Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 1407–1410, February 1991
Medical Sciences

Tissue acylation by the chlorofluorocarbon substitute 2,2-dichloro-1,1,1-trifluoroethane

(hepatic metabolism/hydrochlorofluorocarbons/trifluoroacetylated proteins/neoantigens/^{19}F NMR)

JAMES W. HARRIS*, LANCE R. POHL†, JACKIE L. MARTIN†‡, AND M. W. ANDERS*§

*Department of Pharmacology, University of Rochester, Rochester, NY 14642; †Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; and ‡Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD 21205

Communicated by Gerald N. Wogan, November 14, 1990

ABSTRACT **Hydrochlorofluorocarbons (HCFCs) are being developed as substitutes for ozone-depleting chlorofluorocarbons (CFCs); because widespread human exposure to HCFCs may be expected, it is important to evaluate their toxicities thoroughly. Here we report studies on the bioactivation of the CFC substitute 2,2-dichloro-1,1,1-trifluoroethane (HCFC-123) to an electrophilic intermediate that reacts covalently with liver proteins. HCFC-123 and its analog halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) were studied in rats by ^{19}F NMR spectroscopy, and we found that a trifluoroacetylated lysine adduct was formed with liver proteins. Also, the pattern of proteins immunoreactive with hapten-specific anti-trifluoroacetylprotein antibodies was identical in livers of HCFC-123- and halothane-exposed rats. Because halothane causes an idiosyncratic, and sometimes fatal, hepatitis that is associated with an immune response against several trifluoroacetylated liver proteins, the present findings raise the possibility that humans exposed to HCFC-123 or structurally related HCFCs may be at risk of developing an immunologically mediated hepatitis.**

Chlorofluorocarbons (CFCs) deplete stratospheric ozone (1), thus increasing the amount of ultraviolet radiation that reaches the earth's surface. Ozone destruction may lead to global, adverse human health effects, including increases in cataract formation (2) and in the incidence of skin cancer (3). Restrictions imposed by The Montreal Protocol on Substances That Deplete the Ozone Layer and by Environmental Protection Agency regulations mandate the urgent development of CFC substitutes (4–6). Hydrochlorofluorocarbons (HCFCs), which have little ozone-depleting potential because they degrade in the troposphere (7, 8), are being developed as substitutes for CFCs currently used as refrigerants, solvents, foam-blowing agents, and, in some countries, aerosol propellants (4–6). 2,2-Dichloro-1,1,1-trifluoroethane (HCFC-123) is a replacement for trichlorofluoromethane (CFC-11) and 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113) (6), whose 1986 U.S. production amounted to 90,000 and 79,000 metric tons, respectively (9). Although the toxicities of HCFCs have not been fully evaluated (5, 10), a preliminary report indicates that HCFC-123 alters lipid concentrations in rats (11).

The HCFC-123 analog halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is metabolized to reactive intermediates that bind irreversibly to liver proteins (12, 13). In susceptible individuals, halothane may cause a fulminant and sometimes fatal hepatitis that is associated with an immune response against neoantigens (14, 15) produced by trifluoroacetylation of liver proteins (16, 17). We now report the identification of the protein adduct formed during HCFC-123 and halothane bioactivation as N^{ϵ}-trifluoroacetyllysine by use of ^{19}F nuclear magnetic resonance (NMR) spectroscopy. Furthermore, we show by immunoblotting the presence of identical N^{ϵ}-trifluoroacetyllysine-containing proteins (TFA-proteins) in the livers of HCFC-123- and halothane-exposed rats. Hence individuals exposed to HCFC-123 may also be at risk of developing an idiosyncratic hepatitis that is unlikely to be detected in animal toxicity studies.

MATERIALS AND METHODS

Instrumental Analyses. NMR spectra were obtained with a Bruker WP-270 instrument equipped with a dedicated 5-mm ^{19}F probe and operating at 254.18 MHz for fluorine. The pulse width was 3 μsec and the interpulse time was 0.7 sec for covalent binding assays (spectral width = 50 kHz). Spectra were acquired at room temperature with sample spinning. Exponential multiplication of the free induction decay was not employed. For the assay of covalent binding, a minimum of 20,000 transients were acquired. Chemical shifts were referenced to external trifluoroacetamide in $^{2}H_2O$ (δ = 0 ppm). Mass spectra were recorded with a Hewlett–Packard 5880A GC equipped with an HP-1 (crosslinked methylsilicone) capillary column and coupled to an HP-5970 mass-selective detector (70 eV, electron impact).

