Metabolic products and pathways of fluorotelomer alcohols in isolated rat hepatocytes

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Introduction

Fluorotelomer alcohols (FTOHs; $CF_3(CF_2)_xC_2H_4OH$; where x=3, 5, 7, 9) are a novel class of polyfluorinated contaminants detected in the North American atmosphere[1] and are possible precursors to the series of perfluoroalkyl carboxylates (PFCAs) in human blood[2,3]. Although the magnitude of human exposure to FTOHs has not been assessed, their widespread distribution in ambient air warranted a comprehensive examination of their metabolic fate. Using 8:2 FTOH (i.e. where x=7) as a model compound, the metabolic products formed by isolated rat hepatocytes were identified, and three synthesized intermediates were incubated separately to elucidate the metabolic pathways.

Materials and Methods

Chemicals. 4:2 (97%), 6:2 (97%), 8:2 (97%), and 10:2 (97%) FTOH were purchased from Oakwood Products, Inc. (West Columbia, SC). The major impurity of 8:2 FTOH was the allylic alcohol ($CF_3(CF_2)_6CF=CHCH_2OH$) [4], and all FTOHs were used without further purification. Other fluorochemical standards, including 8:2 fluorotelomer carboxylic acid (8:2 FTCA), 8:2 a/b-unsaturated fluorotelomer acid (8:2 FTUCA) and fluorotelomer aldehyde (8:2 FTAL) were synthesized in our laboratory according to methods described elsewhere [4], and their purities were all >95% [4]. The 8:2 a/b-unsaturated fluorotelomer aldehyde (8:2 FTUAL) was also synthesized in this study for spectral comparison to a metabolite but was not purified or used for pathway elucidation. A small portion of this impure 8:2 FTUAL material was added to a 10 mL saturated solution of Na_2CO_3 containing 200 mg of GSH and left to react overnight to form the GSH conjugate. An authentic standard of the 2,4-dinitrophenylhydrazine (DNPH) derivative of 8:2 FTAL was prepared by combining 1 mL of 0.018 M 8:2 FTAL in methanol with 0.9 mL of 0.018 M DNPH in hydrochloric acid

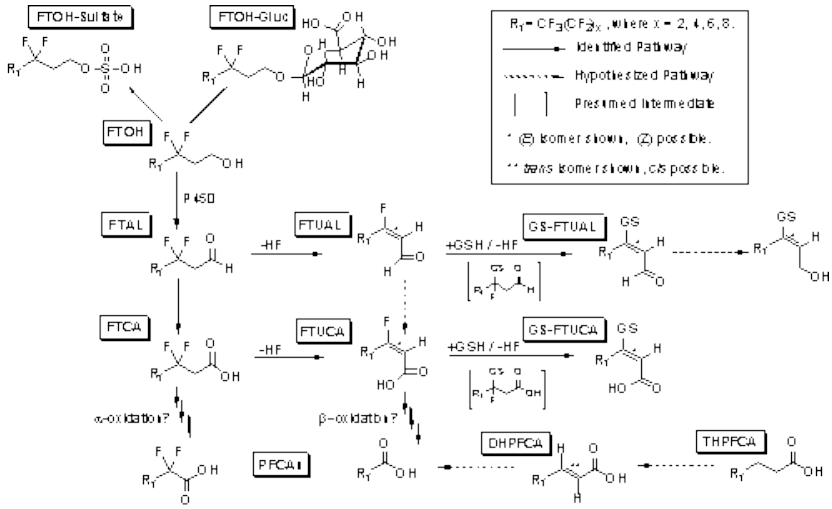
***In Vitro* Hepatocyte Studies.** Hepatocytes were isolated from rat liver perfused with collagenase as described previously by Moldéus *et al*. [5]. Isolated hepatocytes were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES (10 mL, 10^6 hepatocytes mL^{-1}) in continuously rotating round-bottomed 50 mL flasks and incubated in a water bath at 37°C. A preincubation period of 1 hr followed the addition of enzyme inhibitors before test compounds were added to the suspensions.

