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Distribution of perfluorooctanesulfonate and perfluorooctanoate into human plasma lipoprotein fractions

John L. Butenhoff[a,*], Elsbeth Pieterman[b], David J. Ehresman[a], Gregory S. Gorman[c], Geary W. Olsen[a], Shu-Ching Chang[a], Hans M.G. Princen[b]

[a] Medical Department, 3M Company, St. Paul, MN 55455, USA
[b] TNO Biosciences, Zernikedreef 9, 2333 CK Leiden, The Netherlands
[c] McWhorter School of Pharmacy, Samford University, Birmingham, AL 35229, USA

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ABSTRACT

Some cross-sectional epidemiological studies have reported positive associations of serum concentrations of non-high density lipoprotein cholesterol with serum perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA). However, the strength of the reported associations is inconsistent for exposure–response across three orders of magnitude of serum PFOS and/or PFOA concentrations. These positive associations are unexpected based on toxicological/mechanistic studies, suggesting that the associations may have a biological, rather than a causal, basis. This study tested the hypothesis that PFOS and PFOA distribute into serum lipoprotein fractions such that increases in serum lipoproteins would result in corresponding increases in serum concentrations of PFOS and PFOA. Based on observed binding of PFOS and PFOA to isolated β-lipoproteins in physiological saline (96% and 40% bound, respectively) in preliminary experiments using ultrafiltration and LC–MS/MS methods, binding to human donor plasma lipoprotein fractions was investigated by two density gradient methods. The majority of PFOS and PFOA recovered masses were found in lipoprotein-depleted plasma. Plasma density gradient fractionation data suggested that maximally 9% of PFOS distributes to lipoprotein-containing fractions, yet only 1% or less of PFOA is so distributed. These data do not support a strong role for plasma lipoprotein fractions in explaining the inconsistent dose–response associations reported in cross-sectional epidemiological studies.

1. Introduction

Perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) are strong surfactants with exceptional chemical stability due to the strength of the carbon–fluorine bonds incorporated into their structure. Their primary use has been as surfactants in applications such as fire-fighting foams and acid mist suppression in the case of PFOS and fluoropolymer manufacturing in the case of PFOA. While this stability allows their use in harsh environments, it also imparts resistance to metabolic and environmental degradation, leading to persistence in the environment. Both PFOS and PFOA can also enter the environment as degradation products of materials that incorporate structures that can degrade to PFOS and PFOA.

Cross-sectional epidemiological studies have found positive associations between serum concentrations of PFOS and/or PFOA and serum non-high-density lipoprotein cholesterol (non-HDL-C) measured in NHANES general population data and in children and adults living in the mid-Ohio River valley community exposed to PFOA in drinking water (Frisbee et al., 2010; Nelson et al., 2010; Steenland et al., 2009). These associations were non-linear, confined to low-density lipoprotein cholesterol (LDL-C), and the strength of the associations appeared to decline and plateau at concentrations approximating 50 ng/mL. In contrast, studies of occupationally exposed workers have found associations of serum PFOS or PFOA with serum cholesterol that are either absent or are much weaker than those observed in non-occupational populations (Costa et al., 2009; Olsen et al., 2000, 2003; Olsen and Zobel, 2007; Sakr et al., 2007a,b). In a recent longitudinal study of a group of workers engaged in the demolition of former fluorochemicals manufacturing facilities, Olsen et al. (2012) have found no association with increasing serum PFOS and PFOA.

* Corresponding author at: 3M Company, 3M Center, Building 0220-06-W-08, St. Paul, MN 55455, USA. Tel.: +1 651 733 1962; fax: +1 651 733 9066.
E-mail addresses: jlbutenhoff@mmm.com (J.L. Butenhoff), elsbeth.pieterman@tno.nl (E. Pieterman), djehresman@mmm.com (D.J. Ehresman), ggorman@samford.edu (G.S. Gorman), gwolsen@mmm.com (G.W. Olsen), s.chang@mmm.com (S.-C. Chang), hans.princen@tno.nl (H.M.G. Princen).

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Moreover, the positive direction of the associations of serum PFOS and/or PFOA with serum non-HDL-C in cross-sectional studies is intuitively opposite that which would be expected based on toxicological and mode-of-action studies with PFOS and PFOA. Reduction in serum total cholesterol is among the earliest clinically evident effects on repeated dosing with PFOS in cynomolgus monkeys (Seacat et al., 2002), rats (Elcombe et al., 2012; Seacat et al., 2003), and mice (Bijland et al., 2011). These PFOS-induced reductions in serum total cholesterol occurred in association with serum PFOS concentrations approximating 100,000 ng/mL and higher. For PFOA, the most relevant data are those from a phase I clinical trial in 41 cancer patients given ammonium PFOA doses of up to 1200 mg/week for a median of 6.5 weeks and in which non-HDL-C was reduced as a treatment effect (MacPherson et al., 2011).

