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The inhibition of human and rat 11β-hydroxysteroid dehydrogenase 2 by perfluoroalkylated substances

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ABSTRACT

11β-Hydroxysteroid dehydrogenase 2 (11β-HSD2) regulates active glucocorticoid access to glucocorticoid and mineralocorticoid receptors by metabolizing it to an inactive form. Perfluoroalkylated substances (PFASs) are man-made polyfluorinated compounds that are widely used and persistent in the environment. We tested the inhibitory potencies of four PFASs including perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), perfluorohexanesulfonate (PFHxS) and perfluorobutane sulfonate (PFBS) on human and rat 11β-HSD2. PFOS was a potent inhibitor of both human (IC_{50} = 48 nM) and rat (IC_{50} = 293 nM) 11β-HSD2 activities. The potencies for the inhibition of human and rat 11β-HSD2 activities were PFOS > PFOA > PFHxS > PFBS. PFASs showed competitive inhibition of both human and rat 11β-HSD2 activities. This observation indicates that PFOS is a potent endocrine disruptor for glucocorticoid metabolism.

Article from the Special issue on Targeted Inhibitors.

1. Introduction

Perfluoroalkyl substances (PFASs) are a group of chemicals that contain perfluoroalkyl groups. Many PFASs contain sulphur (perfluoroalkyl sulphonates) including perfluorooctyl sulphonate (PFOS, eight-carbon chain length), perfluorohexyl sulfonate (PFHxS, six-carbon chain length) and perfluorobutyl sulphonate (PFBS, four-carbon chain length) (Fig. 1). PFASs also contain chemicals without sulphur such as perfluorooctanoic acid (PFOA, eight-carbon chain length). PFASs are chemically very stable and are neither fat- nor water-soluble. Therefore, they are used in a broad spectrum of consumer and industrial products including textiles, paper and upholstery and as reaction additives in various processes. There is increasing concern over the public health of these chemicals because several PFASs have very long elimination half-lives ($t_{1/2}$) in the human body and potential toxicities in animal models [1–3]. For example, the half-life of elimination ($t_{1/2}$) of three common PFASs, PFOA, PFOS and PFHxS, is 3.8, 5.4 and 8.5 years, respectively [4]. Although 3M Company (St. Paul, MN) has phased out the production of PFOS, its resistance to environmental breakdown results in its persistence in the environment. Furthermore, the short carbon chain PFBS has been introduced to replace PFOS and PFOA because of its shorter $t_{1/2}$ life in human blood [5].

Toxicity studies in rodents have showed that PFASs interfere with many systems including reproductive, immune and endocrine systems [1–3]. The pituitary–adrenal axis may also be the target of PFASs. Rats and mice exposed to PFOS had increased levels of the circulating glucocorticoid, corticosterone (CORT) [6,7]. Glucocorticoid excess could have adverse effects on many systems including the reproductive and immune system [8]. The level of glucocorticoids is regulated not only by its synthesis in the adrenals but also by its metabolism. The endogenous glucocorticoid level is controlled by 11β-hydroxysteroid dehydrogenase (11β-HSD). Two isoforms of 11β-HSD have been identified: an NADP+/NADPH dependent 11β-HSD1 oxidoreductase and an NAD+ dependent 11β-HSD2 oxidase. 11β-HSD1 is a low affinity enzyme that primarily acts as a reductase to generate active 11β-hydroxy glucocorticoids (CORT in rodents and cortisol in human) from biologically inert 11keto steroids (11dehydroCORT, 11DHC, in rodents and cortisone in human) in liver and fat tissues [9]. In the contrast, 11β-HSD2 is a

