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The effects of perfluorinated chemicals on adipocyte differentiation *in vitro*

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ABSTRACT

The 3T3-L1 preadipocyte culture system has been used to examine numerous compounds that influence adipocyte differentiation or function. The perfluoroalkyl acids (PFAAs), used as surfactants in a variety of industrial applications, are of concern as environmental contaminants that are detected worldwide in human serum and animal tissues. This study was designed to evaluate the potential for PFAAs to affect adipocyte differentiation and lipid accumulation using mouse 3T3-L1 cells. Cells were treated with perfluorooctanoic acid (PFOA) (5–100 μM), perfluorononanoic acid (PFNA) (5–100 μM), perfluorooctane sulfonate (PFOS) (50–300 μM), perfluorohexane sulfonate (PFHxS) (40–250 μM), the peroxisome proliferator activated receptor (PPAR) PPARα agonist Wyeth-14,643 (WY-14,643), and the PPARγ agonist rosiglitazone. The PPARγ agonist was included as a positive control as this pathway is critical to adipocyte differentiation. The PPARα agonist was included as the PFAA compounds are known activators of this pathway. Cells were assessed morphometrically and biochemically for number, size, and lipid content. RNA was extracted for qPCR analysis of 13 genes selected for their importance in adipocyte differentiation and lipid metabolism. There was a significant concentration-related increase in cell number and decreased cell size after exposure to PFOA, PFHxS, PFOS, and PFNA. All four PFAA treatments produced a concentration-related decrease in the calculated average area occupied by lipid per cell. However, total triglyceride levels per well increased with a concentration-related trend for all compounds, likely due to the increased cell number. Expression of mRNA for the selected genes was affected by all exposures and the specific impacts depended on the particular compound and concentration. Acox1 and Gapdh were upregulated by all six compounds. The strongest overall effect was a nearly 10-fold induction of Scd1 by PFHxS. The sulfonated PFAAs produced numerous, strong changes in gene expression similar to the effects after treatment with the PPARγ agonist rosiglitazone. By comparison, the effects on gene expression were muted for the carboxylated PFAAs and for the PPARα agonist WY-14,643. In summary, all perfluorinated compounds increased cell number, decreased cell size, increased total triglyceride, and altered expression of genes associated with adipocyte differentiation and lipid metabolism.

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1. Introduction

Perfluoroalkyl acids (PFAAs) are straight chain organofluorine chemicals that are used in commercial applications including paint additives, fire-fighting foams, surfactants, and water and stain repellants. Many PFAAs persist in the environment and numerous studies have demonstrated the environmental accumulation and toxicity of PFAAS (Lau et al., 2007; Lindstrom et al., 2011). The majority of work on PFAAs has been done on perfluorooctanoic acid (PFOA) and perfluorooctane sulfate (PFOS), but data from the National Health and Nutrition Examination Survey (NHANES) indicate that in addition to PFOA and PFOS, perfluorononanoic acid (PFNA) and perfluorohexane sulfate (PFHxS) are routinely found in human serum (Kato et al., 2011).

Peroxisome proliferator activated receptors (PPARs) are a class of nuclear receptors with three subtypes, PPARα, PPARβ, and PPARγ, each with distinct expression and physiological roles (Escher and Wahli, 2000). The PPARγ pathway is a major regulator of adipocyte differentiation and lipid metabolism (Casals-Casas and Desvergne, 2011). The PPARα pathway plays a role in maintaining lipid homeostasis directly regulating genes involved in fatty acid uptake and

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metabolism (Yumuk, 2006). PFAAs have been shown to activate human, mouse and rat PPARs, predominantly PPARα in cultured Cos-1 and 3T3-L1 cells (Shipley et al., 2004; Vanden Heuvel et al., 2006; Wolf et al., 2008, 2012). In Cos-1 luciferase reporter studies, PFAA carboxylates and sulfonates with carbon chains from 4 to 12 carbons long were examined for their ability to activate PPARα. PPPARα activity was found to be highest for perfluorinated compounds with backbones 8–9 carbons long, and carboxylates were more active than sulfonates (Wolf et al., 2008, 2012). The activity of PFAAs, however, is not limited to PPARα. Studies in PPARα knockout mice have uncovered PPARα independent effects (Rosen et al., 2010). Also, in cultured mouse and human hepatocytes, PFAAs induced expression of genes that are characteristic of a PPARα response, but that were also representative of PPARγ and other nuclear receptors, such as constitutive androstane receptor (CAR) activity (Rosen et al., 2013).

Interestingly, epidemiological studies have found associations between the perfluorinated chemicals and human health outcomes associated with lipid metabolism (Halldorsson et al., 2012). PFOA and PFOS were associated with elevated serum levels of cholesterol and low density lipoprotein (LDL) in individuals from highly exposed communities and workers with occupational PFAA exposure, but not high density lipoprotein (HDL) or triglycerides (Steenland et al., 2010). A cross-sectional study investigating the link between perfluorinated chemical exposure and lipid levels using 2003–2004 NHANES data found a positive association between PFOA and PFOS, total cholesterol, and non-high density cholesterol (Nelson et al., 2010), but a larger study incorporating NHANES data from 1999 to 2006 did not report an association between serum lipid and PFAAs (Patel et al., 2012). Animal studies on gene expression in 6 month old mouse liver exposed to PFAAs for 7 days indicate that PFAA exposure results in increased activation of genes regulated by PPARα and involved in fatty acid oxidation (Kudo et al., 2006; Rosen et al., 2008). Such changes in gene expression in the rodent suggest that there may be effects at the cellular level in response to PFAAs that impact lipid metabolism, though mouse studies are typically conducted at higher levels than those seen in the general population.

