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Characterization of non-radiolabeled Thyroxine (T_4) uptake in cryopreserved rat hepatocyte suspensions: Pharmacokinetic implications for PFOA and PFOS chemical exposure

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Abstract

The alteration of thyroxine (T_4) cellular uptake by an environmental chemical can serve as a contributing factor in thyroid hormone (TH) disruption. This study describes a non-radiolabeled (LC-MS/MS) oil-filtration technique designed to characterize the mechanism(s) responsible for T_4 cellular uptake in cryopreserved rat hepatocyte suspensions. The environmental chemicals perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) that are known thyroid-disrupting agents were evaluated for their potential effect on T_4 cellular uptake. At 37°C, hepatic T_4 uptake demonstrated saturable kinetics with increasing T_4 concentrations (0.1–15 μM), while a linear uptake rate was detected at 4°C that is consistent with passive diffusion. Michaelis-Menten analysis of carrier-mediated (enzymatic) T_4 uptake (37–4°C) displayed a V_{max} value of 3.06 pmoles/10^6 cells/sec and a K_M value of 2.93 μM and was determined to be the predominant uptake process (>70%) versus passive diffusion. Cyclosporin A (CsA) chemically inhibited T_4 uptake rates with no discernable effect on passive diffusion, whereas PFOA/PFOS showed no inhibitory effect on T_4 uptake. However, T_4 uptake assays conducted with PFOA/PFOS and the T_4 serum carrier protein transthyretin (TTR) displayed a dose-response increase in hepatic T_4 uptake rates correlating with increased T_4 free fraction values. Our findings indicate the kinetic determinant of T_4 hepatic uptake was predominantly carrier-mediated as demonstrated by an enhanced first-order rate of T_4 uptake compared to passive diffusion. These *in vitro* findings provide new mechanistic and physiological insight regarding decreased T_4 serum concentrations (hypothyroxinemia) previously observed within *in vivo* rodent studies following perfluorinated chemical exposure.

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INTRODUCTION

Thyroid hormones (THs) play a crucial role in a vast array of physiological processes including regulation of cellular metabolism, growth and neural development (Dong and Wade, 2017). The thyroid gland predominantly produces and secretes the prohormone thyroxine (T_4) into circulation, where the majority (>99%) is bound to serum proteins and only a small fraction of free T_4 is dissociated (Richardson et al., 2015). The free T_4 fraction in blood is then available for cellular uptake by the target tissue and metabolized via an intracellular deiodination reaction to the biologically active hormone, triiodo-L-thyronine (T_3) (Mendel, 1989). Thus, the process of T_4translocation across the plasma membrane is required for cellular action (Visser et al., 2011).

Characterizing the mechanism(s) responsible for T_4 uptake across the plasma membrane is critical for pharmacokinetic assessment of T_4 serum levels during environmental chemical exposures. Historically,THs were generally believed to enter target cells solely by cellular diffusion due to their highly lipophilic nature and this assumption was highly accepted and persisted for decades without further investigation (for a historical overview, see Hennemann et al., 2001). Under this premise, environmental chemical assessment on T_4 cellular uptake poses little concern as passive transcellular transport is a concentration-gradient dependent process not subject to chemical interaction (Sugano et al., 2010). However, it is now understood through results from a multitude of in vitro primary and transfected cell line studies that carrier-mediated (enzymatic) transport influences T_4 cellular uptake (Jayarama-Naidu et al., 2015; Friesema et al., 2003; Visser et al., 2011). Unlike passive diffusion, T_4 carrier-mediated uptake can undergo substrate saturation due to a finite number of transporter-enzyme binding sites. Importantly, T_4 carrier-mediated uptake processes are also susceptible to environmental chemical interaction(s), which presents a viable mode of action for potential TH disruption that receives little attention in human health risk assessment (Marchesini et al., 2008; Connors et al., 2010).

Under physiological conditions, the rate of TH uptake from blood serum is influenced by the dissociation of free TH from the fraction bound to serum carrier proteins (Mendel, 1989). In humans, there are three main TH serum carrier proteins that include albumin, transthyretin (TTR) and thyroxine-binding globulin(TBG), whereas albumin and TTR are primarily found in adult rodent (Richardson et al., 2015). Among these proteins, TBG has the highest affinity for T_4, followed by the intermediate TTR, and the low binding affinity of albumin. TTR is of particular importance due to its prominent role in T_4 tissue delivery and its highly conserved nature between both human and rodent species (Richardson et al., 2015; Robbins, 2000). TTR is also found as the primary TH serum protein secreted in cerebrospinal fluid, which plays a critical role in fetal neurodevelopment and function (Palha, 2002). Thus, environmental chemical alteration of free T_4 dissociation from the TTR protein-binding complex serves as a potential mode for TH disruption regarding both cellular diffusion and carrier-mediated transport.

The liver plays a major role in the metabolism and excretion of TH and strongly influences systemic blood TH concentrations (Richardson et al., 2013). Primary hepatocytes serve as an in vitro system of choice for assessing hepatocellular uptake as they maintain a multitude of

endogenous transport proteins including: organic anion transporting polypeptide (OATP), organic anion transporter (OAT), monocarboxylate transporter (MCT) and sodium taurocholate *co*-transporting polypeptide (NTCP) families that have been previously characterized for TH uptake (Soars et al., 2007). Rodent hepatocytes provide advantages over human cells due to minimal inter-donor variability, standardized tissue storage protocols and accessibility to in vivo testing for optimization of physiologically-based pharmacokinetic models (Yabe et al., 2011). The establishment of rodent models provides valuable mechanistic information needed for further species extrapolation and variability assessment of TH hepatic uptake within advanced human systems. However, the application of diverse hepatocyte methodologies, as well as the complexity of investigating the simultaneous contributions of passive diffusion and carrier-mediated transport, has led to variable results in characterizing TH uptake processes (Hennemann et al., 2001; Rao and Rao, 1983). Furthermore, the historical radioactive method for evaluation of TH uptake is compromised by safety and handling issues and the availability of a limited number of TH substrates as well as the inability to monitor for metabolites of interest (Jayarama-Naidu et al., 2015).