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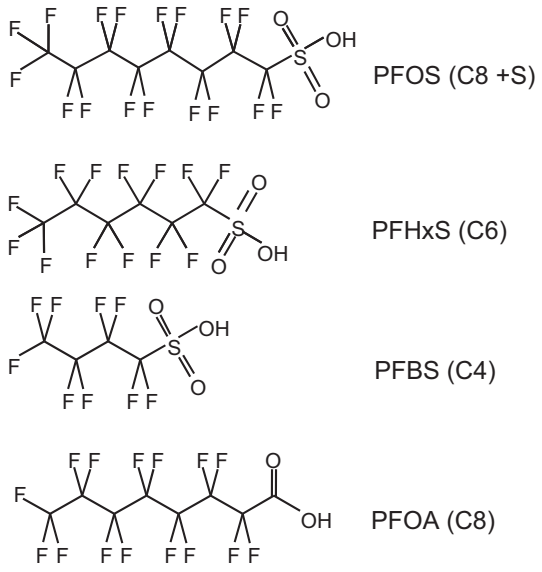

Fig. 1. Structures of perfluoroalkyl substances (PFASs). PFOA: perfluorooctanoic acid; PFOS: perfluorooctane sulfonate; PFHxS: perfluorohexane sulfonate; PFBS: perfluorobutane sulfonate.

unidirectional high affinity (K_m = 10 nM) oxidase to eliminate 11β-hydroxy glucocorticoid and is predominantly present in kidney and placentum [10]. In the kidney, 11β-HSD2 eliminates active CORT and cortisol thereby protecting mineralocorticoid receptors from glucocorticoid occupation [10]. Null mutation of 11β-HSD2 gene causes a syndrome of apparent mineralocorticoid excess manifested with hypokalemia and hypertension [11]. In the placentum, 11β-HSD2 eliminates active maternal CORT or cortisol thereby preventing glucocorticoid access to the fetus which would disrupt fetal development. Null mutation of the 11β-HSD2 gene and exposure to chemical inhibitors has shown that loss of 11β-HSD2 function would reduce birth weight in the newborn [12,13]. Apparently, the inhibition of 11β-HSD2 could lead to the elevated serum CORT or cortisol levels [14], thus causing many pathological changes. We hypothesize that the elevated serum CORT levels after exposure to PFOS is caused by direct inhibition of kidney 11β-HSD2 activity. In the present study, we compared the potencies of inhibiting human and rat 11β-HSD2 activities with PFASs according to the length of their alkane chain.

2. Materials and methods

2.1. Chemicals and animals

[1,2,6,7-N-^{3}H]-Corticosterone (^{3}H-CORT, specific activity, 88 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, MA). [^{3}H]11 Dehydrocorticosterone (^{3}H-11DHC) was prepared from labeled ^{3}H-CORT as described earlier [15]. Unlabelled CORT and 11DHC were purchased from Steraloids (Newport, RI). The following PFASs were purchased from Sigma–Aldrich Company (St. Louis, MO, USA): PFOA, PFOS, PFHxS potassium salt and PFBSK potassium salt. Male Sprague-Dawley rats (250–300 g) were purchased from Charles River Laboratories (Wilmington, MA). All animal procedures were approved by the Institutional Animal Care and Use Committee of Rockefeller University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Human kidney microsomes were purchased from Gentest (Woburn, MA).

2.2. Preparation of microsomal protein

Rat kidney microsomes were prepared as described previously [16]. In brief, rat kidney was homogenized in 0.01 mM phosphate buffered saline (PBS, pH 7.4) containing 0.25 M sucrose, and nuclei and large cell debris were removed by centrifugation at 700 × *g* at 4 °C for 30 min. The post-nuclear supernatants were centrifuged at 14,500 × *g* at 4 °C for 30 min to remove mitochondria, and the resulting supernatants were further centrifuged twice at 105,000 × *g* at 4 °C to collect microsomes. The resultant microsomal pellets were resuspended. Protein contents were measured by Bio-Rad Dye Reagent Concentrate according to manufacturer's instructions. The concentration of microsome protein was adjusted to 2 mg/ml. Microsomes were used for measurement of 11β-HSD2 activities.