Animal Treatment and Tissue Preparation. HCFC-123 was pure by ^{19}F NMR and GC/MS. Adult male Fischer-344 rats (Charles River Breeding Laboratories) were exposed by inhalation to 0.7 ± 0.0% (n = 2) or 1.1 ± 0.1% HCFC-123 (n = 7), to 1.3 ± 0.1% halothane (n = 4), or to air for 2 hr. Concentrations are expressed on a volume/volume basis. The inhalation chamber used has been described (18), except that $CaCl_2$ was used to absorb water and that consumed oxygen was replaced via an attached spirometer. Chamber HCFC-123 and halothane concentrations were measured by GC/MS. The treated animals were immediately placed in metabolism cages and kept on a 12-hr light/dark cycle. Urine was collected into vessels maintained at −78°C and stored frozen until analyzed. After 15 hr, livers were removed from ether-anesthetized rats, and microsomal and cytosolic fractions were isolated by differential centrifugation: the supernatant from a 10,000 × g, 20-min centrifugation of the liver homogenate was centrifuged for 60 min at 100,000 × g, yielding the cytosolic fraction as the supernatant and the microsomal fraction as the pellet. These fractions were dialyzed to remove noncovalently bound metabolites (19). Liver subcellular fractions were dialyzed (Spectrapor 3 tubing, Spectrum Medical Industries, 3500 molecular weight cutoff) in 10 mM phosphate buffer, pH 7.0/0.1% $NaDodSO_4$ for at least 48 hr at 4°C.

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Abbreviations: CFC, chlorofluorocarbon; HCFC, hydrochlorofluorocarbon; HCFC-123, 2,2-dichloro-1,1,1-trifluoroethane; TFA-proteins, N^{ϵ}-trifluoroacetyllysine-containing proteins.

§To whom reprint requests should be addressed.

NMR Analysis of Covalent Binding. Dialyzed protein was lyophilized, dissolved in ^2H_2O, and analyzed by ^{19}F NMR. Protein concentrations in the NMR tube were kept as high as possible consistent with maintaining a liquid, albeit viscous, solution; a minimum of 30 mg of protein per ml was used. After initial NMR analysis, liver cytosolic fractions from HCFC-123- and halothane-treated rats were subjected to proteolysis. Samples were dissolved in 10 mM Tris buffer, pH 7.8/0.5% $NaDodSO_4$ containing proteinase K (Sigma; 6.25 mg/ml) and incubated at 37°C for at least 48 hr.

Protein Immunoblotting. Proteins were separated by $NaDodSO_4$/PAGE and were immunoblotted with hapten-specific anti-TFA-protein serum as previously described (16), except that the goat secondary antibody was conjugated with alkaline phosphatase instead of horseradish peroxidase and that standard-sized resolving gels were used instead of minigels. Lanes contained 120 μg of microsomal proteins or 100 μg of cytosolic proteins.

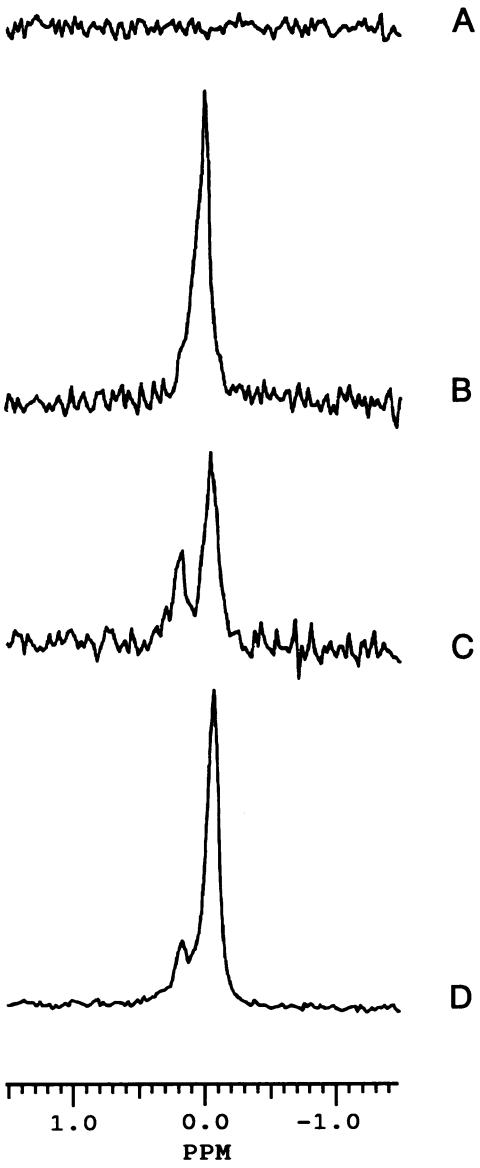

FIG. 1. ^{19}F NMR spectra of dialyzed hepatic cytosolic fractions. (*A*) Control rats. (*B*) Rats exposed to 1.1% HCFC-123. (*C*) Sample in *B* after proteolysis. (*D*) Sample in *C* after addition of N^{ϵ}-trifluoroacetyllysine. In the absence of protein, the chemical shifts of authentic trifluoroacetic acid and N^{ϵ}-trifluoroacetyllysine are the same as shown in *C* and *D*.