The environmental chemicals PFOA and PFOS are potential thyroid disrupting agents, which have received special attention regarding chemical interaction with T_4-TTR binding affinities and overall impact on TH homeostasis (Lee and Choi, 2017). PFOA and PFOS are members of a broad chemical class of per-fluorinated alkyl substances (PFASs) characterized by fully or partially fluorinated alkyl chains containing an anionic charged functional group. PFASs unique physical and chemical surfactant properties that allows for oil and water repellency has led to widespread industrial and consumer applications. Human exposure to PFOA and PFOS from general household products and their persistence in the environment has led to significant detection levels in the general population (Coperchini et al., 2017; Washington et al., 2015).

Previous in vitro studies have examined the displacement of free T_4 from TTR upon PFAS exposure, which then supports PFAS in vivo rodent findings of the transient increase of free T_4 available for liver metabolism and excretion (Ramhoj et al., 2018; Chang et al., 2008; Weiss et al., 2009). However, delineation of the transport mechanism(s) responsible for T_4 hepatic cellular uptake in the presence of PFAS chemicals are not well defined. The objective of this study is to characterize T_4hepatic uptake using cryopreserved rat hepatocyte suspensions to provide mechanistic insight during PFOA and PFOS chemical exposures.

MATERIALS AND METHODS

Chemicals and Reagents

L-thyroxine (T_4; (2*S*)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5diiodo-phenyo] propanoic acid), (T_3; (2*S*)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl] propanoic acid), cyclosporin A (CsA), PFOS, octanoic acid, silicone oil, and mineral oil were purchased from Sigma Aldrich (St. Louis, MO). PFOA was purchased from Oakwood chemicals (Estill, SC) (Fig. 1).

$^{13}C_6$-labeled T4 ($^{13}C_6$-T_4) was purchased as an internal standard from Cambridge Isotope Laboratories (Tewksbury, MA). Williams' media, hepatocyte thaw media (Invitrogen; CM7500) and hepatocyte cell maintenance supplement (CM4000) were purchased from Invitrogen (Waltham, MA). The hepatocytes were maintained in Williams' media supplemented by the following (final concentration): Dexamethasone: 0.1 μM, Penicillin/Streptomycin: 0.5%, human recombinant insulin: 6.25 μg/mL, human transferrin: 6.25 μg/mL, selenous acid: 6.25 ng/mL, bovine serum albumin (BSA): 1.25 mg/mL, linoleic acid: 5.35 μg/mL, GlutaMAX: 2 mM, HEPES, pH 7.4: 15 mM). The serum protein transthyretin (TTR) derived from human plasma was purchased from Athens Research & Technologies (Athens, GA). Evolute Express (CX, 10 mg) 96-well solid-phase extraction (SPE) plates were purchased from Biotage (Charlotte, NC). Microcon® centrifugal filter devices with Ultracel YM-10 membrane (10,000 Da nominal molecular weight limit) were purchased from Millipore (Bedford, MA). All solvents and acids used in extraction and elution were of Optima-Grade performance and purchased from Thermo Fisher (Pittsburgh, PA).

T_4 Hepatocyte Uptake Assay

Oil-filtration tube preparation.—Fisherbrand 0.4 mL polyethylene micro centrifuge tubes set in a 96-well format were used for all oil-filtration experiments. A 100 μL aliquot of 0.5 M cesium chloride(CsCl) prepared in deionized water was placed in the bottom of the 0.4 mL tubes. Next, a 100 μL aliquot of mineral oil and silicon oil (1:5 *v*/v, respectively) was carefully layered on top of the CsCl layer (Li et al., 2013). The tubes were then centrifuged at room temperature at 14,000 rpm for 30s to even the oil layer and disperse all air bubbles.

Cryopreserved hepatocyte thawing.—Pooled male cryopreserved Sprague-Dawley rat hepatocytes were purchased from Bioreclamation IVT (Baltimore, MD) and stored in liquid nitrogen until use. Cryopreserved rat hepatocytes were removed from liquid nitrogen and immediately thawed in a 37 °C water bath until a small ice pellet remained in the bottom of the vial. Contents of the vial were then directly transferred into 37 °C hepatocyte thaw media in a 50-mL conical tube, the cells were then gently inverted and placed in a centrifuge at room temperature to be spun at 86 × g for 6 min. The supernatant was then aspirated off not to disturb the pellet, and the cells were gently re-suspended in 3 mL of freshly prepared (37 °C) Williams' medium containing the hepatocyte maintenance supplement, henceforth referred to as running buffer. Cell counts and viability were then performed using trypan blue exclusion and disposable cell counting chamber (Nexcelom Bioscience) and were brought to a cell concentration of 1.0×10^6 live cells/mL (− TTR) and 2.0×10^6 live cells/mL (+TTR) with viability criteria >80%. The hepatocyte suspension was then placed in a 37 °C orbital shaking water bath at 50 RPM for 10 min or placed on ice for 4 °C assays.

Chemical preparation.—All T_4 (5 mM) and chemical stock solutions (20 mM) were prepared in DMSO. For experiments containing TTR, lyophilized TTR powder was first solubilized in freshly prepared running buffer (37 °C) on the day of experimentation at a concentration of 1 mg/mL. T_4 solutions were then prepared in running buffer (±TTR) in a glass vial and then dispensed into low-binding 1.5 mL microcentrifuge tubes (Midsci™, MAXYmum Recovery), where upon addition of individual test chemicals, both T_4 and the

test chemical resulted in 4(x) the final desired concentration. DMSO was used for all vehicle control experiments and maintained <0.5% of the final concentration in all chemical mixtures. All T_4 chemical solutions with TTR present were gently mixed to not disturb protein integrity and then placed on a heating block at 37 °C for a 30 min equilibration timeframe.