2.3. Enzyme activity assay

The oxidation of CORT by 11β-HSD2 was determined in a mixture containing 0.2–0.5 μM CORT (plus 30,000 cpm [^{3}H]-CORT), 0.2 mM NAD+, 10 mM DTT and 2% ethanol in 0.1 mM potassium phosphate buffer (pH 7.2, 250 μL total volume) at 37 °C. Because 11β-HSD2 in kidney microsomes is present as a dimer, 10 mM DTT (a reducing agent) was added to reaction buffer to disrupt kidney microsomes and the disulfyl group of 11β-HSD2 to convert 11β-HSD2 to as a monomer to increase its activity [17]. Reactions were initiated by the addition of microsomes and NAD+ and terminated by the addition of 2 ml ice-cold ether. The steroids were extracted by vigorous mixing for 1 min, and the organic layer was dried under nitrogen. The extraction efficiency for CORT and 11DHC were 105.8 ± 8.8% (mean ± SD) for CORT and 104.2 ± 14.8% for 11DHC. The steroids were separated chromatographically on thin layer plates in chloroform and methanol (90:10, v/v), and the radioactivity was measured using a scanning radiometer (System AR2000, Bioscan Inc., Washington, DC). The percentage conversion of CORT to 11DHC and was calculated by dividing the radioactive counts identified as 11DHC by the total counts associated with CORT plus 11DHC as previously described [16], and the velocity of the 11β-HSD2 was calculated according to the percentage of substrate conversion, substrate concentration, enzyme amount and reaction time. The formation of product was determined at 4 time points within the linear portion of the reaction.

2.4. Enzyme inhibition studies

Inhibition of the oxidation of CORT catalyzed by 11β-HSD2 was measured using varying concentrations of each inhibitor with the substrate concentrations set to about 2× K_m to calculate the half maximal inhibitory concentration (IC_{50}) values. The mode of inhibition was assayed by adding different fixed CORT concentrations in the presence of various concentrations of each inhibitor. Initial velocity data were fit to competitive, noncompetitive, uncompetitive, and mixed inhibition modes. The inhibition constant K_i was determined.

2.5. Statistics

Each experiment was repeated two to four times. Data were subjected to nonlinear analysis by GraphPad (Version 4, GraphPad Software Inc., San Diego, CA) for IC_{50}. Data were subjected to analysis by one-way ANOVA followed by DUNCAN multiple comparison testing to identify significant differences between groups when three and more groups were calculated. All data are expressed as means ± SEM. Differences were regarded as significant at $P < 0.05$.

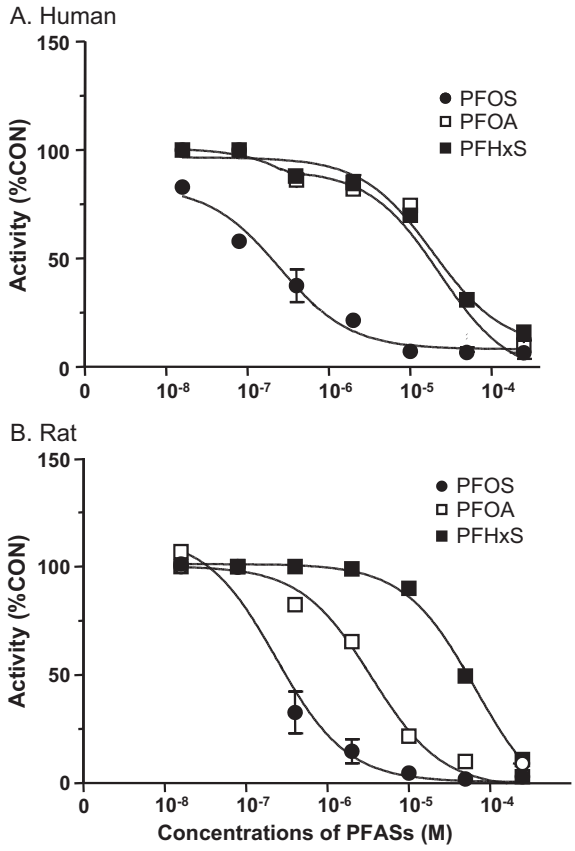

Fig. 2. The concentration-dependent inhibition of perfluoroalkyl substances (PFASs) on human and rat kidney 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2). Panel A: Human kidney microsome; Panel B: Rat kidney microsome. 11β-HSD2 was measured by the conversion of corticosterone to 11-dehydrocorticosterone in the presence of difference concentrations of PFASs. PFOS: perfluorooctane sulfonate; PFHxS: perfluorohexane sulfonate; PFOA: perfluorooctanoic acid. Mean ± SEM (n = 4–6).

