsequently to obtain plasma. The animals were decapitated and their kidneys were removed for measurement of kidney-to-body weight ratios. For biochemical assessment of toxicity, plasma urea, plasma alanine aminotransferase (ALT), urinary protein and urinary glucose were determined as described previously [24]. Statistical significance of differences in plasma and urinary parameters was calculated using the two-tailed Student's *t*-test.

RESULTS

In vivo *metabolism of mercapturic acids and cysteine S-conjugates*

After i.p. treatment of rats with deuterium-labeled mercapturic acids, for all compounds tested both unchanged (deuterium-labeled) and unlabeled mercapturic acids were detected in urine, Table 1. The main fraction (>90%) was excreted within 8 hr following treatment of the rats (data not shown). Only traces of mercapturic acids were detected on urine collected in the 24–48 hr period following treatment.

Both TFE-NAc-d_3 and CTFE-NAc-d_3 were excreted unchanged only at 0.4 ± 0.2% and 0.5 ± 0.2% of the dose respectively, indicating very efficient metabolism of these compounds. The appearance of unlabeled TFE-NAc and CTFE-NAc, 1.6 ± 0.5% and 1.0 ± 0.3% of the dose respectively, indicates that deacetylation to the corresponding cysteine *S*-conjugates, followed by reacetylation occurred *in vivo*.

DCDFE-NAc-d_3 and DBDFE-NAc-d_3 appeared to be excreted unchanged at significantly higher amounts, 17 ± 3% and 31 ± 4% of the dose respectively, in 24 hr urine, Table 1. This points to less efficient metabolism of these mercapturates when compared to TFE-NAc-d_3 and CTFE-NAc-d_3. Next to unchanged mercapturic acids, also relatively high amounts of unlabeled mercapturic acid of DCDFE and DBDFE were present in 24 hr urine, 48 ± 5% and 28 ± 6% of the dose, respectively. These results indicate that deacetylation to the cysteine *S*-conjugates followed by reacetylation are quantitatively important routes of metabolism for these mercapturates.

When rats were treated with TFE-Cys and CTFE-Cys also very low amounts, 3 ± 1% and 4 ± 2% of the dose respectively, were excreted in urine as the corresponding mercapturic acids, Table 1. Treatment with DCDFE-Cys and DBDFE-Cys, however, resulted in much higher excretion of mercapturic acid, 57 ± 8% and 45 ± 10% of the dose respectively, Table 1. For all compounds tested, excretion of cysteine *S*-conjugates in urine was found to be negligible (data not shown). By using direct ^{19}F-NMR analysis of the 24 hr urine fractions, the only *S*-conjugates measured in urine were the mercapturic acids; the presence of *S*-oxides and *S*-thiolactic acid conjugates could not be detected (data not shown).

Metabolism of cysteine S-conjugates and mercapturic acids of fluorinated ethylenes by renal and hepatic subcellular fractions

The activities of renal and hepatic cytosolic

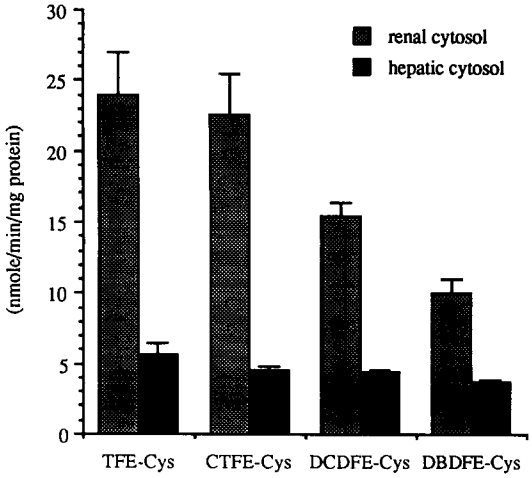

Fig. 2. Activity of cysteine conjugate β-lyase. Cysteine conjugates (4 mM) of 1,1-difluoroethylenes were incubated in potassium borate buffer pH 8.6 at 37°, in the presence of 0.1 mM α-keto-γ-methiolbutyrate and renal or hepatic cytosol (1 mg protein/mL). β-Lyase activity was assessed by measuring the amount of pyruvate formed after 10 min of incubation.