Kinetic assessment of T_4 uptake.—Following pre-incubation of the hepatocytes at 37 °C for 10 min, 150 μL of cell suspension was added to 300 μL of running buffer in a low binding microcentrifuge tube and gently inverted to mix. The hepatic uptake assay was then initiated by the addition of 150 μL of T_4 (± test chemical; ±TTR) with a gentle pipetting motion up and down to ensure proper mixing. Single aliquots (100 μL) were then subsampled from the microcentrifuge tube (25 K cells/aliquot; −TTR and 50 K cells/aliquot; +TTR), and layered on top of the previously prepared oil-filtration tubes and immediately centrifuged (14,000 rpm for 30s) at each of the designated time points (15, 30, 60, and 90s) to separate the cells from the media and cease the reaction. The experimental procedure was conducted in the sequence of vehicle control (DMSO) timeline followed by the chemical test article timeline(s), which represents one replicate. This sequence was then repeated in triplicate allowing for control replicates to account for any variability in the hepatocyte suspension over the time required to complete the experimentation.

For passive diffusion (4 °C) assays, hepatocytes and chemical reagents were placed on ice for a minimum of 15 min prior to conducting the assay. TTR chemical solutions were initially incubated at 37 °C for 30 min to ensure the same T4-TTR equilibration timeframe before being placed on ice for 15 min for the 4 °C experiments. Designated time points used for 4 °C samples were (30, 60, 90, and 120 s) due to slower uptake rates. All oil filtration tubes were then placed in a − 80 °C ultra-freezer overnight before extraction.

Free T_4 concentrations—To determine free T_4 concentration levels present within the running buffer solutions without hepatocytes, triple aliquots (100 μL) of the running buffer (diluted to 1×) were transferred into individual Microcon® centrifugal ultrafiltration devices and centrifuged at 12,000 rpm for 5 min. The filtrate was then prepared and analyzed for T_4 following the protocol applied for hepatocyte suspensions.

T_4 Quantification

Sample preparation.—Frozen oil-filtration tubes (−80 °C) were clipped through the middle of the oil layer using a plastic tubing cutter (Fisher Scientific; 22–088245). The lower CsCl layer containing the pelleted hepatocytes was then collected into a microcentrifuge tube, while carefully discarding the upper frozen level to avoid sample contamination of the test article. The CsCl layer was thawed at room temperature and digested using 400 μL of 80:20% (acetonitrile:water) containing 4% formic acid (4% FA). The samples were vortexed and placed on a mini orbital shaker (Versa-ORB) at 150 rpm for 10 min at room temperature, then centrifuged at 14,000 rpm for 10 min to pellet any residual oil or cell debris. A 200 μL aliquot of supernatant was removed and placed into a clean microcentrifuge tube, spiked with 100 μL internal standard ($^{13}C_6$-T_4,100 ng/mL, 0.1 M NaOH), and diluted to a 2 mL final volume with water (4% FA) for solid phase extraction.

T_4 solid phase extraction.—Samples were processed through solid phase extraction (SPE) using a 96-well plate (Evolute Express CX, 10 mg, 1 mL, Biotage, Charlotte, North Carolina) and a vacuum filtration manifold (Multiscreen HTS, Millipore). The SPE 96-well plate was conditioned with 400 μL of methanol followed by 400 μL of water (2% FA). Sample extracts (2 mL) were then loaded onto the SPE and vacuumed to dryness, each well was then washed with 400 μL of water (2% FA) followed by 400 μL methanol. T_4 was then eluted from the SPE sorbent using a triplicate rinse (100 μL) of 50:50% (methanol:acetonitrile) containing 5% NH_4OH for a 300 μL final volume collection into a 96 well plate. The 96 well plate contents were then evaporated to dryness using Microvap nitrogen dryer (Organomation, Berlin, MA), reconstituted in 100 μL of 60:40% (methanol:water) and sonicated for 5 min before LC/MS/MS analysis.

LC/MS/MS analysis.—The reconstituted samples were analyzed for T_3 and T_4 using an Agilent 1200 Ultra-performance liquid chromatograph (UPLC) coupled to a 6420 triple quad mass spectrometer (Agilent, Santa Clara, CA). Injections (5 uL) at a 1.0 ml/min flow rate were made into an Agilent Zorbax XDB-$_{C18}$ column (4.6 mm × 50 mm, 1.8 um particle diameter; Santa Clara, CA) maintained at 40°C. Gradient elution with methanol (solvent A) and water (solvent B) with 0.2% FA was applied under the following conditions: 60% A for 0.5 min, followed by a linear gradient to 70% A at 3.0 min, increasing to 100% A at 3.2 min, and held for a 6.5 min stop time. The column was then allowed to re-equilibrate under the original conditions for a 3 min post-time. MS/MS detection was conducted using ESI+ in multiple reaction mode under the following conditions: T_4 quantifying ion transition *m/z* 777.7→731.5, with qualifying ion transitions *m/z* 777.7→633.5 (collision energies 25V, respectively), with the fragmenting voltage set to 160 V and the cell accelerator at 7 V. T_3 quantifying ion transition *m/z* 651.8→605.9, with qualifying ion transition *m/z* 651.8→478.7 (collision energies 30 V and 35 V, respectively), with the fragmenting voltage set at 120 V and the cell accelerator at 7 V. The internal standard, L-Thyroxine ($^{13}C_6$ -T_4) was quantified based on the transition *m/z* 783.8→737.8 with the fragmenting voltage, collision energy, and cell accelerator set to 160 V, 25 V, and 7 V, respectively (Wang and Stapleton, 2010). ESI source parameters were applied according to the following: source gas temperature 350°C, gas flow 12 L/min, nebulizer 55 psi, capillary 4000 V. T_3 and T_4 standard curves (0.1–125 ng/mL) were prepared in (60:40, methanol:water) using L-thyroxine ($^{13}C_6$ -T_4) as an internal standard and verified during analysis with a check standard every 12 samples, followed by a blank sample for carryover assessment. Data processing was performed using Agilent MassHunter software (version B.04.01) and sample concentrations were determined using an internal-standard response factor.