A growing set of studies has looked at pollutants that affect adipocytes using *in vitro* models. The 3T3-L1 model is a fibroblast derived cell line that undergoes adipocyte differentiation (Green and Meuth, 1974) and, in the presence of medium containing high levels of serum, the differentiated adipocytes accumulate lipid. The model has been used in metabolic disease research for nearly 40 years and more recently to explore the obesogenic capacity of various chemicals. The organotin, tributyl tin, and its ability to induce adipocyte differentiation has been studied extensively in the 3T3-L1 system (Grun et al., 2006). Phthalates, which have been linked to increased waist circumference in men (Stahlhut et al., 2007), have also been found to stimulate the proliferation of adipocytes in this system (Feige et al., 2007). Recently, an endocrine disruptor study treated 3T3-L1 cells with a panel of chemicals including 2,2′,4,4′-tetrabrominated diphenyl ether (BDE-47), bisphenol A (BPA), PFOA and PFOS (Bastos Sales et al., 2013). BDE-47 increased adipocyte differentiation in a dose dependent manner and BPA increased differentiation at concentrations above 10 μM, however, no effect was seen for the PFOA or PFOS at 10 μM.

This study was designed to evaluate the ability of PFAAs to affect adipocyte differentiation and lipid accumulation in an *in vitro* system. Cells were treated with the four perfluorinated compounds most highly prevalent in human serum: PFOA, PFNA, PFHxS and PFOS. The doses selected were previously shown to activate PPAR reporters in Cos-1 cells in studies in our laboratory (Wolf et al., 2012). Cells were also treated with the PPARα agonist, WY-14,643, and the PPARγ agonist, rosiglitazone. At the end of the culture period, cells were assessed for number, size, and lipid content. The expression of genes important in adipogenesis and regulation of lipid homeostasis was examined using quantitative PCR (qPCR). While the PPARα and PPARγ pathways have a good deal of overlap, for this study genes were selected that are generally unique to one pathway.

2. Materials and methods

2.1. Chemicals

Wyeth-14,643 (4-chloro-6-(2,3-xylidine)-pyrimidinylthioacetic acid) (WY-14,643) and rosiglitazone were purchased from Sigma Chemical (St. Louis, MO) and prepared in dimethyl sulfoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ). WY-14,643 and rosiglitazone were prepared as 25 mM stocks. Each positive control was adjusted to a final DMSO concentration of 0.1%. PFOS (potassium salt; purity > 98%) and PFOA (ammonium salt; purity > 98%) were purchased from Fluka Chemical (Steinheim, Switzerland). PFNA (purity 97%) was obtained from Sigma, and PFHxS (potassium salt; purity 98.6%) was provided as a gift from 3M Corporation (St. Paul, MN). All PFAA stocks were prepared in sterile filtered, distilled, deionized water (ddH_2O), and working solutions were prepared fresh each day of treatment with incubation at 65 °C in a sonicating water bath until completely dissolved. Since the PFAA stocks were made in ddH_2O and the positive controls were prepared in DMSO, a pilot study was run comparing ddH_2O and 0.1% DMSO over the full 17 day culture period. No treatment effect was found for DMSO in 3T3 cells (data not shown), and cells treated with medium spiked to 0.1% DMSO were used as our zero effect control.

2.2. 3T3 preadipocyte cell culture and treatment

All culture medium and mouse 3T3-L1 preadipocytes were purchased as a frozen stock (500,000 cells/vial) from Zenbio (Research Triangle Park, NC). An experimental flask was cultured for 7 days in preadipocyte medium to allow cells to reach confluence. Cells were then trypsinized, counted and plated in 48-well plates at a density of 3000 cells per well in preadipocyte medium. Culture in 48-well plates took place for a period of 17 days (Fig. 1). Culture days 1–7 were in preadipocyte medium. Culture days 8–10 were in PPARγ-agonist-free differentiation medium. PPARγ-agonist-free medium was used to optimize the ability to detect effects of PFAA chemicals. A pilot study found that the traditional differentiation medium offered by Zenbio, which contains a potent proprietary PPARγ agonist, strongly induced differentiation and lipid accumulation, making it difficult to detect any influence of PFAAs on these endpoints. On culture days 11–17 cells were treated in adipocyte maintenance medium. Cells were allowed to adhere for a period of 24 hours after plating and then received the first treatment exposure. Medium was changed every 2–3 days with treatment concentrations remaining constant and switching medium type as described above (Fig. 1). Due to the presence of other substances in the medium that can induce adipogenesis, including insulin, isobutylmethylxanthine, and dexamethasone, all metrics were compared to control cells treated with the same medium that was used to prepare each treatment solution. Each 48-well plate received 6 wells of control medium, 6 wells of the positive control rosiglitazone, and 6 different concentrations of a PFAA treatment with 6 wells per concentration. The exception to this plate layout was PFNA, for which the plate was treated with control medium and 7 PFNA concentrations instead of rosiglitazone.

Cultures were treated with a range of concentrations for each compound. The carboxylates, PFOA and PFNA, were administered at concentrations of 0, 5, 20, 40, 80, 100 μM and 0, 5, 10, 20, 40, 60, 80, 100 μM, respectively. The sulfonates, PFHxS and PFOS, were administered at concentrations of 0, 40, 80, 100, 150, 200, 250 μM and 0, 50, 100, 150, 200, 250, 300 μM, respectively. The PPARα agonist WY-14,643 was administered at concentrations of 0, 5, 10, 20, 40,