Based on the above observations from epidemiological and toxicological studies, the association of serum PFOS and/or PFOA with serum total cholesterol in cross-sectional studies could be inferred as non-causal. Indeed, both Frisbee et al. (2010) and Steenland et al. (2009) speculated on non-causal factors that may result in their observed positive associations, including the possibility of a saturated threshold response. One hypothesis for a non-causal basis for the observed association in cross-sectional studies of serum PFOS or PFOA with serum cholesterol is that PFOS and PFOA distribute into serum lipoprotein fractions. If so, increases in serum lipoproteins would result in increased serum concentration of PFOS and PFOA. If such a process were saturable, the resulting association would weaken with increasing serum PFOS and PFOA concentrations. To further explore this non-causal hypothesis of PFOS and PFOA with serum cholesterol and to determine additional binding affinities of PFOS and PFOA, protein binding evaluations were initially conducted to investigate if PFOS and PFOA would bind to human β-lipoproteins in isolation in physiological saline. This was followed by investigating the effect of plasma concentration of PFOS and PFOA on the proportion of plasma PFOS and PFOA bound to the plasma lipoprotein fractions very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL).

2. Materials and methods

2.1. Fluorochemicals

Potassium perfluorooctanoate (K^+PFOA, L-15822, 98.2% purity) and potassium perfluorooctanesulfonate (K^+PFOS, FC-95, Lot 217, 87.6% purity) were supplied by 3M Company, St. Paul, MN, USA.

2.2. Preliminary investigation of PFOS and PFOA binding to isolated human plasma lipoproteins in physiological saline

The potentials of PFOS and PFOA to bind to isolated human-derived β-lipoproteins in physiological saline (0.9% NaCl, pH = 7.4) were evaluated initially. Also included in the initial evaluation were the potentials of PFOS and PFOA to bind to isolated human albumin, γ-globulin, α-globulin, fibrinogen, α-2-macroglobulin, and transferrin in physiological saline. These experiments were conducted at PFOS and PFOA concentrations of 10,000 ng/mL (20 μM and 24 μM, respectively) with human serum binding proteins at approximately 10% and 100% of their respective physiological concentrations. Details of this preliminary investigation are presented in the supplementary material, with results presented in Supplementary Table S1. Albumin was found to bind the largest amount (≥99.7%) for both PFOS and PFOA at 100% physiological concentration. Isolated β-lipoproteins also bound 95.6% of added PFOS and 39.6% of added PFOA. The latter observation established a basis for investigating the potential distribution of PFOS and PFOA into plasma lipoprotein fractions from actual human plasma.

2.3. Distribution of PFOS and PFOA in human plasma lipoprotein fractions

Blood for the experiments was obtained from a single healthy donor via venipuncture using lithium heparinized Vacutainer™ tubes. Plasma was prepared by centrifugation at 4 °C followed by stabilization with the addition of sodium azide (final concentration 0.01%). Collected plasma was analyzed for total cholesterol and triglycerides (Roche Diagnostics enzymatic kits Nos. 11489437 and 11488872, respectively, Almere, The Netherlands). Plasma was snap frozen in liquid nitrogen and stored at −80 °C.

During method developments, the largest proportions of added PFOS and PFOA were found to distributed into the lipoprotein-depleted plasma (LPDP) fractions containing albumin (data not shown). The pilot studies revealed difficulties in adequately separating albumin from the high-density-lipoprotein (HDL) fraction using either the standard Redgrave density gradient ultracentrifugation (UCF) (Redgrave et al., 1975) or fast-protein liquid chromatography methods. Therefore, it was necessary to develop methods that provided optimal separation between HDL and albumin-containing LPDP fractions. This was accomplished by modification of the Redgrave gradient to provide a full separation between HDL and LPDP (see below for details). In addition, an iodixanol density gradient ultracentrifugation method for serum lipoprotein fractionation reported by Yee et al. (2008) was applied as an alternative to the Redgrave method, thus avoiding the high ionic-strength conditions present in the Redgrave method. The iodixanol method was modified to ensure good separation between VLDL, LDL, HDL, and LPDP. Thus, both modified Redgrave and iodixanol methods, detailed below, were then used in conducting the experiments.