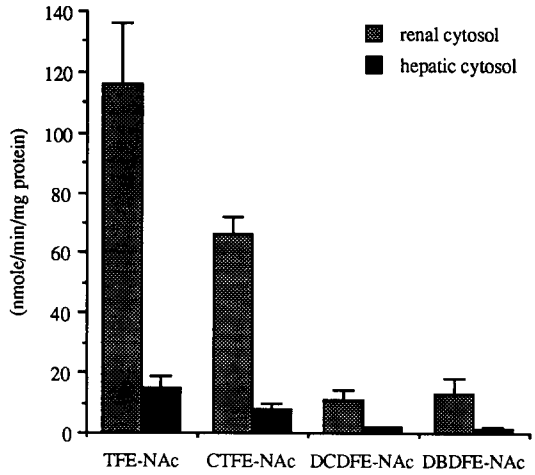

Fig. 3. Activity of *N*-deacetylase. Mercapturic acids (4 mM) of 1,1-difluoroethylenes were incubated in potassium phosphate buffer pH 7.4 at 37°, in presence of 0.2 mM aminooxyacetic acid and renal or hepatic cytosol (4 mg protein/mL). *N*-Deacetylase activity was assessed by measuring the amount of cysteine conjugate formed after 5 min of incubation.

cysteine *S*-conjugate β-lyase towards the cysteine *S*-conjugates of the four fluorinated ethylenes are shown in Fig. 2. Specific activities (nmol/min/mg protein) of β-lyase in renal cytosol decreased in the order (means ± standard deviation, N = 3): TFE-Cys (24 ± 3) ≈ CTFE-Cys (23 ± 3) > DCDFE-Cys (15 ± 1) > DBDFE-Cys (10 ± 1). Specific activities of β-lyase in hepatic cytosol were 3–5-fold lower when compared to the corresponding activities in renal cytosol; a similar structure activity relationship was observed: TFE-Cys (5.7 ± 0.8) > CTFE-Cys (4.6 ± 0.1) > DCDFE-Cys (4.4 ± 0.1) > DBDFE-Cys (3.7 ± 0.1).

The specific activities of *N*-deacetylase towards the *N*-acetylcysteine *S*-conjugates of the four flourinated ethylenes in renal and hepatic cytosol are shown in Fig. 3. Specific activities (nmol/min/mg protein) of *N*-deacetylase in renal cytosol decreased in the order (means ± SD, N = 3): TFE-NAc (116 ± 20) > CTFE-Cys (66 ± 6) ≫ DCDFE-Cys (11 ± 3) ≈ DBDFE-Cys (13 ± 5). Specific activities in hepatic cytosol showed a similar structure activity relationship, specific activities however were 7–9 times lower when compared to the corresponding activities in renal cytosol: TFE-NAc (15 ± 4) > CTFE-Cys (8 ± 2) ≫ DCDFE-Cys (1.7 ± 0.3) ≈ DBDFE-Cys (1.5 ± 0.4).

No significant differences were observed between the rates of N-deacetylation of trideuterium-labeled mercapturic acids and the corresponding unlabeled mercapturic acids (data not shown).

Figure 4 shows the specific activities of rat kidney and liver microsomal enzymes for N-acetylation of the cysteine *S*-conjugates of the four fluorinated ethylenes investigated. Formation of mercapturic acid was linear for at least 10 min. In absence of microsomes or cofactor acetyl coenzyme A, formation of mercapturic acids did not occur. The

