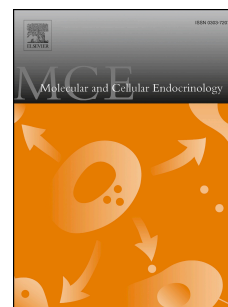


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Credit Author Statement

Amalia Conti: Data curation; Formal analysis; Investigation; Methodology; Supervision; Writing - review & editing. **Chiara Strazzeri:** Investigation; Methodology; Writing - review & editing. **Kerry J Rhoden:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Visualization; Roles/Writing - original draft; Writing - review & editing.

Perfluorooctane sulfonic acid, a persistent organic pollutant, inhibits iodide accumulation by thyroid follicular cells *in vitro*

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Abstract

Poly- and perfluoroalkyl substances (PFAS) are a class of endocrine disrupting chemicals (EDCs) reported to alter thyroid function. Iodide uptake by thyroid follicular cells, an early step in the synthesis of thyroid hormones, is a potential target for thyroid disruption by EDCs. The aim of the present study was to evaluate the acute effects of perfluorooctane sulfonic acid (PFOS) and perfluorooctane carboxylic acid (PFOA), two of the most abundant PFAS in the environment, on iodide transport by thyroid follicular cells *in vitro*. Dynamic changes in intracellular iodide concentration were monitored by live cell imaging using YFP-H148Q/I152, a genetically encoded fluorescent iodide biosensor. PFOS, but not PFOA, acutely and reversibly inhibited iodide accumulation by FRTL-5 thyrocytes, as well as by HEK-293 cells transiently expressing the Sodium Iodide Symporter (NIS). PFOS prevented NIS-mediated iodide uptake and reduced intracellular iodide concentration in iodide-containing cells, mimicking the effect of the NIS inhibitor perchlorate. PFOS did not affect iodide efflux from thyroid cells. The results of this study suggest that disruption of iodide homeostasis in thyroid cells may be a potential mechanism for anti-thyroid health effects of PFOS. The study also confirms the utility of the YFP-H148Q/I152 cell-based assay to screen environmental PFAS, and other EDCs, for anti-thyroid activity.

Key words: endocrine chemical disruptor, thyroid follicular cell, iodide, Sodium Iodide Symporter, poly- and perfluoroalkyl substances (PFAS), perfluorooctane sulfonic acid (PFOS)

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of man-made chemicals that have raised world-wide concern as persistent organic pollutants that threaten ecosystems and human health (ITRC, 2018; Wang et al., 2017). Thanks to their unique physicochemical properties - resistance to oil, water, heat and degradation - PFAS have been extensively used in manufacturing processes since the 1950's. They are present in a vast array of industrial and consumer products, including fire-fighting foams, hydraulic systems, electronic components, building materials, paints, textiles, food packaging, non-stick cookware, cleaning agents and cosmetics, among others. At least 3000 PFAS are on the global market (KEMI, 2015), of which perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) have historically been the most widely used.

The same physicochemical properties that underlie their industrial and consumer utility have also led to the ubiquitous and persistent occurrence of PFAS in the environment and biota. PFAS are bioaccumulated thanks to their ability to interact with both phospholipids and proteins (Ng and Hungerbühler, 2014). Partitioning to cell membranes, and noncovalent binding to transporters and serum proteins, accounts for the broad tissue distribution of PFAS in wildlife and man, and to their slow elimination from the body (Ng and Hungerbühler, 2014). Indeed, PFOS and PFOA have extremely long serum elimination half-lives in man of 4.8 and 3.5 years, respectively (Olsen et al., 2007).

Human exposure to PFAS has been linked to a variety of health effects, including altered hormone and cholesterol levels, impaired pre- and post-natal development, reproductive dysfunction, immunotoxicity, liver damage, and certain types of cancer (EFSA, 2018; USEPA, 2016). The World Health Organization has included both PFOS and PFOA in its assessment of endocrine disrupting chemicals (EDCs) (WHO, 2013). Epidemiologic and laboratory studies have reported thyroid disruption by both compounds, although not

consistently (Coperchini et al., 2017; EFSA, 2018; USEPA, 2016). Reported effects are highly heterogeneous, and may involve different steps in the synthesis, metabolism, clearance and action of thyroid hormones. Given that PFAS and PFOA exist in the environment in their anionic form, it is reasonable to hypothesize that, in the body, these chemicals may interfere with the homeostasis of physiological anions, and in the case of the thyroid gland, with that of iodide.

Iodine is necessary for the synthesis of thyroid hormones, thyroxine (T4) and triiodothyronine (T3). The thyroid gland concentrates circulating iodide thanks to the Sodium Iodide Symporter (NIS; SLC5A5), a secondary active transporter present on the basolateral surface of thyroid follicular cells (Portulano et al., 2014). Iodide uptake by NIS is followed by diffusion across the apical membrane into the lumen of thyroid follicles for incorporation into thyroglobulin, the precursor of thyroid hormones. Using a high throughput screening assay, the U.S. EPA's Endocrine Disruptor Screening Program (EDSP) identified PFOS as a NIS inhibitor in HEK293T cells expressing human NIS and FRTL-5 thyroid cells (Buckalew et al., 2020; Wang et al., 2019, 2018). The aim of the present study was to evaluate the effect of PFOS and PFOA on intracellular iodide concentration in thyroid cells *in vitro* in order to address impairment of iodide homeostasis as a potential mechanism for the anti-thyroid effect of PFAS.

2. Materials and Methods

2.1. Materials

Chemicals and reagents, unless otherwise specified, were obtained from Sigma-Aldrich, including PFOS (catalog #77283) and PFOA (catalog # 71468). Cell culture media, unless otherwise specified, were obtained from Euroclone (Italy). FRTL-5 cells were kindly provided by Dr. F. Curcio and Dr. F.S. Ambesi-Impiombato of the University of Udine (Italy). Plasmids pcDNA3.1-YFP-H148Q/I152L (Galiotta et al., 2001) and pcDNA3-hNIS (Smanik et al., 1996) were kindly provided by Dr. LGV Galiotta (TIGEM, Italy) and Dr. S.M. Jhiang (Ohio State University), respectively. Other materials and their sources are described in the relevant Methods section.

2.2. Cell culture and transfection

FRTL-5 normal rat thyroid follicular cells were cultured in Coon's modified nutrient mixture F-12 Ham (Sigma-Aldrich) supplemented with 5% newborn calf serum, 1 mg/ml insulin, 3.6 ng/ml hydrocortisone, 5 mg/ml apotransferrin, 10 ng/ml gly-his-lys acetate, 10 ng/ml somatostatin, 1 mU/ml thyroid-stimulating hormone (TSH), 100 U/ml penicillin and 100 mg/ml streptomycin (Ambesi-Impiombato et al., 1980). HEK-293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. All cells were maintained at 37°C in humidified air containing 5% CO₂.

A clonal population of FRTL-5 cells with stable expression of YFP-H148Q/I152L (referred to hereafter as FRTL5-YFP) was previously generated and used to monitor intracellular iodide (Rhoden et al., 2008, 2007). HEK-293 cells were transiently co-transfected with pcDNA3.1-YFP-H148Q/I152L and pcDNA3-hNIS, using Lipofectamine 3000 (ThermoFisher Scientific) according to manufacturer's instructions. Transfection efficiency,

estimated as the proportion of fluorescent cells 48h post-transfection, was typically >80%. hNIS expression in HEK-293 cells was confirmed by Western blotting using anti-hNIS(KELE) antibody.