3. Results

3.1. Effects of PFASs on human and rat 11β-HSD2 activities

We examined the inhibition of human and rat 11β-HSD2 by a series of PFASs at 250 μM. This highest concentration was selected based on the report that the highest serum PFOA level was 114.1 mg/L (~250 μM) in occupational workers [18]. At this concentration, PFOS, PFHxS, PFBS and PFOA generated 90.50 ± 1.04%, 87.50 ± 2.06%, 1.25 ± 2.56% and 89.25 ± 0.94% inhibition for human and 98.75 ± 0.71%, 92.050 ± 1.51%, 3.00 ± 0.77% and 98.78% ± 0.71% for rat 11β-HSD2 activities. Therefore, PFBS had almost no inhibition for both human and rat 11β-HSD2 activities. We then determined the IC_{50}s for PFOS, PFHxS and PFOA for both human and rat enzymes (Fig. 2A and B). IC_{50}s showed the potencies for inhibition of human and rat 11β-HSD2 activities (Fig. 2A and B) were: PFOS (0.048 ± 0.027 μM, mean ± SE) > PFHxS (18.97 ± 3.03 μM) > PFOA (24.41 ± 0.09 μM) for human enzyme activity and PFOS (0.293 ± 0.177 μM) > PFOA (3.80 ± 0.09 μM) > PFHxS (62.87± 5.21 μM) for rat enzyme activity. PFOS was the most potent (IC_{50} = 48 nM for human and 293 nM for rat enzymes). Compared with PFOA, PFOS was about 500 times more potent in the inhibition of human 11β-HSD2. Structurally, we observed that the potencies of inhibition declined when the carbon length of the alkane chain became shorter: PFOS (C8 + S) > PFOA (C8) > PFHxS (C6) > PFBSK (C4).