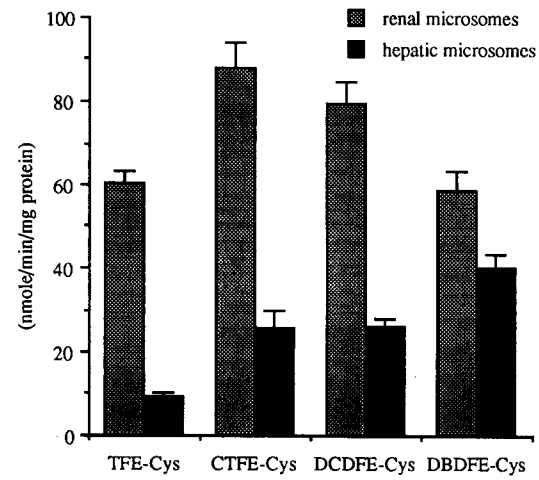

Fig. 4. Activity of *N*-acetyltransferase. Cysteine conjugates (4 mM) were incubated in potassium phosphate buffer pH 7.4 at 37°, in presence of 1 mM acetyl-CoA and renal or hepatic microsomes (1 mg protein/mL). *N*-Acetyltransferase activity was assessed by measuring the amount of mercapturic acid formed after 10 min of incubation.

specific activities for all conjugates tested were higher in renal microsomes when compared to the corresponding activities in hepatic microsomes, Fig. 4. In renal microsomes, the highest rate of N-acetylation was observed with CTFE-Cys (88 ± 6 nmol/min/mg protein) and DCDFE-Cys (80 ± 5) as substrates, while both TFE-Cys (60 ± 3) and DBDFE-Cys (58 ± 5) were N-acetylated at a slightly but significantly lower rate. In hepatic

Table 2. Dose dependence of nephro- and hepatotoxicity of the *N*-acetylcysteine conjugate of chlorotrifluoroethylene (CTFE-NAc) in the rat

	Dose of CTFE-NAc (μmol/kg)*			
	0	10	25	50
Plasma urea (mg %)	25 ± 15	15 ± 10	120 ± 50†	350 ± 100†
Urine glucose (mg/24 hr)	3 ± 2	2 ± 2	110 ± 40†	80 ± 35†
Urine protein (mg/24 hr)	12 ± 2	15 ± 5	80 ± 35†	48 ± 20†
Kidney : body wt (× 100)	0.83 ± 0.04	0.85 ± 0.04	0.92 ± 0.04†	1.05 ± 0.07†
Plasma ALT (units/L)	15 ± 2	10 ± 4	16 ± 3	16 ± 3

* Values are means ± SD of groups of four rats. Biochemical parameters were measured 48 hr after treatment with CTFE-NAc.

† Denotes statistically significant differences from control rats ($P < 0.01$, Student's *t*-test).

microsomes, however, the rate of N-acetylation increased in the order TFE-Cys (10 ± 1) < CTFE-Cys (26 ± 4) ≈ DCDFE-Cys (26 ± 2) < DBDFE-Cys (40 ± 3). In incubations with the corresponding mercapturic acids, N-deacetylation was observed neither with kidney nor liver microsomal fractions (data not shown).

Toxicity of CTFE-NAc in vivo

The effects of different i.p. doses of CTFE-NAc on various blood and urinary parameters, indicative for nephrotoxicity and hepatotoxicity, are shown in Table 2. The significant elevation of plasma urea, urine glucose, urine protein and relative kidney weight at doses equal to and higher than 25 μmol/kg all point to a strong nephrotoxicity. Histological examination of the kidneys prepared 48 hr after treatment also showed a necrotic band in the region of the inner cortex. Plasma ALT activities were not elevated at all doses, indicating no toxic effect of this mercapturic acid on the liver.

DISCUSSION

The metabolism of the cysteine conjugates and the mercapturic acids of four structurally related 1,1-difluoroalkenes was studied both *in vivo* and *in vitro*, in order to explain the relative differences in nephrotoxicity caused by these *S*-conjugates. Previously, we showed that the threshold dose necessary to cause nephrotoxicity increased in the order: TFE-NAc, 50 μmol/kg < DCDFE-NAc, 75 μmol/kg < DBDFE-NAc, 100 μmol/kg [20]. The additional toxicity data presented in the present study show that CTFE-NAc, causing nephrotoxicity at a dose as low as 25 μmol/kg, Table 2, is even significantly more nephrotoxic than TFE-NAc. Different factors may play a role in determining the relative toxicity of these mercapturic acids: biotransformation, transport mechanisms or nature of reactive intermediates.