2.3. Dynamic monitoring of intracellular iodide concentration

Intracellular iodide concentration was monitored by microscopic live cell imaging with YFP-H148Q/I152L, a fluorescent genetically-encoded iodide biosensor, as previously described (Cianchetta et al., 2010; Di Bernardo et al., 2011; Rhoden et al., 2007, 2008). Cells were sub-cultured on 25 mm diameter round glass coverslips in 6-well culture dishes. Coverslips were mounted on a thermostatically-controlled RC-21BR imaging chamber connected to a VC-8 perfusion valve control system and TC-344B temperature controller (Warner Instruments). Fluorescence intensity (excitation 500 ± 12.5 nm, emission 545 ± 17.5 nm) was monitored with an Axiovert 200 inverted fluorescence microscope (Carl Zeiss) equipped with a 40x oil immersion objective and Lambda 10C optical filter changer (Sutter Instrument Company; Crisel Instruments, Rome, Italy). Image acquisition was performed with a Coolsnap HQ² CCD (Photometrics; Crisel Instruments, Rome, Italy) and Metafluor Imaging Software (Molecular Devices; Crisel Instruments, Rome, Italy). Images were acquired with an exposure of 25 ms every 10 seconds.

Imaging chambers were perfused continuously with a serum-free balanced salt solution (BSS) composed of 137 mM NaCl, 2.7 mM KCl, 0.7 mM CaCl₂, 1.1 mM MgCl₂, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄ and 10 mM glucose (pH 7.4) at 37°C. Baseline fluorescence was recorded for at least 5 min at the beginning of each experiment, after which cells were exposed to 10 μ M NaI in order to induce iodide accumulation. The effects of test reagents (PFOS, PFOA, NaClO₄, or ouabain) on intracellular iodide concentration were evaluated according to one or more of the following protocols: Protocol I, 2 min pre-treatment +/- test

135 reagents, followed by 10 min exposure to 10 μ M NaI +/- test reagents; Protocol II, 10 min
136 exposure to 10 μ M NaI, followed by 10 min exposure to 10 μ M NaI +/- test reagents;
137 Protocol III, 10 min exposure to 10 μ M NaI, followed by 10 min exposure to BSS +/- test
138 reagents. Stock solutions of test reagents were prepared in distilled water and diluted
139 appropriately in BSS.

140 Intracellular iodide concentration was quantified from iodide-induced changes in cellular
141 YFP-H148Q/I152L fluorescence, as described previously (Cianchetta et al., 2010; Di
142 Bernardo et al., 2011; Rhoden et al., 2008, 2007). Average fluorescence intensity was
143 quantified within a region-of-interest (ROI) containing 20-50 cells, paying careful attention
144 to select an ROI without cell loss during the entire duration of the experiment. Background
145 fluorescence of a cell-free area was subtracted. To control for the decline in cellular
146 fluorescence over time due to photo-bleaching of the fluorophore, baseline fluorescence in
147 iodide-free BSS measured during the first 5 min of each experiment, was fit by non-linear
148 regression to a one-phase exponential decay curve. Cellular fluorescence (F) at each time
149 point of the experiment was then normalized to the corresponding best-fit value of baseline
150 fluorescence (F_0) to obtain a measure of relative fluorescence ($RF=F/F_0$). Intracellular
151 iodide concentration was calculated according to the equation $[I^-] = K_{0.5}(RF_{max}-RF)/(RF-$
152 $RF_{min})$ where $K_{0.5}$ is the affinity of YFP-H148Q/I152L for iodide, RF is relative fluorescence
153 at each time point during the experiment, and RF_{max} and RF_{min} represent the maximal and
154 minimal values of RF in the absence and presence of saturating iodide, respectively.
155 RF_{max} is 1, by definition, and mean values for RF_{min} (0.036) and $K_{0.5}$ (1.372 mM) were
156 determined experimentally using FRTL5-YFP cellular extracts (see section 2.4). The
157 maximal rate of iodide uptake, defined as the maximal rate of change of intracellular iodide
158 concentration ($\Delta[I^-]/\Delta t$), was estimated by fitting intracellular $[I^-]$ to a one-phase exponential
159 association equation with respect to time, and obtaining the derivative of the best fit curve.

The rate constant of iodide efflux was estimated by fitting intracellular $[I^-]$ to a one-phase exponential decay equation with respect to time.

2.4. YFP-H148Q/I152L fluorescence in cellular extracts

In order to evaluate whether PFAS interfere with the ability of YFP-H148Q/I152L to detect iodide, experiments we carried out using FRTL5-YFP cellular extracts. Cells were cultured in T75 flasks till near confluence, detached in PBS and micro-centrifuged at 12000g for 10 min at 4°C. Cellular pellets were disrupted with a pestle in 10 mM HEPES pH 7 (200 µl per pellet) and passed repeatedly through a 28-gauge needle. Lysates were centrifuged, and supernatants containing liberated YFP-H148Q/I152L were collected on ice. Pellets were re-suspended in 10 mM HEPES pH 7, homogenized and centrifuged to recover further YFP-H148Q/I152L. Samples of pooled supernatants were diluted 1:1 with HEPES 10 mM pH 7 containing NaI and/or PFOS to achieve a range of final concentrations (0.1-100 mM NaI, 0.1-10 mM PFOS). Replicate 10 µl aliquots were deposited on a coverslip, and fluorescence images were acquired with the same imaging station used for cellular experiments. Average fluorescence intensity within a constant ROI was quantified and normalized against the mean value obtained in the absence of NaI, to obtain a measure of relative fluorescence ($RF=F/F_0$). The affinity of YFP-H148Q/I152L for iodide ($K_{0.5}$) was defined as the concentration of NaI causing a 50% of maximal decrease in relative fluorescence intensity.

2.5. SDS-PAGE electrophoresis and Western blotting

Cells were lysed in ice-cold buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 1 % Triton-X, 1 mM EDTA), supplemented with Roche cOmplete Protease Inhibitor cocktail. Lysates were passed repeatedly through a 20-gauge needle on ice. Protein concentration was measured colorimetrically using the DC Protein Assay (Bio-Rad) according to the

manufacturer's instructions. Lysates were mixed 1:1 with a modified Laemmli sample buffer (125 mM Tris pH 6.8, 20% glycerol, 20% SDS 20%, 200 mM dithiothreitol, and 0.2% bromophenol blue), and incubated for 30 min at 37°C. Samples (40 µg protein) were subjected to SDS-PAGE electrophoresis using hand-cast 9% gels in a Mini-Protean Tetra Vertical Electrophoresis Cell (Bio-Rad). Samples were transferred onto 0.2 µM nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). For Western blotting, membranes were incubated with WesternBreeze Blocker/Diluent (ThermoFisher) for 1h at room temperature, primary antibody overnight at 4°C, and peroxidase-conjugated secondary antibody for 1h at room temperature, with frequent washing in Tris-buffered saline containing 0.1% Tween. Bound antibodies were detected by enhanced chemiluminescence using WESTAR Supernova substrates (Cyanagen, Bologna, Italy) and a ChemiDoc™ XRS+ Imaging System (Bio-Rad). The following antibodies were used: rabbit polyclonal anti-hNIS(KELE) antibody (Tazebay et al., 2000) generously provided by Dr. N. Carrasco (Yale University) and Dr. C. Portulano (formerly of Albert Einstein College of Medicine, USA), mouse monoclonal anti-γ-tubulin antibody clone GTU-88 (Sigma-Aldrich), goat anti-rabbit IgG-peroxidase antibody (Sigma-Aldrich), and goat anti-mouse IgG-peroxidase (Sigma-Aldrich).