Statistical analyses.—Total (37°C) and passive diffusion (4°C) rates of T4 uptake (pmol/(10^6 cells*s)) were determined from the slope of the regression line ($r^2 > 0.7$, quality criteria for 37°C control) of least squared error in plots of sample concentration (pmol/10^6 cells) vs time (s). Differences in uptake rates between treatment and control were determined using a Student's t-test for differences in regression slopes having a common variance (Steel & Torrie, 1980):

$$t = \frac{R_r - R_c}{\sqrt{s_p^2\left[1/\Sigma\left(X_{rj} - \overline{X}_{r*}\right)^2 + 1/\Sigma\left(X_{cj} - \overline{X}_{c*}\right)^2\right]}} \quad (1)$$

where R_r and R_c designates uptake rate for treatment and control, respectively, s_p^2 is the pooled estimate of the common variance, X_j is the time at which sample j was collected and $\overline{X}_*$ is the mean time of sample collection. The pooled estimate of variance is defined as (Steel & Torrie, 1980):

$$s_p^2 = \frac{\Sigma\left(Y_{rj} - \widehat{Y}_r\right)^2 + \Sigma\left(Y_{cj} - \widehat{Y}_c\right)^2}{n_r + n_c - 4} \quad (2)$$

where Y_j is the concentration of sample j, $\widehat{Y}$ is the regression estimate for the time at which Y_j was drawn and n is the number of samples included in the regression. Significant differences in treatment vs control were evaluated by comparing the t statistic to two-tailed critical values of t at p=0.05 and 0.01 for n_r+n_c-4 degrees of freedom (df).

The presence of differences between treatments and controls in free T4 was determined with Students t-test with unequal variances (Steel & Torrie, 1980).

The Michaelis-Menten constants V_{max} and K_M were determined based on nonlinear regression of T_4 uptake velocities (pmoles/sec) versus concentration using Sigma Plot (version 13.0, Systat Software,San Jose, CA) using a one-site saturation component.

RESULTS

A linear rate of T_4 uptake was determined using oil filtration of cryopreserved male rat hepatocytes in suspension at both 37 °C and 4 °C. A representative kinetic profile of T_4 hepatic uptake (37 °C) versus time (15, 30, 60, and 90 s) determined from individual timelines conducted in triplicate is depicted in Fig. 2. The rate of T_4hepatic uptake remained linear up to approximately 120 s at physiological temperature (37 °C) before beginning to plateau. A positive y-intercept was observed from the linear regression of the measured T_4 uptake time course, indicating a rapid phase of T_4 cellular partitioning during the initial lag period (<15 s) of the cell separation procedure. All T_4 hepatic uptake rates reported were determined from individual time lines conducted in triplicate.

The potential deiodination of T_4 resulting in T_3 was not observed as T_3 levels remained below the level of detection. The coefficient of variation (CV) for all 37 °C control hepatic uptake rates with and without TTR, representing daily and batch LOT variability (Table 1), remained below 30% for all experimentation. All T_4hepatic uptake activity ceased via disruption of cellular function and cell membrane integrity following a freeze thaw cycle (−80 °C for 30 min) of hepatocytes.

Hepatic T_4 uptake velocities conducted at 37 °C demonstrated saturable enzymatic (carrier-mediated + passive diffusion) kinetics with respect to increasing T_4substrate concentrations

(0.1–15 μM), whereas assays conducted at 4 °C displayed a nonsaturable linear relationship to increasing T_4 concentrations representative of passive diffusion (Fig. 3).

All chemical inhibition assays were conducted with T_4 (1.0 μM) initial concentration to ensure substrate levels were maintained well below the determined K_M value. Cyclosporin A (CsA), a broad range chemical inhibitor for membrane bound transporting proteins (transporters), was examined in a dose-dependent (0.1–10 μM) manner for inhibition of T_4 uptake (Karlgren et al., 2012). At 37 °C, a significant decrease ($p < .05$) in T_4 total hepatic uptake was observed at low CsA (0.1 μM) concentration levels in comparison to controls (Fig. 4A). No statistical difference in T_4 uptake rates were observed at 4 °C while varying CsA levels (0.1–10.0 μM). In contrast to the chemical inhibition displayed by CsA at 37 °C, T_4 uptake assays conducted in the presence of the environmental chemicals PFOA (0.1–10 μM) and PFOS (10 μM; data not shown) displayed no statistical difference in T_4 hepatic uptake rates at either 37 °C or 4 °C indicating no effect on T_4 membrane transport (Fig. 4B).

PFOA and PFOS are known to competitively bind to the thyroid hormone serumcarrier protein transthyretin (TTR) resulting in the displacement of the biologically active free T_4 available for uptake (Weiss et al., 2009). To assess the binding potency of PFOA to TTR and the influence on T_4 hepatocyte membrane transport, we first assessed the rate of T_4 uptake in the presence of varying TTR concentration levels (0–0.125 μg/mL) (Fig. 5A). Results demonstrated increasing TTR levels significantly decreased ($p < .05$) the rate of T_4 hepatic uptake at 37 °C, which correlated with free T_4 analysis determined within the initial running buffer solution as less free T_4 was detected with increasing TTR concentration (Fig. 5B). T_4 hepatic uptake rates at 4 °C also demonstrated a declining trend with increasing TTR levels, but no statistical significance was achieved likely due to the slower rate of passive diffusion. A temperature dependency on free T_4 levels versus T_4 -TTR bound was observed as less free T_4 was detected in 4 °C samples compared to 37 °C. Calculations of T_4-K_{ow} values using the SPARC physical-chemical parameter model predicted a higher K_{ow} value for T_4 (log K_{ow} 8.82) at 4 °C in comparison to 37 °C (log K_{ow} 7.42), which corresponds with an increased T_4 binding affinity for TTR at lower temperature values (Hilal and Karickhoff, 2004). In control samples containing no TTR, the decrease in free T_4 at lower temperatures is likely attributed to increased T_4 binding to BSA (1.25 mg/mL) and other constituents present within the running buffer.

To assess PFOA influence on T_4 (1.0 μM) uptake rates in the presence of TTR, PFOA concentrations were varied (0.1–10 μM) while maintaining a constant TTR (62.5 μg/mL) level (Fig. 6A). At both 37 °C and 4 °C, an upward trend in T_4 uptake rates was observed in a dose-response manner with increasing PFOA concentrations. At 37 °C, a significant increase in T_4 uptake rates were measured within the 8 μM ($p < .05$) and 10 μM ($p < .01$) PFOA assays, while displaying a 3 to 4 fold increase above the 4 °C T_4 uptake assays. Free T_4 concentrations trended upward at both 37 °C and 4 °C in the presence of increasing PFOA concentration with less free T_4 being detected in 4 °C samples versus 37 °C (Fig. 6B). The evaluation of free T_4 levels at 4 °C containing PFOA (8–10 μM) show the free T_4 levels at 4 °C exceed those within the 37 °C control; however, the T_4 uptake rates at 4 °C remained well below (~50%) the 37 °C control. These results indicate an increased first-order rate associated with T_4 carrier-mediated transport remained the predominant process at 37 °C.