In the present study, major differences in the metabolism of the mercapturic acids and cysteine *S*-conjugates of TFE, CTFE, DCDFE and DBDFE could be observed *in vivo*, Table 1. By administration of *N*-trideuteroacetyl-labeled mercapturic acids, excretion of unchanged mercapturic acid could be distinguished from excretion of mercapturic acid resulting from deacetylation/reacetylation cycling, Fig. 1. DCDFE-NAc and DBDFE-NAc both appeared to be excreted unchanged at relatively much higher amounts (17–30% of the dose) than TFE-NAc and CTFE-NAc (0.4–0.5% of the dose). The relatively high amounts of unchanged DCDFE-NAc and DBDFE-NAc in urine most likely can be explained by the much lower activities of renal and hepatic *N*-deacetylases; the rates of deacetylation of these two mercapturic acids by renal and hepatic subcellular fractions were 10–12-fold lower when compared to the rates of deacetylation of TFE-NAc and CTFE-NAc, Fig. 3. Alternatively, higher rates of urinary excretion of DCDFE-NAc and DBDFE-NAc may also play a role; however, no experimental data of excretion rates are available.

Although only traces of cysteine *S*-conjugates could be detected in urine, deacetylation appears to be an important route of metabolism of the deutero-labeled mercapturic acids *in vivo*. The occurrence of significant amounts of unlabeled mercapturic acids in urine of rats treated with DCDFE-NAc-d_3 and DBDFE-NAc-d_3, Table 1, points to deacetylation to the cysteine *S*-conjugates followed by reacetylation. When comparing the amounts of mercapturic acids in urine of rats treated with DCDFE-NAc-d_3 and DBDFE-NAc-d_3, the total amount of mercapturic acid excreted in urine was comparable, 65 vs 59% of the dose. With the *S*-conjugates of DCDFE and DBDFE as substrate, the activities of *N*-acetyltransferase and *N*-deacetylase were comparable when measure *in vitro* (Figs 3 and 4). However, after treatment with DCDFE-NAc-d_3 a significantly higher ratio of unlabeled vs labeled mercapturic acid in urine was observed than after treatment with DBDFE-NAc-d_3, Table 1. The reason for the discrepancy between these *in vitro* and *in vivo* data remains to be established.

When comparing the excretion of mercapturic acids in urine of rats treated with the corresponding cysteine *S*-conjugates, it appears that DCDFE-Cys and DBDFE-Cys are N-acetylated to a much higher extent than TFE-Cys and CTFE-Cys, Table 1. The relative amount of mercapturic acids excreted is determined by the activity of *N*-acetyltransferases as well as by the activity of the competing enzymes such as *N*-deacetylase, β-lyase, cysteine *S*-conjugate transaminases, L-amino oxidases and *S*-sulfoxidases.

All cysteine S-conjugates tested were actively N-acetylated by hepatic as well as by renal microsomal fractions, Fig. 4, the renal fractions having higher specific activities than the hepatic fractions, similarly to previous results with different cysteine S-conjugates [18, 28]. Activity of N-acetyltransferase towards CTFE-Cys and DCDFE-Cys was comparable in both renal and hepatic microsomes, Fig. 4. However, the relative amount of excretion of CTFE-NAc was much lower than that of DCDFE-NAc, 4% vs 57% of the dose respectively, Table 1. This difference most likely results from the large difference in N-deacetylation activities; CTFE-NAc is N-deacetylated at a 5–6-fold higher rate than DCDFE-NAc both in hepatic and renal reactions, Fig. 3.

Differences in activities of cytosolic cysteine S-conjugate β-lyase, which competes with N-acetyltransferase for cysteine S-conjugate, were much smaller than differences in N-deacetylation activities, Fig. 2, and therefore probably play a less prominent role in the highly different excretion patterns. β-Lyase activity has also been demonstrated in mitochondrial fractions [29–31]. With CTFE-Cys as substrate, β-lyase activity appears to be equally distributed between cytosol and mitochondria [29]. β-Lyase activity with TFE-Cys as substrate is comparable to that with CTFE-Cys as substrate, both in cytosol (Fig. 3) and in mitochondria [31]. Activity of β-lyase with TFE-Cys as substrate, therefore, is probably also equally distributed between cytosol and mitochondria. Whether the β-lyase activity towards the other cysteine-conjugates used in this study, DCDFE-Cys and DBDFE-Cys, has a similar organelle-distribution, is presently under investigation. Because mitochondria have been proposed as primary targets of toxicity [29–31], mitochondrial β-lyase activity may be more important in the onset of toxicity than the cytosolic enzyme. However, at present the relative contribution of the cytosolic and mitochondrial β-lyase activities to toxicity remains to be established.