2.6. Cytotoxicity

Cell viability was assessed using a Quick Cell Proliferation Colorimetric Assay Kit Plus (BioVision) based on the cleavage of water-soluble tetrazolium salt to formazan by cellular mitochondrial dehydrogenase. FRTL-5 cells were exposed to PFOS or PFOA (1-100 µM) for 1h, and viability was assayed according to manufacturer's instructions. Absorbance was read at 450 nm using a Model 680 Microplate Reader (Bio-Rad).

208 **2.7. Data analysis**

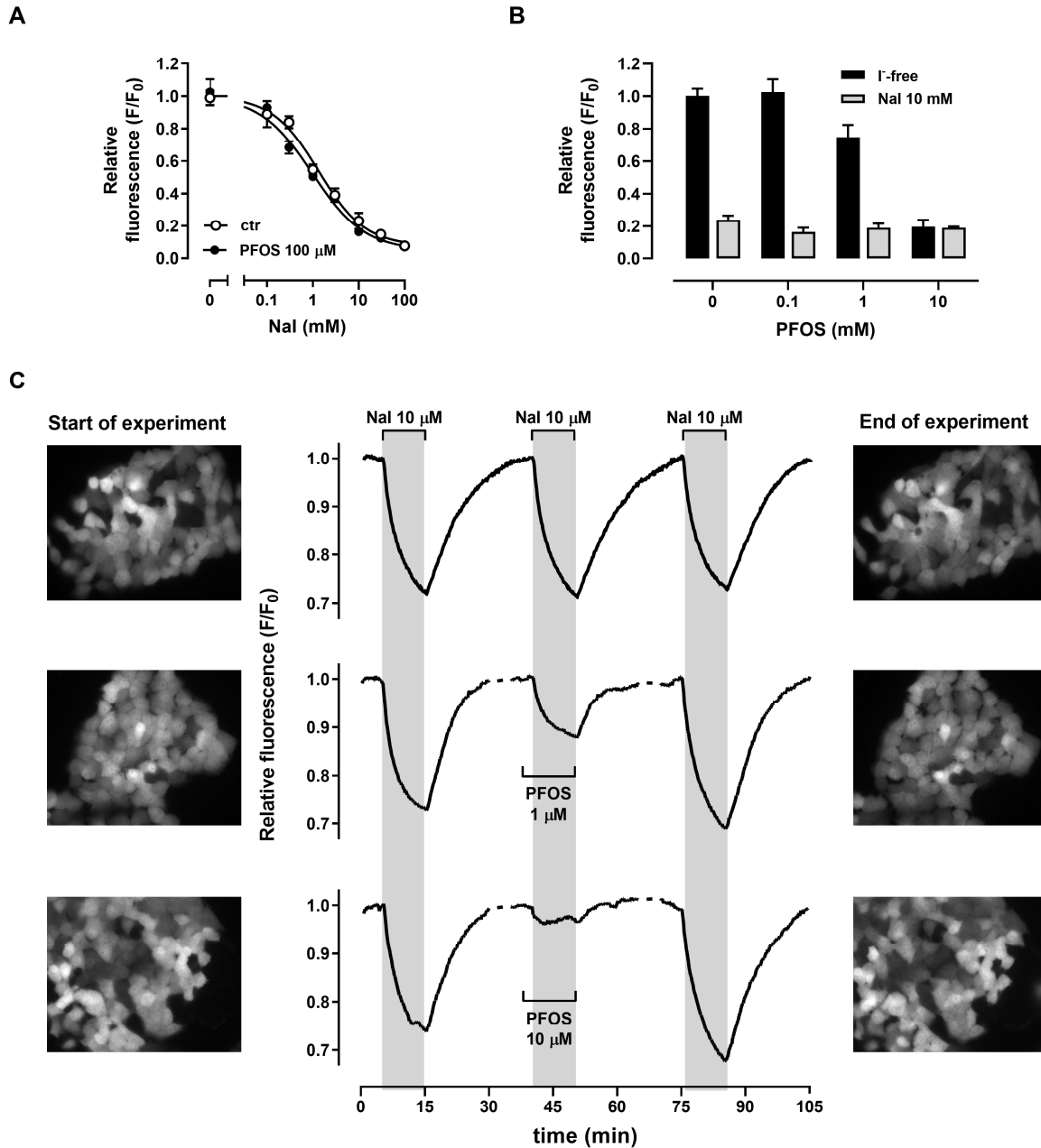
209 Data analysis, including curve fitting, iodide quantification and statistical analyses, were
210 performed using Graphpad Prism Software. Data are expressed as mean values +/- SEM
211 of n independent experiments performed on cells from different passages. Concentration-
212 response data were fit by non-linear regression to a four-parameter logistic equation.
213 PFOS potency was expressed as the half-maximal inhibitory concentration (IC_{50}).
214 Statistical differences between treatment groups were determined by ANOVA followed by
215 Dunnett's multiple comparison tests.

3. Results

3.1. Effects of PFOS and PFOA on iodide accumulation in FRTL-5 thyroid cells

YFP-H148Q/I152L was used as an iodide biosensor in FRTL-5 thyroid cells. To exclude the possibility that PFOS may interfere with the fluorophore's ability to detect iodide, its effect on YFP-H148Q/I152L fluorescence was first measured in a cellular extract following lysis of FRTL5-YFP cells. PFOS (100 μ M) had no effect on the fluorescence intensity of FRTL5-YFP extracts, nor did it alter the sensitivity of YFP-H148Q/I152L to NaI ($K_{0.5}$ = 1.3 vs. 1.0 mM in the absence and presence of PFOS respectively, $n=4$) (Figure 1A). Higher concentrations of PFOS than those used for functional studies (1-10 mM) reduced the fluorescence of FRTL5-YFP extracts, suggesting a direct interaction of PFOS with the fluorophore at such high concentrations (Figure 1 B).

Perfusion of intact FRTL5-YFP cells with 10 μ M NaI resulted in a rapid decline in cellular fluorescence that was both reversible and reproducible over time (Figure 1 C). We have previously demonstrated this response to be due to cellular uptake of iodide via the transporter NIS (Rhoden et al., 2007). The response to NaI was diminished in the presence of 1 or 10 μ M PFOS but was fully restored following removal of PFOS from the perfusate (Figure 1 C). Baseline cellular fluorescence was not affected by PFOS, nor was cellular morphology altered from the beginning to the end of experiments lasting up to 1 h. A higher concentration of PFOS (100 μ M) caused some cells (<10%) to detach from coverslips during a 10 min exposure period.