To further assess T_4 membrane transport and chemical displacement of T_4 in the presence of TTR, T_4 (1.0 μM) hepatic assays were screened at 37 °C in the presence of (10 μM) chemical inhibitor CsA, PFOA, PFOS and the natural free fatty acid octanoic acid (Fig. 7). Perfluorinated chemical results indicate increasing T_4 uptake rates correlated to free T_4 levels with PFOA > PFOS, while both compounds demonstrated significantly higher T_4 uptake rates ($p < .01$) versus control. Octanoic acid also displayed a significant increase ($p < .05$) in T_4 hepatic uptake, however, unlike PFOA/PFOS no discernable effect on free T4 level was observed suggesting an alternative mode of action. Previous studies indicate octanoic acid can serve as a membrane-disrupting agent that may affect cell membrane fluidity and surface potential allowing for increased chemical penetration (Tan et al., 2017; Fu et al., 2015). The chemical inhibitor CsA showed no displacement of free T_4 from TTR in comparison to control and significantly decreased ($p < .05$) T_4 uptake activity, further confirming T_4 carrier-mediated transport.

DISCUSSION

Adequate levels of TH are necessary for proper cellular function. The alteration of T_4 translocation across the cellular membrane may serve as a contributing factor in TH disruption. Due to its high lipophilicity, T_4 was historically believed to enter target cells solely by passive diffusion, a process that is not susceptible to chemical perturbation (Hennemann et al., 2001). However, it is now understood from a multitude of in vitro studies that T_4 cellular uptake is influenced by carrier-mediated (enzymatic) transport, which requires further evaluation for potential environmental chemical interaction (Visser et al., 2011; Jayarama-Naidu et al., 2015; Friesema et al., 2003).

In this study, we applied an oil-filtration technique using LC-MS/MS to characterize the mechanism(s) responsible for T_4 cellular uptake using cryopreserved rathepatocytes in suspension. At physiological temperature (37 °C), a saturable kinetic profile for T_4 cellular uptake was observed with increasing substrate concentration. These results indicate the presence of a T_4 carrier-mediated uptake component as saturation occurs when the number of T_4 molecules exceeds the number of enzymebinding sites present (Sugano et al., 2010). At 37 °C, both carrier-mediated transport and passive diffusion occur simultaneously, whereas under low temperatures enzymatic activity generally ceases. The coexistence of a T_4 passive component was observed within our 4 °C assays as T_4 uptake rates remained linear with respect to substrate concentration. Further validation of T_4 passive transport was noted as the slope of the 4 °C T_4 uptake rates were consistent with 37 °C rates observed at experimentally high T_4 concentrations (>10 μM), where once saturation is achieved passive diffusion serves as the prevalent process (Li et al., 2013). The evaluation of the difference in T_4 uptake rates at (37–4 °C) solely defines the carrier-mediated component, which fitted via nonlinear regression analysis displayed a K_M value of 2.3 μM. The comparison of total T_4 uptake rates (37 °C) versus carrier-mediated (37–4 °C) at substrate levels within the first-order region (<K_M), demonstrated T_4 carrier-mediated uptake was the predominant kinetic determinant accounting for >70% of total T_4 hepatic uptake. Based on these in vitro results, the mechanistic criteria for physiologically-based modeling of TH hepatic uptake (clearance) at low physiological T_4 serum levels suggests parameterization of both carrier-mediated and passive diffusion processes.

In previous studies, the application of diverse experimental protocols using primary hepatocytes has led to varying results in the quantitative assessment of T_4 uptake transport (Hennemann et al., 2001). Hepatocyte uptake assays are often performed using a single incubation time point while assuming linearity. However, the linear rate of uptake can be compromised by multiple factors such as cell membrane partitioning, bidirectional passive diffusion and decrease in extracellular substrate concentration (Poirier et al., 2008). Our use of non-radiolabeled T_4 with LC-MS/MS detection allows for highly quantitative analysis while simultaneously monitoring for potential TH metabolic products. The in vitro technique we applied of rapid subsampling of hepatocytes from suspension optimized at an appropriate cell concentration, allowed for multiple measured time points (full timeline) to determine T_4 kinetic uptake rates based on a linear slope of regression. A positive y-intercept was observed and is consistent with a rapid membrane partitioning phaseof the highly hydrophobic T_4 during the lag period in the cell separation procedure (15 s) (Hallifax and Houston, 2006). It is important to note, the incorporation of the partitioning phase using a single time point assessment may lead to variable results in characterizing T_4 uptake processes (Chalmers et al., 1993; Rao and Rao, 1983; Riley and Eales, 1993).

Primary hepatocytes contain a vast array of endogenous transport proteins that can potentially mediate T_4 hepatic uptake. Further characterization of the T_4 carrier-mediated transport component was conducted within our hepatic assay system using the chemical inhibitor CsA. Clinical studies have shown CsA serves as a broad-based chemical inhibitor for the OATP transporter family (Takahashi et al., 2013; Karlgren et al., 2012). Previous in vitro studies indicate that approximately fifteen different OATP's among rodent and human have been identified to facilitate thyroid hormone uptake transport; however, limited time-course data exist delineating the role of T_4 carrier-mediated and passive diffusion processes within native hepatocytes (Visser et al., 2011). Our CsA results further confirmed T_4 carrier-mediated transport as total T_4 uptake rates (37 °C) that displayed a saturable component were chemically inhibited by CsA at low inhibitor concentration levels (0.1 μM), while showing no discernable effect on passive diffusion. These results suggest further investigation with the specific transporter rat Oatp1b2 (Human orthologs OATP1B3/1B1), which is one of the most predominantly expressed rodentOatp transporters in the liver known to mediate T_4 uptake (Hagenbuch and Meier, 2004). However, it should be noted that delineation of the role of specific transport proteins in primary hepatocytes is difficult due to the lack of specific chemical inhibitors and that multiple transporters may be functioning simultaneously. Furthermore, cell line studies conducted with overexpression of individual transporters indicate that the majority of hepatic T_4 uptake transporters are non-specific to TH, as they functionally accept a wide variety of other endogenous ligands essential for cellular function (Visser et al., 2011). A notable exception is the presence of monocarboxylate transporter (MCT8) in the liver, which has been identified to only selectively transport T_4 and other iodothyronine related compounds (Friesema et al., 2003). To the best of our knowledge, CsA chemical inhibition of MCT8 mediated T_4 uptake has not yet been investigated.