Test substances used in pathway elucidation included 8:2 FTOH, 8:2 FTAL, 8:2 FTCA, 8:2 FTUCA (Scheme 1), perfluorooctanoic acid (PFOA), and perfluorononanoic acid (PFNA). The concentrations of test substances used in suspensions ranged from 20-200 µM, and a control incubate was used on every day to monitor baseline cell toxicity and contamination with background perfluoroalkyl contaminants. No toxicity was observed at these concentrations for any chemical, as determined by Trypan Blue exclusion, in the experimental time frame (2-4 hrs).

Sample Preparation. Acid metabolites were ion-pair extracted from blood, liver, kidney, or hepatocytes using tetrabutyl ammonium hydrogen sulfate as previously described [6]. Glucuronide and sulfate conjugates were detected by the same procedure, and also by 1:1 addition of methanol to an aliquot of the hepatocyte suspension, followed by centrifugation and filtration. GSH conjugates were examined for by extraction of the hepatocyte suspension with acetonitrile, and subsequent filtration before HPLC/MS/MS analysis. Aldehydes were derivatized to DNPH.

HPLC/MS/MS Identification of 8:2 FTOH and Metabolites. All compounds were identified using reversed phase chromatography on a Genesis C8 column (2.1 x 50mm, Jones Chromatography, Lakewood, CO, USA) and mass spectral detection using a Micro LC (Micromass, Manchester, UK) triple quadrupole mass spectrometer equipped with an electrospray source operating in negative ion mode. Data was acquired in full scan MS mode, or in MS/MS mode (daughter scan, parent scan, or multiple reaction monitoring).

Results



Scheme 1. Generalized FTOH metabolic products and pathways in isolated rat hepatocytes. GSH conjugates, THPFCA, and DHPFCA were only examined in 8:2 FTOH incubates but are shown as generalized structures.

Based on the observed metabolites and conjugates of FTOHs, and metabolites observed in incubations with synthesized intermediates, the metabolic pathways for FTOHs are summarized in Scheme 1.

For 8:2 FTOH a major fate was direct conjugation to form the *O*-glucuronide and *O*-sulfate. Identification was based on observation of the expected molecular ion in full scan MS, and diagnostic interpretation of their product ion spectra (Fig 2). For the 8:2 FTOH-glucuronide, the expected molecular ion appeared at *m/z* 639 (i.e. $[M-H^+]^-$) and yielded product ions corresponding to glucuronate (*m/z* 193) and its dehydrate (*m/z* 175). For the sulfate, the expected pseudomolecular ion was detected at *m/z* 543 and yielded an abundant product ion at *m/z* 97, corresponding to sulfate. As with most polyfluorinated metabolites, a neutral loss of 20 was apparent in the product spectra of the glucuronide and sulfate at *m/z* 619 and 523, respectively, corresponding to neutral loss of HF in both instances. The corresponding FTOH-sulfate for each FTOH was detected and confirmed by MS/MS in separate hepatocyte incubations, whereas the FTOH-glucuronide was only confirmed in 4:2, 6:2, and 8:2 FTOH incubations. Using DNPH trapping, the immediate oxidation product of 8:2 FTOH was identified as 8:2 FTAL (Scheme 1). A precursor scan experiment for *m/z* 163 in extracts of hepatocytes incubated with 8:2 FTOH revealed two distinct chromatographic peaks corresponding to precursor ions at *m/z* 641 and 621, respectively. Neither peak was present in control hepatocytes derivatized with DNPH. The first of these peaks at *m/z* 641 corresponded to the mass of the expected 8:2 FTAL derivative, and produced a product spectrum that matched the authentic standard product spectrum. The later eluting peak at *m/z* 621 produced a product ion spectrum that was very similar to 8:2 FTAL, except that only one neutral loss of HF was evident for the deprotonated molecular ion. Although no authentic standard was available for comparison, the overall mass spectral evidence strongly suggested that this metabolite was 8:2 FTUAL (Scheme 1). 8:2 FTAL was transient and eliminated HF non-enzymatically to yield 8:2 FTUAL which was also short-lived and reacted GSH and perhaps other endogenous nucleophiles.