237
 238 **Figure 1.** Effect of PFOS on YFP-H148Q/I152L fluorescence of FRTL5-YFP cells. (A)
 239 Fluorescence intensity of cellular extracts in the absence/presence of 100 μ M PFOS and 0.1-100
 240 mM Nal. (B) Fluorescence intensity of cellular extracts in the absence/presence of 10 mM Nal and
 241 0.1-10 mM PFOS. (C) Representative tracings of YFP-H148Q/I152L fluorescence in living cells
 242 exposed to Nal and PFOS, and corresponding images captured at the beginning (left) and end
 243 (right) of each experiment. Repeated exposure of cells to 10 μ M Nal induced reproducible changes
 244 in fluorescence intensity (upper tracing), with recovery of resting fluorescence between each
 245 exposure. Nal-induced fluorescence changes were reduced in the presence of 1 μ M PFOS (middle
 246 tracing) or 10 μ M PFOS (lower tracing) and were restored following PFOS washout. Symbols and
 247 bars in A and B represent mean $\pm$ SEM of n=4 replicates.

Intracellular iodide concentration in FRTL5-YFP cells was estimated from NaI-induced changes fluorescence intensity. Consistent with the known ability of NIS to concentrate iodide within thyroid cells (Portulano et al., 2014), exposure to 10 μ M NaI resulted in a rapid increase in intracellular iodide concentration, reaching close to 500 μ M within 10 min (Figure 2 A). PFOS (0.1-100 μ M), added 2 min before and during NaI perfusion (Protocol I), caused a concentration-dependent decrease in iodide uptake (Figure 2 A-C). The concentration-dependence of PFOS obeyed a log sigmoidal relationship relative to both the maximal rate of iodide uptake ($\max \Delta[I^-]/\Delta t$) and the concentration of intracellular iodide reached after 10 min of perfusion with NaI ($[I^-]_{10\text{min}}$). Thus, PFOS inhibited iodide uptake with an IC_{50} value of 1.0 μ M ($\log IC_{50}$ -5.99 $\pm$ 0.20) and Hill slope of -0.99 for $\Delta[I^-]/\Delta t$, and an IC_{50} value of 0.65 μ M ($\log IC_{50}$ -6.19 $\pm$ 0.16) and Hill slope of -0.96 for $[I^-]_{10\text{min}}$ (n=6-9). The lowest concentration of PFOS causing a detectable decrease in intracellular iodide concentration was 0.3 μ M or 150 ng/ml ($p < 0.05$ Dunnett's multiple comparison test versus no PFOS control). At the highest concentration used (100 μ M or 50 μ g/ml), PFOS caused a near-complete (>95%) inhibition of iodide uptake, as did the NIS inhibitor $NaClO_4$ (Figure 2 D-F). In contrast, PFOA (100 μ M, 41 μ g/ml) had no significant effect on iodide uptake (Figure 2 D-F).

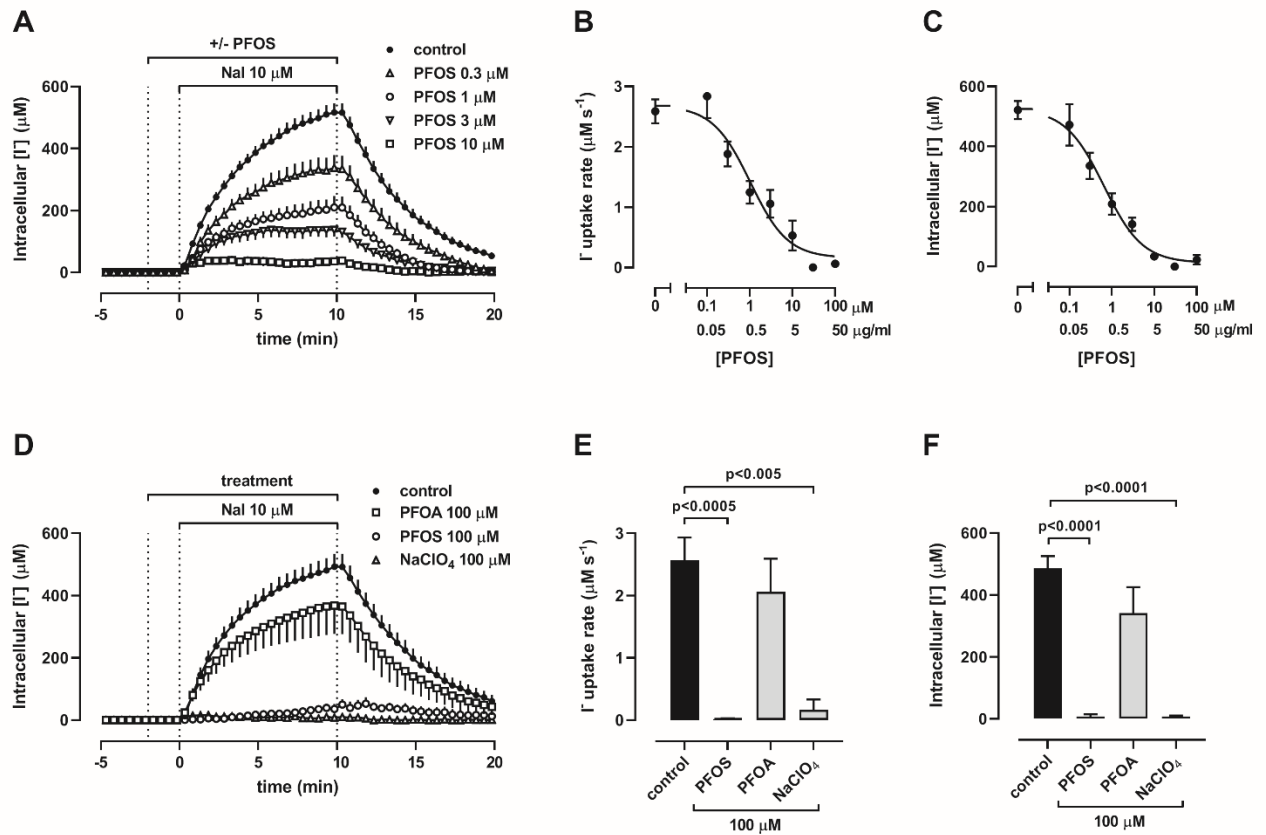


Figure 2. PFOS, but not PFOA, decreases iodide uptake by FRTL5-YFP cells. Cells were exposed to PFOS, PFOA or NaClO₄, added 2 min before and during exposure to 10 μM NaI for a further 10 min. Graphs A-C show the effects of PFOS (0.1-100 μM) on (A) the time-course of iodide uptake, (B) the maximal rate of iodide uptake, and (C) the intracellular iodide concentration at t=10 min. Graphs D-E show the effects of 100 μM PFOA, PFOS or NaClO₄ on (D) the time-course of iodide uptake, (E) the maximal rate of iodide uptake, and (F) the intracellular iodide concentration at t=10 min. Symbols and bars represent mean $\pm$ SEM of n=6-9 experiments.

PFOS was also able to reduce iodide concentration in iodide-containing FRT5-YFP cells, evidenced as a reversal of NaI-induced fluorescence changes (Figure 3 A). In this case, cells were first exposed to 10 μ M NaI for 10 min to pre-load them with iodide, followed by PFOS in the continued presence of NaI for a further 10 min (Protocol II). To control for differences in iodide uptake between replicate experiments that could mask the effect of PFOS, intracellular iodide concentration in each experiment was normalized to the level reached after 10 min of iodide uptake, immediately before PFOS addition (i.e. $[I^-]_{10\text{min}}$ is defined as 100%). PFOS caused a time- and concentration-dependent decrease in iodide concentration (Figures 3 B-C), with an IC_{50} of 0.92 μ M ($\log IC_{50}$ -6.04 $\pm$ 0.14) and Hill slope of -0.94 ($n=4-8$). The effect of PFOS (100 μ M) mimicked that of $NaClO_4$ (100 μ M), whereas PFOA (100 μ M) had no significant effect (Figures 3 D-E).