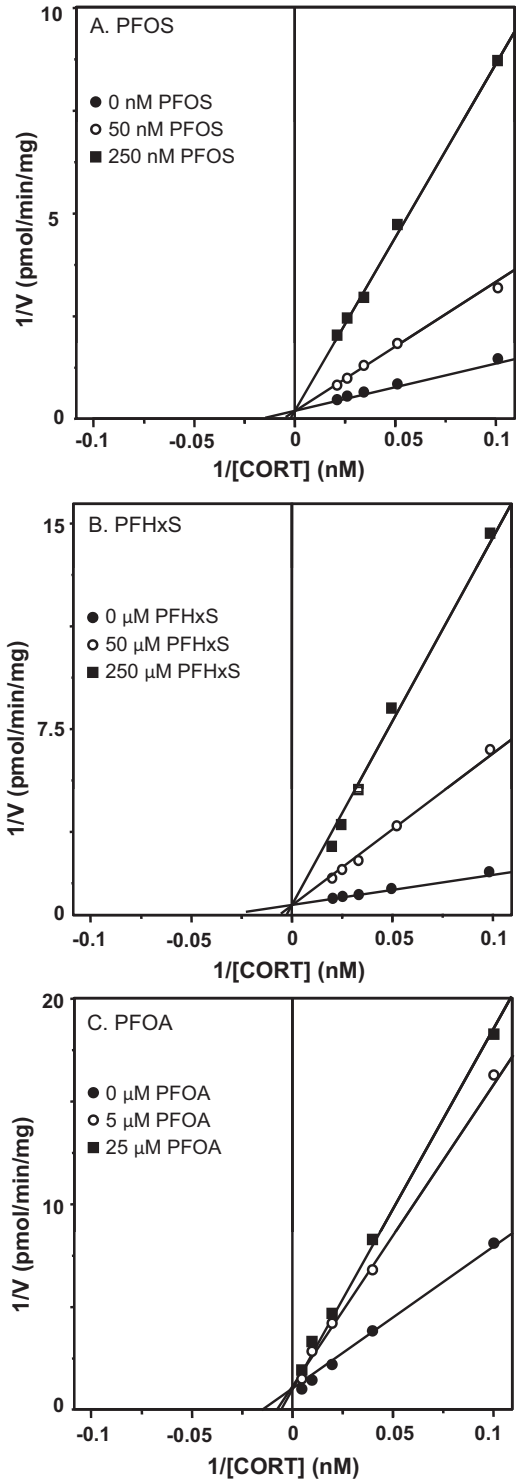

Fig. 3. Lineweaver–Burk plot of rat kidney microsomal 11β-HSD2 in the presence of PFOS, PFHxS and PFOA. 11β-HSD2 was measured by the conversion of corticosterone to 11-dehydrocorticosterone in the presence of difference concentrations of PFASs. PFOS: perfluorooctane sulfonate; PFHxS: perfluorohexane sulfonate; PFOA: perfluorooctanoic acid. The experiments were repeated twice.

3.2. Enzyme kinetic analysis

The patterns of inhibition by PFASs for 11β-HSD2 were examined. Lineweaver–Burk's plot analysis showed that PFOS, PFHxS and PFOA were competitive inhibitors of rat 11β-HSD2 (Fig. 3). PFOS, PFHxS and PFOA were also competitive inhibitors for human

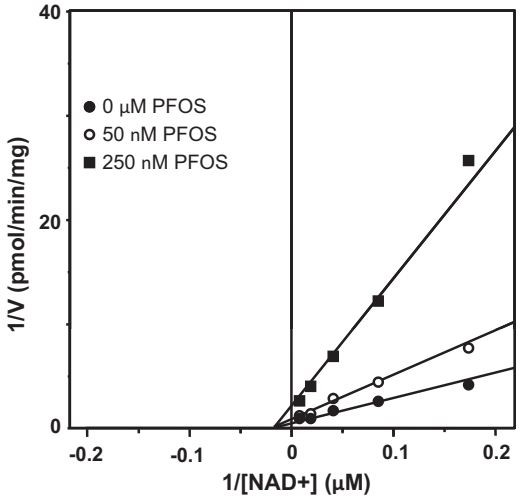

Fig. 4. Lineweaver–Burk plot of kidney microsomal 11β-HSD2 in the presence of PFOS. 11β-HSD2 was measured by the conversion of corticosterone to 11-dehydrocorticosterone in the presence of difference concentrations of PFOS with different fixed NAD+ concentrations. The experiments were repeated twice.

11β-HSD2 (data not shown). Rat 11β-HSD2 was assayed in the presence of PFOA and different cofactor (NAD^+) concentrations. Lineweaver–Burk plot showed that PFOA was noncompetitive inhibitor at the NAD+ cofactor binding site (Fig. 4). Other PFASs showed the similar mode of inhibition (data not shown). Our data indicate that PFASs are noncompetitive inhibitors of NAD+ binding site of human and rat 11β-HSD2.

4. Discussion

Little is understood about the mechanism of PFASs, although there are significant accumulation of PFASs, especially PFOS and PFOA, in human blood and wild-life tissues. In the present study, we showed that PFOS was a potent inhibitor of human and rat kidney 11β-HSD2. The IC_{50} of PFOS for human 11β-HSD2 was 48 nM, and that for rat 11β-HSD2 was 293 nM.

The inhibition of 11β-HSD2 is specific for PFASs since they have clear structure activity relationships. Structural changes in the alkane chain length may account for the different potencies for inhibition of both human and rat 11β-HSD activities by PFASs: PFOS (8 carbons) > PFHxS (6 carbons) > PFBS (4 carbons). Although FPOS and PFOA have the same carbon chain length, PFOS is more potent than PFOA in the inhibition of the enzyme. This difference could be explained by the additional sulphur atom in the PFOS compared to PFOA. PFOA is still more potent than PFHxS and PFBS since it has a longer carbon chain. Structural differences also accounted for the degree of adverse effects for *in vitro* models. The rank order of developmental neurotoxicity was PFOS > PFBS = PFOA [19]. PFOS altered cell membrane fluidity at 5–15 mg/L, but PFHxS and PFBS had no effect on cell membranes [20]. PFBS is a recent introduction to the market and is less persistent than other PFASs. For example, the $t_{1/2}$ of PFBS is 3.1 h in male rats [21] and it is far shorter compared to PFOA (6–8 days).