Taken together, the *in vitro* and *in vivo* results indicate that the higher activity of the activating enzymes β-lyase and, in particular, N-deacetylase may be important factors in determining the lower amounts of excretion as well as the higher nephrotoxic potential of TFE-NAc and CTFE-NAc, when compared to DCDFE-NAc and DBDFE-NAc.

CTFE-NAc appears to be a more potent nephrotoxin than TFE-NAc. However, activity of the activating enzyme N-deacetylase as well as β-lyase was higher towards the TFE-derived S-conjugates when compared to the CTFE-derived conjugates, Figs 2 and 3. In addition, the activity of the deactivating enzyme N-acetyltransferase is significantly lower towards TFE-Cys, Fig. 4. An explanation may be a higher activity of alternative deactivation pathways for TFE-Cys, such as deamination reactions by cysteine S-conjugate transaminases [32] and amino acid oxidases [33]. However, the activities of deamination of TFE-Cys and CTFE-Cys by hepatic and renal reactions were very low (unpublished results). Alternatively, differences in S-oxidation by renal and hepatic microsomal S-oxygenases may play a role [34]; the sulfoxides of the cysteine S-conjugates and mercapturic acids of the four fluorinated ethylenes, however, can not be observed in urine after ^{19}F-NMR analysis (data not shown).

Another explanation of the higher toxicity of CTFE-NAc may be the formation of a more toxic reactive intermediate by β-lyase-catalysed cleavage of the cysteine S-conjugates. By Green and Odum [35], the toxicity of fluorinated cysteine S-conjugates was attributed to the formation of thiol compounds whose sulfur atoms would have electrophilic properties due to the electron-withdrawing activity of the fluorine atoms. However, it was recently demonstrated that TFE-Cys is bioactivated to high amounts of electrophilic thioacylating reactive intermediates, probably thionoacyl fluorides, with high reactivity towards primary amines [23]. CTFE-Cys has been shown to be bioactivated to corresponding thioacylating species [36], however, at much lower amounts when compared to TFE-Cys ([20], Commandeur *et al.*, manuscript in preparation). For the thiol-compound derived from CTFE-Cys, the formation of a thiirane compound seems to be favoured over formation of a thioacyl fluoride (Commandeur *et al.*, manuscript in preparation). Recently, it was shown that covalent binding to mitochondria, presumably the primary targets of nephrotoxic cysteine S-conjugates, was only slightly higher with CTFE-Cys than with TFE-Cys [31]. With CTFE-Cys a high fraction of covalent binding had disulfide character, in contrast to covalent binding of TFE-Cys which was relatively insensitive to dithiothreitol [31]. These data support the hypothesis that the higher toxicity of CTFE-NAc when compared to TFE-NAc, may in part be attributed to formation of different types of reactive intermediates. As a consequence of this, the cellular targets for covalent binding may differ between the different nephrotoxic cysteine S-conjugates [37].

In conclusion, the results of the present study indicate that next to bioactivation by cysteine S-conjugate β-lyase, the balance of N-deacetylation and reacetylation also plays an important role in determining the relative nephrotoxicity of the mercapturic acids of four structurally related fluorinated ethylenes in rats. The relative nephrotoxicity decreased in the order CTFE-NAc > TFE-NAc > DCDFE-NAc > DBDFE-NAc. The higher degree of N-deacetylation, as seen with the more potent nephrotoxins TFE-NAc and CTFE-NAc may result in a higher availability of the corresponding cysteine S-conjugates to the bioactivating enzyme cysteine S-conjugate β-lyase in the kidney. The activity of β-lyase was higher towards the more potent nephrotoxic S-conjugates. Formation of reactive intermediates with different reactivities toward cellular constituent, however, may also contribute to the observed differences in relative toxicity.

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