The environmental chemicals PFOA and PFOS are potential thyroid disrupting chemicals that exist as anionic surfactants at physiological pH 7.4. Research emphasis has been placed on these PFAS chemicals as they are known to reduce circulating serum levels of TH's in

vivo by increasing their metabolic clearance rate (Lee and Choi, 2017; Lau et al., 2007). In contrast to our CsA chemical inhibition results, hepatic assays conducted in the presence of increasing PFOA exposure did not alter T_4 hepatic uptake. To the best our knowledge, we believe this is the first report demonstrating PFOA does not alter T_4 carrier-mediated transport processeswithin primary hepatocytes. Furthermore, a distinguishable factor under physiological conditions is that TH's are extensively bound (>99%) to blood serum proteins, which serves as a reservoir to buffer changes in the small free T_4 fraction available for hepatic uptake (Palha, 2002). Therefore, a potential rate-altering step regarding T_4 hepatic uptake in vivo is the dissociation of free T_4 from the protein-binding complex (Mendel, 1989).

It has been previously reported that the unique physical-chemical properties of PFOA and PFOS affect the dissociation rate constant that exists between the T_4bound versus free T_4 fraction with the serum protein TTR (Weiss et al., 2009). To explore this potential interaction, we first amended our hepatocyte suspensions with the serum protein TTR. Accordingly, our results showed the increased addition of TTR serum protein resulted in decreased T_4 hepatic uptake rates due to detection of less available free T_4. A temperature dependency of lower free T_4 levels with decreasing temperature (37 °C vs 4 °C) was observed and is likely attributed to an increased T_4 protein binding affinity due to increased T_4-K_{ow} values at lower temperatures (Hilal and Karickhoff, 2004). To further characterize potential PFAS interaction on T_4 hepatic uptake in the presence of the serum protein TTR, a PFOA dose-response relationship was evaluated while maintaining constant TTR levels throughout experimentation. Under these conditions, our results demonstrated a significant increase in total T_4 hepatic uptake rates occurred with increasing PFOA exposure levels, which correlated to an increased free T_4 fraction. The rate of T_4hepatic uptake remained first-order with respect to carrier-mediated transport as depicted by an enhanced response to free T_4 levels under physiological temperature (37 °C) versus the passive diffusion component (4 °C). Subsequently, our screening of PFOS at high concentration levels (10 μM) with TTR displayed similar results as PFOA resulting in a significant increase in T_4 uptake rates due to increased free T_4values.

The identification of T_4 carrier-mediated transport as the predominant kinetic determinant in the presence of TTR was reaffirmed as CsA chemically inhibited T_4hepatic uptake while not affecting free T_4 levels. It was previously reported in a (non-cellular) TTR-binding assay that the naturally occurring octanoic fatty acidserves as a control for assessing T_4 displacement from TTR by displaying no increase in free T_4 levels (Weiss et al., 2009). While our analysis confirmed no free T_4displacement from TTR in the presence of octanoic acid, hepatic suspensions amended with octanoic acid displayed a significant increase in T_4 hepatic uptake rate, suggesting an alternate mode of T_4 translocation. Thus, previous cellular studies have shown octanoic acid can serve as a known membrane-disrupting agent that may affect cell membrane fluidity and surface potential allowing for increased chemical penetration (Tan et al., 2017; Fu et al., 2015). Our results suggest alteration of membrane fluidity was not a significant factor regarding PFOA amendments as no significant increase in T_4 hepatic uptake was observed in experiments without TTR present (Fig. 4B).

Our in vitro assessment of increased T_4 hepatic uptake during PFOA and PFOS exposure with TTR present provides new mechanistic insight required for in vivo physiological assessment of TH serum levels. We demonstrated that T_4 hepatic uptake predominantly followed first-order carrier-mediated transport within hepatocyte suspensions and the carrier-mediated process was not altered (inhibited) by PFOA and PFOS following chemical exposure. Therefore, the increase of free T_4levels from PFOA and PFOS displacement of T_4 bound to TTR resulted in increased hepatic uptake rates, leading to a predicted decrease in physiological T_4 serum levels. Results from our in vitro technique supports in vivo rodent toxicological studies reporting a reduction in TH serum levels related to PFOA and PFOS exposure (Martin et al., 2007; Chang et al., 2008). The decrease in total rodent T_4serum levels (hypothyroxinemia) observed in vivo were attributed to increased tissue availability and hepatic metabolism due to the transient increase in free T_4 levels (Lau et al., 2003). Hence, understanding the mechanistic basis for T_4 hepatic uptake is critical for accurate pharmacokinetic assessment of T_4 blood serum levels during PFAS chemical exposure. If physiological modeling applications of T_4 hepatic uptake in the presence of PFOA/PFOS exposure are based solely on T_4 passive diffusion coefficients, the predicted impact on T_4 serum levels may be underestimated due to the omission of enhanced T_4 carrier-mediated transport. Furthermore, our results indicate TH chemical risk assessment requires evaluating the susceptibility of T_4 carrier-mediated transport to potential environmental chemical interaction.