To determine whether altered sodium gradients could contribute to PFOS-induced responses, the effect of the Na-K ATPase inhibitor ouabain (100 μ M) on intracellular iodide concentration was also examined (Protocol II). Compared to the time control, ouabain reduced intracellular iodide concentration in iodide-containing cells by only 23% (figure 4 A), in contrast to the 82% inhibition induced by 10 μ M PFOS over the same 10 min time frame (from figure 3 B).

To determine whether PFOS stimulates iodide efflux, FRTL5-YFP cells were exposed to 10 μ M NaI for 10 min to pre-load them with iodide, followed by washout in NaI-free BSS with or without 10 μ M PFOS (Protocol III). Intracellular iodide concentration decreased upon NaI-removal with a similar rate constant in the presence ($0.014 \pm 0.001 \text{ s}^{-1}$) and absence ($0.012 \pm 0.002 \text{ s}^{-1}$) of PFOS (figure 4 B).

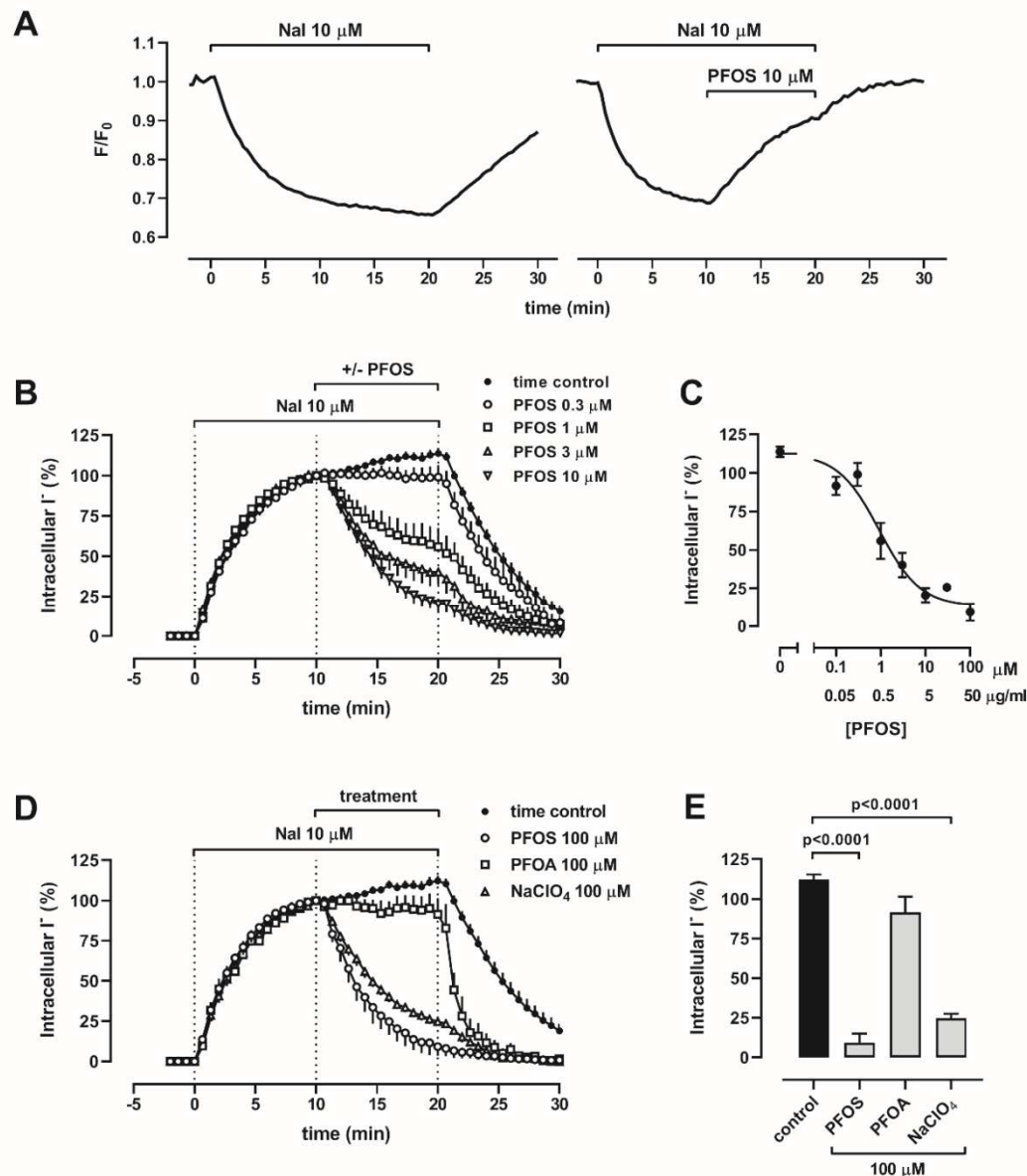


Figure 3. PFOS, but not PFOA, decreases iodide concentration in iodide-containing FRTL5-YFP cells. Cells were exposed to 10 μM Nal for 10 min, followed by 10 μM Nal +/- PFOS, PFOA or NaClO_4 for a further 10 min. (A) Representative tracing of normalized YFP-H148Q/I152L fluorescence (F/F_0). (B) Time-dependent changes in intracellular iodide concentration induced by 0-10 μM PFOS. (C) PFOS concentration-dependence of intracellular iodide concentration measured at $t=20$ min. (D) Time-dependent changes in intracellular iodide concentration induced by 100 μM PFOS, PFOA or NaClO_4 . (E) Effect of 100 μM PFOS, PFOA and NaClO_4 on intracellular iodide concentration measured at $t=20$ min. In graphs B-E, intracellular iodide concentration was normalized within each individual experiment to the level reached after 10 min of uptake, before the addition of test agents. Symbols and bars represent mean $\pm$ SEM of $n=4-8$ experiments.

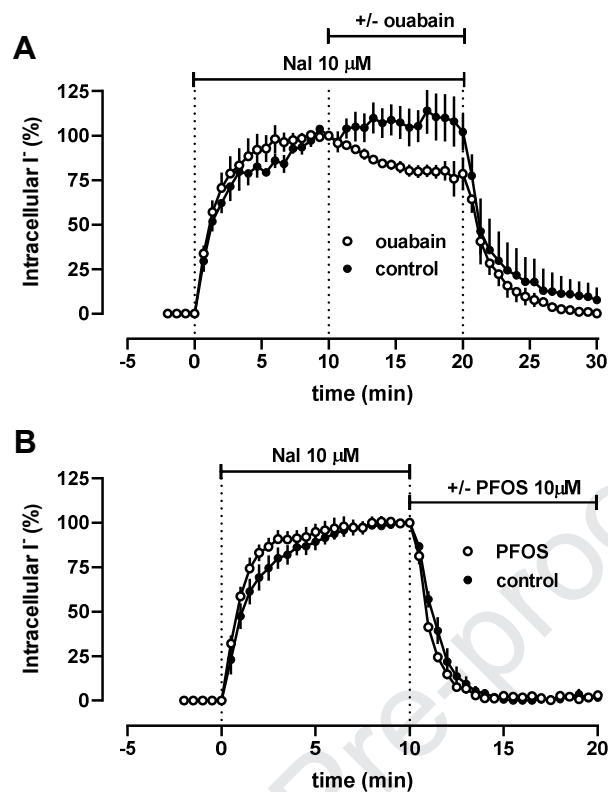


Figure 4. Effect of ouabain and PFOS on intracellular iodide concentration in FRTL5-YFP cells. (A) Cells were exposed to 10 μM Nal for 10 min, followed by 10 μM Nal +/- 100 μM ouabain for a further 10 min. (B) Cells were exposed to 10 μM Nal for 10 min, followed by Nal-free BSS +/- 10 μM PFOS for a further 10 min. Intracellular iodide concentration was normalized within each individual experiment to the level reached after 10 min of uptake, before the addition of test agents. Symbols and bars represent mean $\pm$ SEM of n=4 experiments.