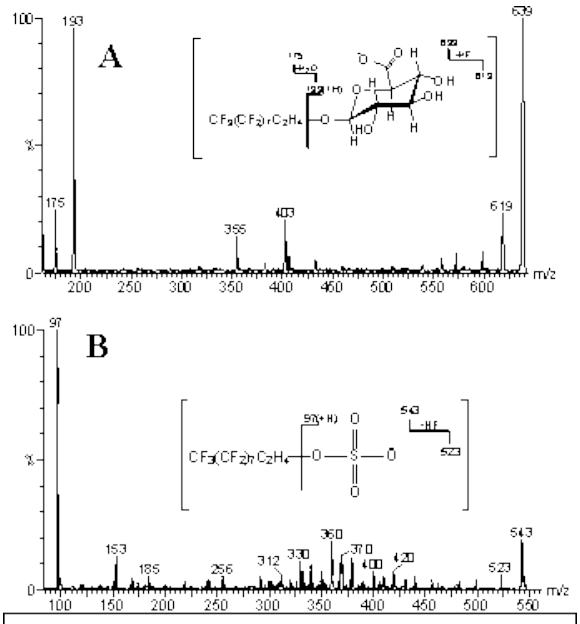


Figure 4. Product spectra of metabolites detected at *m/z* 639 and 543 in hepatocytes incubated with 8:2 FTOH. Products were identified as the (A) *O*-glucuronide, and (B) *O*-sulfate, respectively.

Three GSH conjugates were detected in isolated rat hepatocytes incubated with 8:2 FTOH. Their identification as GSH conjugates was based on precursor ion scans for *m/z* 306, 272, and 254, which are highly specific and diagnostic ions produced by dissociation of the GSH moiety. This approach identified the precursors ions at *m/z* 728, 744, and 730 as GSH conjugates, and interpretation of their product spectra suggested that *m/z* 728 and 744 were the dehydrofluorinated 1,4 addition products of both unsaturated metabolites: GS-FTUAL and GS-FTUCA, respectively. A product having a *m/z* of 728 was synthesized by reacting an authentic (yet impure) standard of 8:2 FTUAL with GSH in sodium bicarbonate buffer, and its product spectrum matched the spectrum for the metabolite detected in hepatocytes at m/z 728. GS-FTUCA was also observed in an incubation of 8:2 FTCA, whereas GS-FTUAL was not observed. There was no spectral evidence for any terminal-carbon GSH addition products. Based on product spectrum interpretation the third GSH conjugate (*m/z* 730) was determined to be the reduced alcohol product of GS-8:2 FTUAL.

Four polyfluorinated acid intermediates were also detected, including 8:2 FTCA, 8:2 FTUCA (Scheme 1), tetrahydroperfluorodecanoate ($CF_3(CF_2)_6(CH_2)_2CO_2^-$; THPFCA), and dihydroperfluorodecanoate ($CF_3(CF_2)_6CH{=}CHCO_2^-$; DHPFCA). The pathways leading to 8:2 FTCA and FTUCA involve oxidation of 8:2 FTAL, however the pathways leading to the latter two polyfluorinated acids remain inconclusive. The fate of the unsaturated metabolites, 8:2 FTUAL and FTUCA, included conjugation with GSH and dehydrofluorination to yield α/β unsaturated GSH conjugates, and GS-8:2 FTUAL was reduced to the corresponding alcohol. PFOA and minor amounts of PFNA were confirmed as metabolites of 8:2 FTOH. The analogous acids, aldehydes, and conjugated metabolites of 4:2, 6:2, and 10:2 FTOH (i.e. where x=3, 5, and 9, respectively) were also detected, and metabolite profiles among FTOHs generally differed only in the length of their perfluoroalkyl chains. Preincubation with aminobenzotriazole (1mM), but not pyrazole (100 µM or 1mM), inhibited the formation of metabolites from all FTOHs, suggesting that their oxidation was catalyzed by a P450 isozyme, not alcohol dehydrogenase.