In conclusion, an area of critical concern regarding PFAS exposure is the role maternal TH levels play during pregnancy, as a decrease in serum T_4 concentrations (hypothyroidism) may lead to impaired fetal neurodevelopment (Hassan et al., 2017; Gilbert et al., 2012). Recently, perfluorohexane sulfonate was shown to decrease T_4levels in rodent dams leading to developmental effects in their offspring (Ramhoj et al., 2018). Thus, further in vitro studies are needed to assess how varying PFAS chain length and functional group composition influence T_4 hepatic uptake. The evaluation of TH transport processes within human hepatocytes along with enhanced analytical detection limits nearing physiological conditions would greatly improve interspecies extrapolation and health risk assessment during PFAS chemical exposure.

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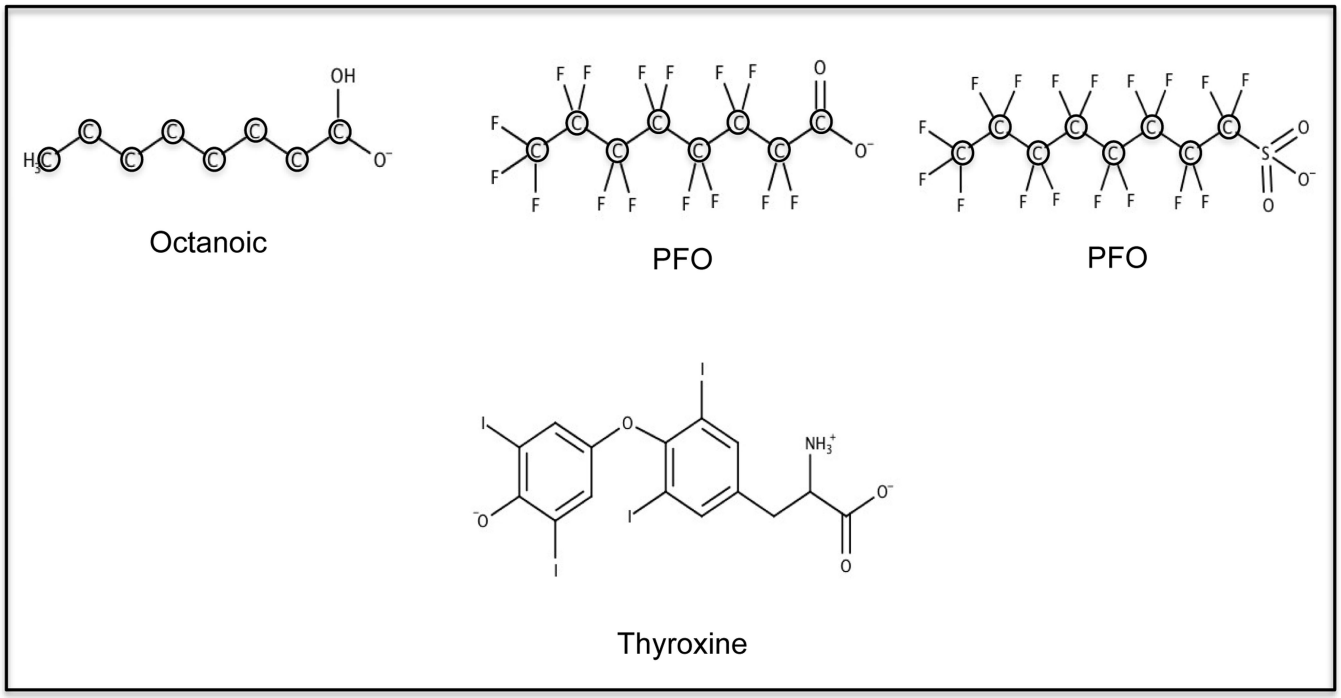

FIG. 1.
Chemical structure and speciation of octanoic acid, PFOA, PFOS and L-thyroxine (T_4).