3.2. Effect of PFOS on iodide accumulation in HEK-293 expressing hNIS

hNIS expression in HEK-293 cells transfected with hNIS cDNA was confirmed by Western blotting (Figure 5 A). NaI (10 μ M) decreased the cellular fluorescence of HEK293-YFP-hNIS cells by 25% over 10 min but had a negligible (<2%) effect on HEK293-YFP cells, consistent with NIS-mediated iodide uptake in the former (Figure 5 B). PFOS (10 μ M) did not alter baseline fluorescence in either cell model, but reduced fluorescence changes induced by NaI in HEK293-YFP-hNIS cells. This effect was reversible, with full recovery of NaI-induced responses following washing with physiological solution. PFOS (10 μ M) also provoked the loss of iodide from iodide-containing HEK293-YFP-hNIS cells, as indicated by the reversal of NaI-induced fluorescence changes. Quantitation of intracellular iodide (Figure 5 C-D) revealed a significant inhibition of iodide uptake by 10 μ M but not 1 μ M PFOS, in terms of both the maximal rate of iodide uptake and the near steady-state concentration reached after 10 min exposure to NaI.

3.3 Effect of PFOS and PFOA on cell viability

Cell viability of FRTL-5 cells exposed to 1-100 μ M PFOS or PFOA for 1h was assessed using a tetrazolium-based assay (Figure 6). Although one-way ANOVA indicated an overall difference ($p < 0.05$) for both compounds, Dunnett's multiple comparisons post-tests failed to reveal any significant differences in the viability of cells exposed to either compound compared to the time control. Tukey's multiple comparison test only revealed a significant decrease in the viability of cells exposed to 100 μ M versus 30 μ M PFOS ($p < 0.05$).

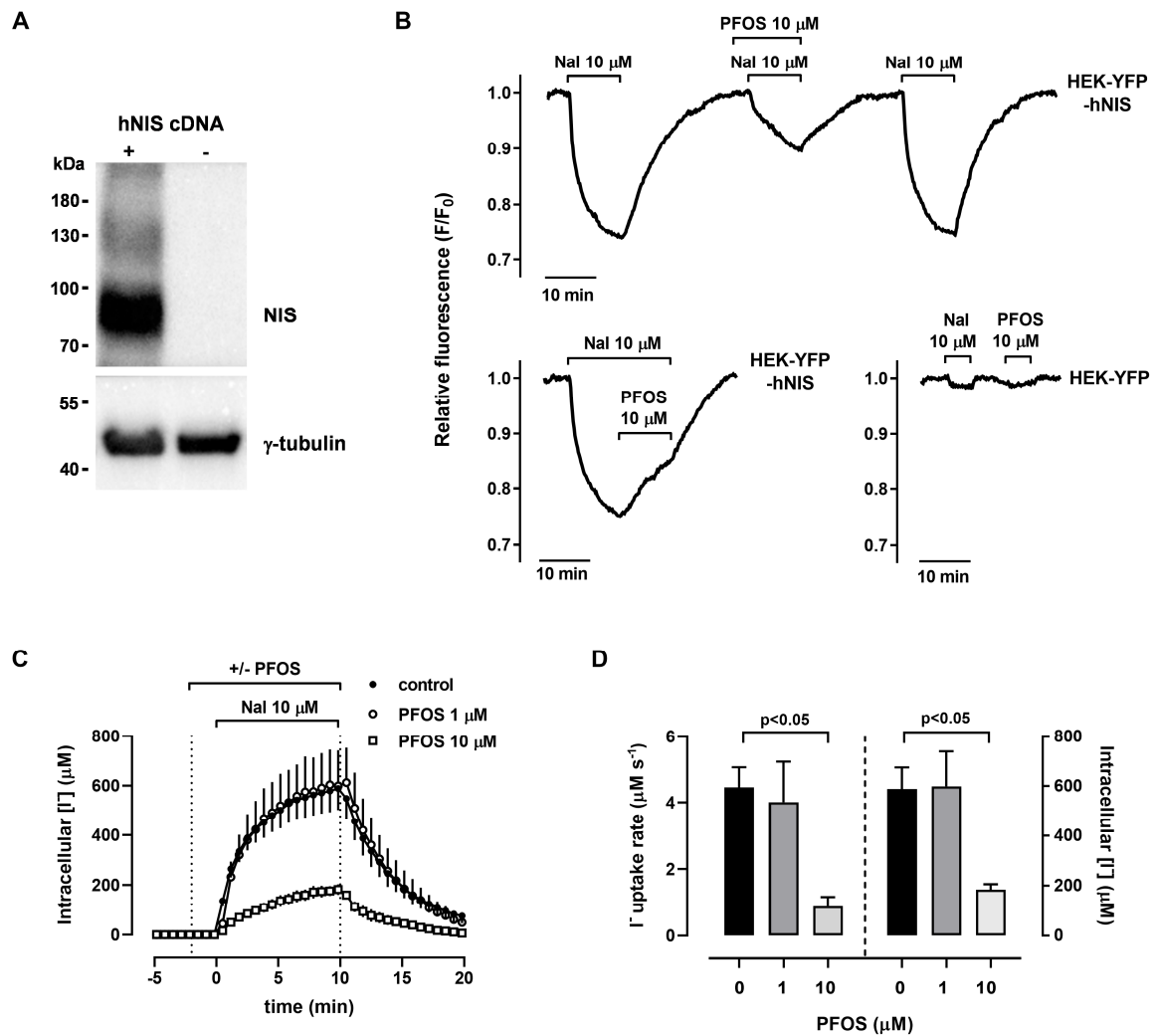


Figure 5. PFOS decreases iodide accumulation by HEK293 cells expressing hNIS. (A) Western blot detection of hNIS and γ -tubulin (loading control) in HEK-293 cell lysates (40 μ g protein), 48h after transfection with/without hNIS cDNA. (B) Representative tracings of normalized YFP-H148Q/I152L fluorescence (F/F_0) in HEK293-YFP-hNIS and HEK293-YFP cells exposed to 10 μ M Nal and/or 10 μ M PFOS. (C) Time course of iodide uptake in HEK293-YFP-hNIS in the presence of 0, 1, or 10 μ M PFOS. (D) Effect of PFOS on the maximal rate of iodide uptake and intracellular iodide concentration at $t=10$ min in HEK293-YFP-hNIS cells. Symbols and bars represent mean $\pm$ SEM of $n=4$ experiments.