RESULTS

Spectral Studies. The ^{19}F NMR spectra of liver cytosol from rats exposed to HCFC-123 contained a single, broadened resonance that was not lost on dialysis (Fig. 1*B*); this resonance was also observed in microsomes (data not shown). Identical results were obtained with halothane-exposed rats (data not shown). No resonances were detected in liver fractions from control rats (Fig. 1*A*). The broadened peak found in HCFC-123- or halothane-exposed rats co-resonated with trifluoroacetamide, which was used as an external reference (δ = 0 ppm). Spectra acquired over a range of 350 ppm, which encompasses the resonances of organofluorine compounds (20), showed only this single resonance after dialysis.

Dialyzed hepatic cytosolic fractions from HCFC-123- or halothane-exposed rats were incubated with proteinase K to increase the rotational freedom of the protein adduct, thus decreasing the observed peak width. The ^{19}F NMR spectrum of the proteolyzed fractions (Fig. 1*C*) revealed two singlets; the intensity of the downfield peak (0.17 ppm) increased and the intensity of the upfield peak (−0.07 ppm) decreased with increasing proteolysis time. The intensity of the upfield singlet was increased by the addition of N^{ϵ}-trifluoroacetyllysine (Schweizerhall, South Plainfield, NJ) (Fig. 1*D*), whereas the downfield singlet was increased by the addition of trifluoroacetic acid (data not shown). Trifluoroacetic acid was detected by GC/MS (21) in exhaustively proteolyzed tissue samples; trifluoroacetic acid was released from authentic N^{ϵ}-trifluoroacetyllysine when incubated with proteinase K under similar conditions. These findings demonstrate that N^{ϵ}-trifluoroacetylated lysine is the major stable adduct formed by the interaction of HCFC-123 or halothane metabolites with proteins. No evidence was found for trifluoroacetylation of other protein nucleophiles (-SH, -OH); such adducts are known to be rapidly hydrolyzed at physiological pH (22). These data do not exclude adducts with N-terminal amino groups of proteins, although such adducts may be comparatively few in number.

Trifluoroacetic acid was the only fluorinated metabolite detected in the urine of HCFC-123- or halothane-exposed

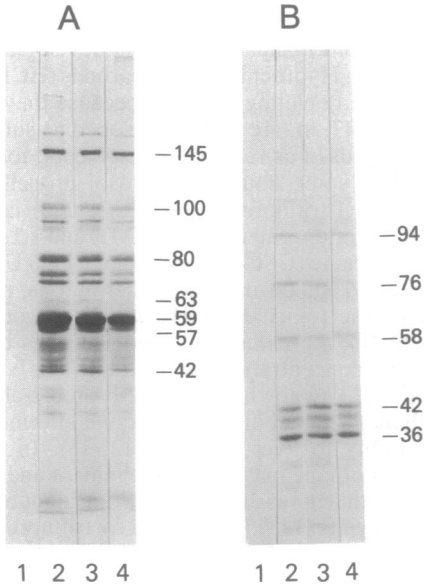

FIG. 2. Immunochemical detection of TFA-proteins in hepatic microsomal (*A*) and cytosolic (*B*) fractions from rats exposed to air (lanes 1), 1.3% halothane (lanes 2), 1.1% HCFC-123 (lanes 3), or 0.7% HCFC-123 (lanes 4). Markers at right indicate molecular mass in kilodaltons.