The distribution of PFOS and PFOA in human plasma lipoprotein fractions was investigated at three nominal concentrations using both the modified Redgrave and modified iodixanol density gradient separation methods. The initial concentrations of PFOS and PFOA evaluated were those endogenous to the donor's plasma and were representative of background concentrations observed in the general population (25 ng/mL and 9–10 ng/mL, respectively (see Table 1)). To evaluate the effect of increasing concentrations of PFOS and PFOA on distribution to plasma lipoprotein fractions, aliquots of donor plasma were spiked separately with the potassium salts of PFOS or PFOA to achieve nominal concentrations of approximately 100 ng/mL and 10,000 ng/mL. These concentrations were selected to be representative of more highly exposed general populations and occupational exposed workers, respectively.

Experiments were performed in triplicate. Each replicate experiment consisted of five samples in 2-mL cryo vials into each of which were pipetted 10 μL of one of the following solutions: (1) methanol (vehicle for endogenous plasma PFOS and PFOA concentrations only); (2) 0.0042 mg/mL potassium PFOA in methanol; (3) 0.42 mg/mL potassium PFOA in methanol; (4) 0.005 mg/mL potassium PFOS in methanol; (5) 0.5 mg/mL potassium PFOS in methanol. The additions of potassium PFOA and potassium PFOS were not corrected for purity, as the actual concentrations of PFOS and PFOA anion were later determined by LC–MS/MS. For all samples, the methanol was evaporated to dryness under a gentle stream of nitrogen gas followed by the addition of 500 μL plasma. Samples were then vortexed for 30 s and incubated for 24 h at 37 °C on a rollerbank. After the incubation step, 2 × 50 μL plasma samples from each tube were snap frozen in liquid nitrogen and stored at −80 °C pending analysis for PFOS and PFOA concentrations by LC–MS/MS (see below for details).

For Redgrave density gradient ultracentrifugation experiments, 300 μL of incubated human plasma from each incubated preparation was mixed with 1.7 mL of 452 g/L KBr in phosphate buffered saline and added to SW40 centrifuge tubes (14 mm × 95 mm, 14 mL). This step was followed by the addition, in the following order, of 2.6 mL of 307 g/L KBr (ρ = 1.210 g/cm^3), 2.5 mL 270 g/L KBr (ρ = 1.180 g/cm^3), 3.0 mL 155 g/L NaCl (ρ = 1.100 g/cm^3), 2.0 mL 30.7 g/L NaCl (ρ = 1.019 g/cm^3), and 1.7 mL H_2O (ρ = 1.000 g/cm^3). The tubes were then centrifuged at 4 °C for 24 h at 285,000 × *g* (40,000 rpm with a SW40 Ti rotor) in a Beckman Optima™ L-70 ultracentrifuge.

For iodixanol density gradient ultracentrifugation experiments, OptiPrep™ Density Gradient Medium (60% iodixanol in sterile water) was purchased from Sigma Aldrich (St. Louis, MO, USA, Catalog number D1556) and all further iodixanol dilutions were made in HEPES buffered saline (100 mM HEPES, pH 7.4, 0.85% NaCl). For each sample, 300 μL of incubated plasma was mixed with 40 μL HEPES buffered saline and 660 μL of OptiPrep™ to yield human plasma mixed in 40% buffered iodixanol at a total volume of 1 mL. This was followed by addition, in the following order, of 2 mL 35% buffered iodixanol, 3 mL 30% buffered iodixanol, 2 mL 20% buffered iodixanol, 1 mL 12% buffered iodixanol, 2 mL 9% buffered iodixanol, and 1.5 mL HEPES buffered saline. Ultracentrifugation was conducted for 4 °C for 44 h at 285,000 × *g* (40,000 rpm with a SW40 Ti rotor) in a Beckman Optima™ L-70 ultracentrifuge.

After centrifugation, a total of 25 0.5 mL fractions were aliquoted into 1.5 mL Eppendorf tubes starting from top to bottom (lowest density to highest density) of the SW40 tubes. From each aliquoted fraction, 40 μL was taken for total cholesterol determination by fraction using using Roche Diagnostics Enzymatic Kit No. 11489437 (Almere, The Netherlands). Fractions representing VLDL, LDL, HDL, and LPDS as well as fractions intermediate to these were pooled based on previously determined albumin, cholesterol, and density measurements. Pooled fractions were stored frozen, shipped on dry ice, and held at −80 °C until analyzed for PFOS and PFOA concentration by LC–MS/MS.

2.4. LC–MS/MS analysis of PFOS and PFOA

Due to the high sensitivity required for the assay, potential reagent contamination with PFOS and PFOA was addressed as described in Sundström et al. (2011). Among other procedures, this included preparation of analytical grade water by first passing deionized water through a Milli-Q® (Millipore Corporation, Billerica, MA, USA) water purification system with further processing through a C-18 HPLC column.