The potency of PFOS for the inhibition of human 11β-HSD2 was comparable to that of carbenoxolone (IC_{50} = 20 nM) and greater than that of glycyrrhetinic acid (IC_{50} = 400 nM) [14,22]. With IC_{50} of 48 nM for human 11β-HSD2, the IC_{50} of PFOS is in the range of average levels of PFOS worldwide (20–30 ng PFOS/ml, 40–60 nM) [23]. Serum PFOS levels in some people were much higher. For example, in the serum of American blood donors, more than 1500 ng PFOS/ml have been observed [24] and industrial workers can have serum levels of PFOS over 100 μg/ml [23]. Thus, we predict that at this level PFOS will inhibit 11β-HSD2. It was true that rats and mice exposed to PFOS had a significant elevation of serum CORT level [6,7]. Although the exact mechanism of the elevated serum CORT level has not been explained in these studies, one of the mechanism could result from the direct inhibition of 11β-HSD2 by PFOS. We have demonstrated that another potent 11β-HSD2 inhibitor glycyrrhetinic acid could increase rat serum CORT by twofold after rats were exposed to 5 mg/kg glycyrrhetinic acid [14]. Much concern has been paid attention to PFOS, because its $t_{1/2}$ is very long, with an estimated 8.5 years [4].

11β-HSD2 regulates certain aspects of development and has important functions in various adult tissues. In the placentum, 11β-HSD2 protects the fetus from the high circulating levels of maternal glucocorticoid [25–27]. The inhibition of 11β-HSD2 by PFOS during pregnancy could result in glucocorticoid-mediated effects with dangerous consequences. For example, gestational exposure of experimental animals, including rats, to PFOS or PFOA results in reduced birth weight and weight gain at maturity [28]. Recently, human cohort studies also showed correlations between serum levels of PFOS plus PFOA and low birth weight [29–31]. Low birth weight is a consequence of exposure to excessive levels of glucocorticoid [25–27], which occurs after inhibition of 11β-HSD2 by mutation of the 11β-HSD2 gene or reduced gene expression [12,13,25,32]. Thus, inhibition of 11β-HSD2 could account for significantly reduced birth weight of pups exposed to PFOS *in utero*.

11β-HSD2 is also localized in classic mineralocorticoid receptor targeted tissues such as kidney [10]. In kidney, 11β-HSD2 eliminates the natural glucocorticoids thereby protecting mineralocorticoid receptors from glucocorticoid occupation. In the human, mutation of the 11β-HSD2 gene causes a syndrome named AME characterized by hypertension and hypokalemia [10]. This AME can also be caused by chemical inhibition of the enzyme [10]. We conjecture that the potent inhibition of kidney 11β-HSD2 in humans by PFOS could lead to AME.

PFASs may also interfere with the development of testis and male reproduction. PFOS plus PFOA have been reported to be associated with the reduced semen quality and low testosterone and luteinizing hormone ratios in 105 Danish men [33]. The low testosterone and luteinizing hormone ratio indicates that Leydig cell function was impaired in the high-PFAS group compared to the low-PFAS group [33]. 11β-HSD2 has been localized to fetal and adult mammalian (human, rat and pig) Leydig cells [34–36]. Therefore, 11β-HSD2 plays an important role in glucocorticoid inactivation in Leydig cells to prevent glucocorticoid-mediated suppression of testosterone production. Suppression of 11β-HSD2 in Leydig cells could lead to the suppression of testosterone production in these cells.

In conclusion, we tested the effects of PFASs on 11β-HSD2 and found that PFASs could inhibit 11β-HSD2. The potencies of inhibiting the enzyme by PFASs depended on carbon length. The potencies were PFOSK (8 carbons) > PFHSK (6 carbons) > PFBSK (4 carbons).

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