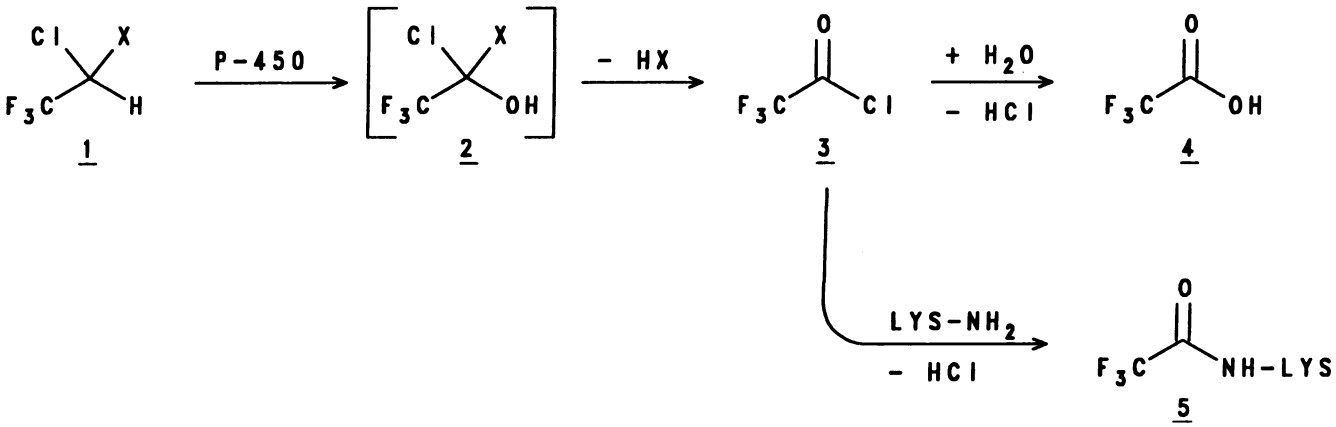

FIG. 3. Proposed metabolic pathway for HCFC-123 (X = Cl) and halothane (X = Br). P-450, cytochromes P-450; LYS-NH_2, lysine-containing proteins.

rats, as determined by GC/MS (21) and NMR (data not shown).

Protein Immunoblotting. Hapten-specific anti-TFA-protein serum was used to detect TFA-proteins. The hapten specificity of these polyclonal antibodies has been described (23). When hepatic microsomal and cytosolic fractions from HCFC-123- or halothane-exposed rats were examined by NaDodSO$_4$/PAGE and immunoblotting, identical patterns of TFA-proteins were detected (Fig. 2). Identical results were obtained when the analyses were repeated with another set of animals.

DISCUSSION

The finding that exposure to HCFC-123 or halothane leads to the formation of TFA-proteins that are identical by ^{19}F NMR (Fig. 1) and by immunoblotting (Fig. 2) is attributed to similar pathways of metabolism: both HCFC-123 and halothane (Fig. 3) are expected to undergo a cytochrome P-450-catalyzed hydroxylation to afford geminal halohydrin, **2** (24); halohydrin **2** may lose HX to give trifluoroacetyl chloride, **3**, which may react with the ε-amino group of lysine to yield protein-bound N^{ε}-trifluoroacetyllysine, **5**. Hydrolysis of trifluoroacetyl chloride would give trifluoroacetic acid, **4**, which was detected in the urine of HCFC-123- and halothane-exposed rats.

Trifluoroacetylation of hepatic proteins in rats exposed to HCFC-123 is significant because a relationship between the covalent modification of proteins by xenobiotic metabolites and cell damage and death has been established for many compounds (25–27). Moreover, hepatic TFA-proteins, which are formed after exposure of animals or humans to halothane (28), have been implicated in halothane-induced liver damage because most patients with this disease possess serum antibodies that react with at least one of these proteins (16, 28, 29). This suggests that halothane hepatitis is caused by an allergic or hypersensitivity reaction directed against the TFA-proteins (14–17). Certain of these TFA-proteins have been identified (29).

The present findings raise the possibility that individuals sensitized to HCFC-123 may also be at risk of developing hepatitis after subsequent exposure to HCFC-123 or after anesthesia with halothane or with enflurane [2-chloro-1-(difluoromethoxy)-1,1,2-trifluoroethane], which cross-reacts with halothane (30). Although animal toxicity studies are being conducted, it is unlikely that HCFC-123-induced allergic hepatitis would be detected during such studies, because drug-induced allergic hepatitis may be expected to be as uncommon in animals as it is in humans. Moreover, because no animal model for drug-induced allergic hepatitis has been developed, it is impossible to predict the lowest limits of HCFC-123 or halothane exposure that are required to produce this disease.

Finally, the CFC substitutes HCFC-124 (CF_3CHFCl), HCFC-125 (CF_3CHF_2), HCFC-225ca ($CF_3CF_2CHCl_2$), and HCFC-225cb (CF_2ClCF_2CHFCl) (4, 5) are structural analogs of HCFC-123; because all of these compounds possess a geminal dihalomethyl group (-CHX_2), they may undergo a similar metabolic fate and afford acylating intermediates that react with liver proteins. Because the potential exists for widespread human exposure to commercially important HCFCs, their metabolism, capacities for protein acylation, and toxicities merit examination.

We thank Charles F. Reinhardt, M.D., Haskell Laboratories, DuPont, for providing a sample of HCFC-123. J.W.H. was supported by National Institute of Environmental Health Sciences Training Grant ES07026; J.L.M. was supported by a Pharmacology Research Associate Fellowship from the National Institute of General Medical Sciences. This research was supported in part by National Institute of Environmental Health Sciences Grant ES05407 to M.W.A.

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