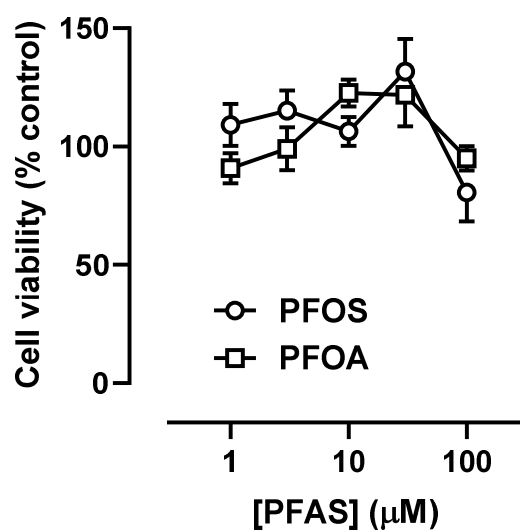


Figure 6. Effect of PFOS and PFOA on cell viability in FRTL5 cells. Cells were exposed to PFOS or PFOA for 1 h, after which cell viability was measured with a tetrazolium-based assay. Symbols and bars represent mean $\pm$ SEM of n=3-4 replicates.

4. Discussion

Cell-based assays are useful tools to investigate the mechanisms by which environmental contaminants disrupt physiological processes. Current screening assays for NIS inhibitors include radiotracer and chemical assays (Hallinger et al., 2017; Lecat-Guillet et al., 2007; Waltz et al., 2010), both of which provide end-point measurements of iodide uptake by NIS-expressing cells. In this study, we used a genetically encoded halide-sensitive biosensor, YFP-H148Q/I152L, to monitor intracellular iodide concentration by live cell imaging (Rhoden et al., 2008, 2007). Iodide quenching of cellular YFP-H148Q/I152L fluorescence is rapid, reversible, and concentration-dependent. The assay requires small numbers of cells and can be used to follow the time course of iodide influx and efflux in a single experiment. Cells can be repeatedly exposed to extracellular iodide, with reproducible changes in YFP-H148Q/I152L fluorescence. Although the technique suffers some limitations (e.g. interference by intracellular pH, not suitable for difficult-to-transfect cells), it is particularly useful to study the disruption of iodide homeostasis by EDCs.

The results of this study demonstrate that PFOS inhibits NIS-mediated iodide accumulation in thyroid follicular cells, as well as in non-thyroid cells with heterologous NIS expression. PFOS mimicked the NIS inhibitor perchlorate in its ability to both prevent iodide uptake by cells and reduce iodide concentration in iodide-containing cells. In the intact thyroid gland, the polarized expression of transport proteins on follicular cells – NIS on the basolateral surface, pendrin, anoctamin-1 and possibly other channels on the apical surface – normally ensures the unidirectional flow of iodide towards the follicular lumen for subsequent oxidation and organification. In contrast, FRTL-5 cells are neither polarized nor able to organify iodide. Since the electrochemical gradient for iodide is strongly outward, NIS inhibition will cause net loss from iodide-containing cells, even in the continued presence of extracellular iodide. PFOS did not alter the rate constant for iodide

efflux, suggesting that the decrease in intracellular iodide concentration induced by PFOS is due only to inhibition of NIS-mediated uptake, and not the activation of efflux channels/transporters.

PFOS inhibited iodide accumulation by FRTL-5 cells with an IC_{50} close to 1 μM (log IC_{50} values of -5.99 for the maximal rate of uptake $\Delta[I^-]/\Delta t$, and -6.19 for the concentration of intracellular iodide reached after 10 min of perfusion with NaI), similar to the value obtained for perchlorate in these cells using the same YFP-based assay (Cianchetta et al., 2010). Several small monovalent anions have been shown to inhibit NIS competitively, with an order of potency of $PF_6^- > ClO_4^- > BF_4^- > SCN^- \gg NO_3^- = ClO_3^- > IO_4^- \gg Br^-$ (Hallinger et al., 2017; Jones et al., 1996; Lecat-Guillet et al., 2007; Tonacchera et al., 2004; Van Sande et al., 2003). Using a high throughput assay, the U.S. EPA's Endocrine Disruptor Screening Program (EDSP) recently demonstrated that PFOS inhibits radioiodide uptake in HEK-293T cells expressing human NIS, but with a more than 10-fold lower potency (log IC_{50} -4.66 to -4.78) (Wang et al., 2019, 2018). In a further study, EDSP reported inhibition of radioiodide uptake in FRTL-5 cells and hNIS-expressing HEK-293T cells by PFOS, with log IC_{50} values of -6.45 and -5.87, respectively (Buckalew et al., 2020). Different potencies may reflect the choice of cell model (heterologous versus endogenous NIS expression), species (human versus rat NIS), detection method (radiotracer versus fluorescent biosensor), and/or other aspects of the experimental protocol (e.g. duration, iodide concentration, and temperature).

PFOA, in contrast to PFOS, had no effect on iodide uptake by thyroid cells. EDSP also found no effect of PFOA on radioiodide uptake in NIS-expressing HEK-293T cells (Wang et al., 2019). Both compounds belong to the perfluoroalkyl acid class of PFAS that consist of a fluorinated carbon chain of variable length attached to a charged functional group (ITRC, 2018). PFOS ($C_8F_{17}SO_3H$) has 8 perfluorinated carbons and a sulfonic acid head,

whereas PFOA ($C_7F_{15}COOH$) has 7 perfluorinated carbons and a carboxylic acid head (ITRC, 2018). Due to their low pK_a , PFOS and PFOA are almost completely ionized at physiological pH, existing in most biologic matrices as perfluorooctane sulfonate ($C_8F_{17}SO_3^-$) and perfluorooctane caboxylate ($C_7F_{15}CO_2^-$) respectively. It is therefore tempting to speculate that inhibition of iodide uptake by PFOS, but not PFOA, may reflect the activity of the perfluoroalkyl sulfonate but not the carboxylate anion, or may be related to the length of the perfluorinated carbon chain.

The mechanism of NIS inhibition by PFOS is unclear. Indirect effects on cell viability or sodium gradients are unlikely to play a major role, if any, since (i) cytotoxicity was not observed at PFOS concentrations lower than 100 μM , and (ii) the Na-K ATPase inhibitor ouabain only reduced intracellular iodide concentration by 23%, compared to the 82% reduction caused by 10 μM PFOS over the same time frame. Alternatively, inhibition of NIS activity by PFOS may involve direct binding to NIS and/or an interaction with NIS's phospholipid environment. PFAS are known to bind proteins and to partition into phospholipid bilayers, both properties contributing to their bioaccumulation (Ng and Hungerbühler, 2014). Our results indicate that NIS inhibition by PFOS involves reversible single-site binding, suggested by Hill slope estimates close to one, and by the complete restoration of iodide uptake following PFOS washout. Further studies that assess the structure-activity relationship of perfluoroalkyl anions as NIS inhibitors, with reference to the functional group and the length of the fluorocarbon chain, could shed light on the mechanism of NIS inhibition by PFOS.