Although significant, a low molar balance for the quantifiable acid metabolites, including PFOA, indicates that they are not the major metabolic fate of FTOHs in rat hepatocytes. The unaccounted molar balance is at least partially explained by the five novel conjugates (3 GSH, 1 glucuronide, and 1 sulfate), and to a lesser extent by DHPFCA and THPFCA. Although we synthesized two polyfluorinated acids to arrive at the partial mass-balance presented here, we were unable to quantify these additional unexpected metabolites due to a general lack of commercially available chemical standards and/or appropriate synthetic starting material, including pure 8:2 FTUAL (a small amount of impure 8:2 FTUAL was generated and the corresponding GSH was synthesized for spectral matching, but this could not be purified to allow quantification). The quantifiable molar balance in hepatocyte incubations of polyfluorinated acids (8:2 FTCA and 8:2 FTUCA) are reasonable (>80%), whereas the quantifiable molar balance in incubations of either 8:2 FTOH or 8:2 FTAL are poor (i.e. less than 10% in both circumstances). While it is possible that these five novel conjugates may explain the unaccounted molar balance, as we argue later on, future studies should also consider non-specific reactions of the electrophilic metabolites (particularly 8:2 FTUAL) with endogenous biological macromolecules or other common cellular nucleophiles.

The two α/β unsaturated metabolites are electrophilic substances based on the observation of their dehydrofluorinated GSH conjugates: GS-8:2 FTUAL and GS-8:2 FTUCA. Observation of GS-8:2 FTUCA in an incubation of 8:2 FTCA demonstrated that GSH reacted directly with the unsaturated acid, and that this was not an oxidation product of GS-8:2FTUAL. Two resonance structures can be drawn for each unsaturated metabolite, whereby the electrophilic centre can be situated on either the β- or carbonyl-carbon. We did not observe a GSH conjugate corresponding to reaction at the carbonyl-carbon, as sometimes occurs when strongly electron withdrawing groups are adjacent to the b-carbon such as two trifluoromethyl groups [7]. For addition of GSH, the overall effect of the

β-carbon fluorine and adjacent C_7F_{15} moiety seems to be a strong potentiation of the β-carbon centred electrophile, and thus 1,4 addition. Conjugation of these electrophilic species to GSH probably aids their biliary excretion *in vivo*. The identification of these GSH conjugates was complicated because of the unexpected dehydrofluorination of the 1,4 addition product. Non-dehydrofluorinated conjugates were not detected in hepatocytes, nor in the synthesized material, suggesting that the 1,4 addition product is unstable. This may be of important toxicological consequence, because the resulting dehydrofluorinated GSH conjugates are themselves α/β unsaturated aldehydes that could, theoretically, react with a second nucleophile.

This work has demonstrated that FTOHs can be metabolized to PFCAs of various chain-lengths, depending on the starting chain-length, and thus exposure to FTOHs is a feasible explanation for the occurrence of long-chain PFCAs in human blood (e.g. 8:2 FTOH, 10:2 FTOH, and possibly 12:2 FTOH). However, the magnitude of human exposure to FTOHs is unknown and only future air monitoring efforts will determine if FTOH exposure can account for the low ng/mL PFCA concentrations in human blood [2]. Given the electrophilic metabolites identified in this study, further metabolic and toxicological investigations are warranted for FTOHs.

Acknowledgements

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