Small competitive anionic NIS inhibitors, such as perchlorate, thiocyanate and nitrate, are transported by NIS (Portulano et al., 2014), and are concentrated within thyroid follicular cells (Di Bernardo et al., 2011; Wolff, 1964). PFOS is known to be a substrate for other SLC transporters that contribute to its hepatic accumulation, renal clearance and placental

transfer (Kummu et al., 2015; Zhao et al., 2017, 2015). Although field and laboratory studies have identified PFAAs in thyroid tissue of several mammalian and non-mammalian species, there is no evidence for active or preferential accumulation in the thyroid gland compared to other tissues (Maestri et al., 2006; Ng and Hungerbühler, 2014). Coperchini et al. (2015) detected PFOS and PFOA in cellular pellets of FRTL-5 thyrocytes grown in the presence of these compounds, and proposed a gradient-based passive diffusion mechanism. In other cell types, PFAA accumulation correlates with phospholipophilicity, suggesting that binding to membrane phospholipids may be the most important factor driving cellular accumulation (Sanchez Garcia et al., 2018). We propose that although PFOS interacts with NIS and/or its environment to inhibit iodide transport, it is not necessarily transported by NIS into cells because of its size or other physicochemical properties.

The lowest concentration of PFOS to inhibit iodide uptake in FRTL-5 thyroid cells was 0.3 μ M (150 ng/ml), a concentration that is only observed in human serum following extreme exposures. PFOS has been detected in the blood of most individuals tested in industrialized nations as a result of occupational, domestic or other accidental exposure. Average serum PFOS levels in the general population are typically <1-30 ng/ml, with maximum individual values occasionally reaching into the 100-1000 ng/ml range (Antonia M Calafat et al., 2007; Antonia M. Calafat et al., 2007; Kannan et al., 2004; Li et al., 2017; Olsen et al., 2003). Thanks to regulatory interventions and voluntary industrial efforts to limit PFAS production, serum PFOS levels in the general population are gradually declining in many countries (Glynn et al., 2012; Kato et al., 2011; Toms et al., 2014). Nevertheless, the widespread use of manufactured goods containing PFAS can result in unexpectedly high exposure levels. High serum PFOS levels (median 345 ng/ml, range 24-1500 ng/ml) were detected in people exposed to contaminated municipal drinking

water, due to the use fire-fighting foams in a nearby military airfield (Li et al., 2018). In a Canadian family, moderately high levels of serum PFOS (range 15.2–108 ng/ml) were linked to dust ingestion and/or inhalation following home carpet treatment with Scotchguard formulations (Beesoon et al., 2012). Although the present study suggests that PFOS inhibits NIS at concentrations that may be relevant during exceptionally high *in vivo* exposures, an important caveat for the interpretation of the inhibitory concentrations we report, is the absence serum during *in vitro* exposures. It remains to be determined whether binding to serum proteins such as albumin may alter the potency of PFOS as a NIS inhibitor.

Epidemiologic and clinical data on the effects of PFAS exposure on thyroid disease risk and thyroid hormone levels are mixed and often inconclusive (reviewed by Coperchini et al., 2017; EFSA, 2018; USEPA, 2016). NHANES studies reported gender-dependent associations of serum PFOS and/or PFOA with treated or subclinical thyroid disease in the US adult population (Melzer et al., 2010; Wen et al., 2013). An association between serum PFAS levels and thyroid disease was also found in exposed chemical plant workers and nearby community residents (Winqvist and Steenland, 2014), but not in another population exposed to high PFAS levels through contaminated municipal drinking water (Andersson et al., 2019). PFAS levels show positive/negative, null or ambiguous associations with TSH and thyroid hormone levels in several types of populations including exposed individuals, the general population, pregnant women and mother-infant pairs (Berg et al., 2015; Blake et al., 2018; Inoue et al., 2019; Li et al., 2017; Seo et al., 2018; Tsai et al., 2017). The lack of consensus may reflect differences in study design, population characteristics (size, developmental stage, gender, ethnicity), thyroid status (undiagnosed thyroid disease, iodine sufficiency), and exposure (concentration, duration, mixture effects). In fact, people are exposed to complex mixtures of PFAS present in the

environment, as well as other endocrine disruptors and stressors targeting the HPT axis at different levels. Webster et al. proposed a “multiple hit hypothesis,” postulating that individuals with multiple thyroid stressors (e.g. pregnancy, low iodine, thyroid antibodies), may be more susceptible to PFAS-induced thyroid disruption. (Webster et al., 2016, 2014). Thus, in pregnant women, serum PFAS levels were positively associated with TSH and negatively associated with free T4, but only in women with high levels of thyroid peroxidase antibodies (TPOAb) suggestive of auto-immune thyroid disease (Webster et al., 2014). Furthermore, in the US adult population, associations between serum PFAS and thyroid parameters occurred only those with high TPOAb and low iodine status (Webster et al., 2016). Recent studies have also addressed the combined effects of multiple PFAS in pregnant women, finding either positive or limited associations of PFAS mixtures with thyroid hormones in maternal and cord sera (Lebeaux et al., 2020; Preston et al., 2020).

In vivo studies in rats have shown that PFOS decreases circulating thyroid hormone levels without changing TSH (reviewed in Coperchini et al., 2017). This is contrary to the expected hypothyroid effect of NIS inhibition, such as that induced by perchlorate (Männistö et al., 1979). Several mechanisms for PFOS-induced hypothyroxemia have been proposed, including competitive binding to thyroid hormone transport proteins, increased conversion of thyroxine (T4) to triiodothyronine (T3) by type 1 deiodinase (Yu et al., 2009), and increased hepatic clearance (Coperchini et al., 2017; Yu et al., 2009). Furthermore, our study evaluated acute, but not long-term, effects of PFOS/PFOA on NIS-mediated iodide accumulation in thyroid cells. Chronic PFOS exposure had no effect on NIS mRNA levels in rats (Yu et al., 2009), but increased them in zebrafish larvae (Shi et al., 2009). Further studies to evaluate the long-term effects of PFAS on NIS expression,

localization and function, at concentrations that mirror serum levels, will be critical to extrapolate *in vitro* findings to the interpretation of *in vivo* responses.

5. Conclusion

Although PFAS disruption of thyroid function is still controversial, potential effects may occur through a multitude of mechanisms. Using a cell-based fluorescence assay, we have shown that PFOS inhibits NIS-mediated iodide uptake by thyroid cells *in vitro*. Although inhibition occurs at PFOS concentrations that are unlikely to impact the general population, it may be relevant in susceptible individuals accidentally exposed to exceptionally high levels. Mechanisms other than NIS inhibition may be involved in thyroid disruption by PFOA. Despite the gradual phase-out of PFOS and PFOA from industrial and commercial applications, the widespread use and persistence of PFAS in the human habitat highlights the need for further studies to address the health effects of this important class of anthropogenic chemicals.

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588 **7. Author contributions**

589 AC: Data curation; Formal analysis; Investigation; Methodology; Supervision; Writing -
590 review & editing. CS: Investigation; Methodology; Writing - review & editing. KJR:
591 Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation;
592 Methodology; Project administration; Resources; Supervision; Visualization; Roles/Writing
593 - original draft; Writing - review & editing.

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Highlights

- Disruption of thyroid iodide homeostasis by environmental contaminants
- Intracellular iodide in thyroid cells monitored with a fluorescent biosensor
- PFOS inhibits iodide uptake in thyroid cells via the Sodium Iodide Symporter
- PFOS does not stimulate iodide efflux from thyroid cells