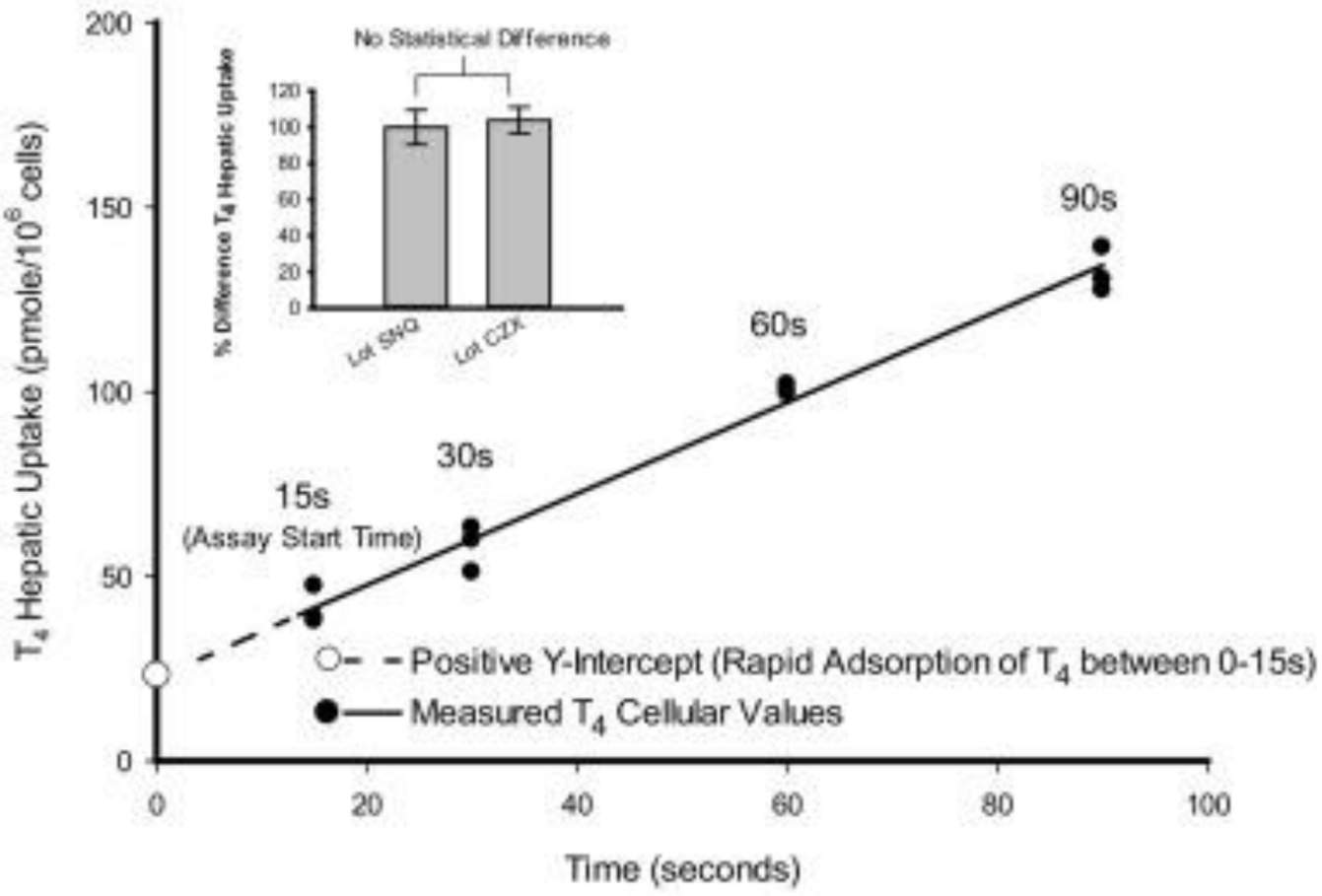

FIG. 2.

Representative kinetic profile of T4 (1.0 μM) uptake at 37 °C in cryopreserved rathepatocytes (Lot CZX) using oil filtration. A linear rate of T4 uptake (pmoles/10^6 cells/s) was determined from individual timelines conducted in triplicate within a single experiment. Inset depiction displays no statistical difference between duplicate pooled cryopreserved hepatocyte Lots (SNQ: 24 donors; CZX: 6 donors) used as a T_4 (1.0 μM) positive control for all experimentation conducted without the serum carrier proteinTTR.

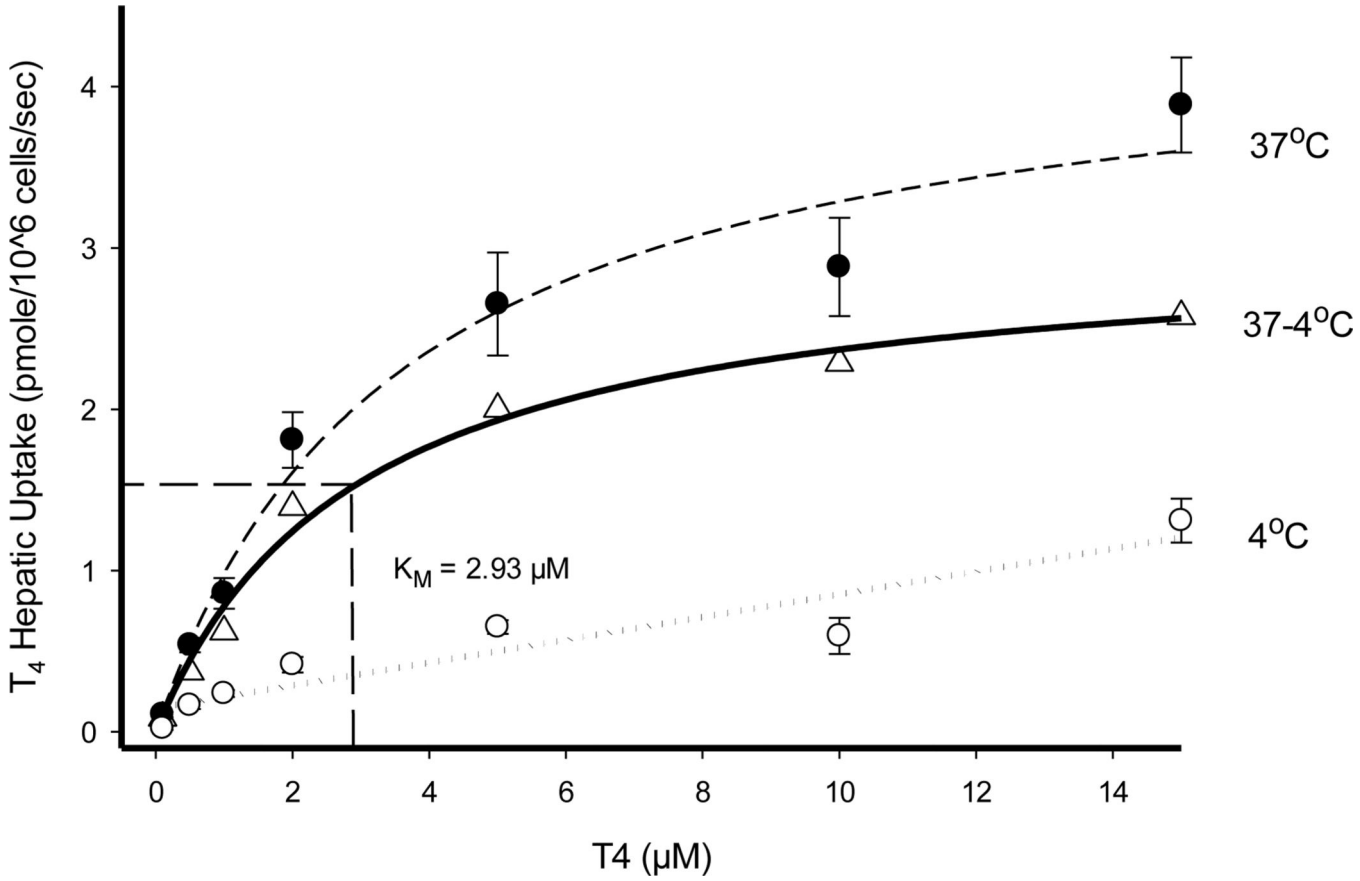

FIG. 3.

Substrate saturation kinetic profiles of T_4 uptake in cryopreserved rat hepatocyte suspensions ● 37°C (carrier-mediated + passive diffusion), Δ 37°C - 4°C (carrier mediated), and ○ 4°C (passive diffusion). A linear rate of T_4 hepatic uptake (pmoles/10^6 cells/sec) was determined from individual timelines conducted in triplicate for each substrate concentration tested. (Error bars represent ± SD.).

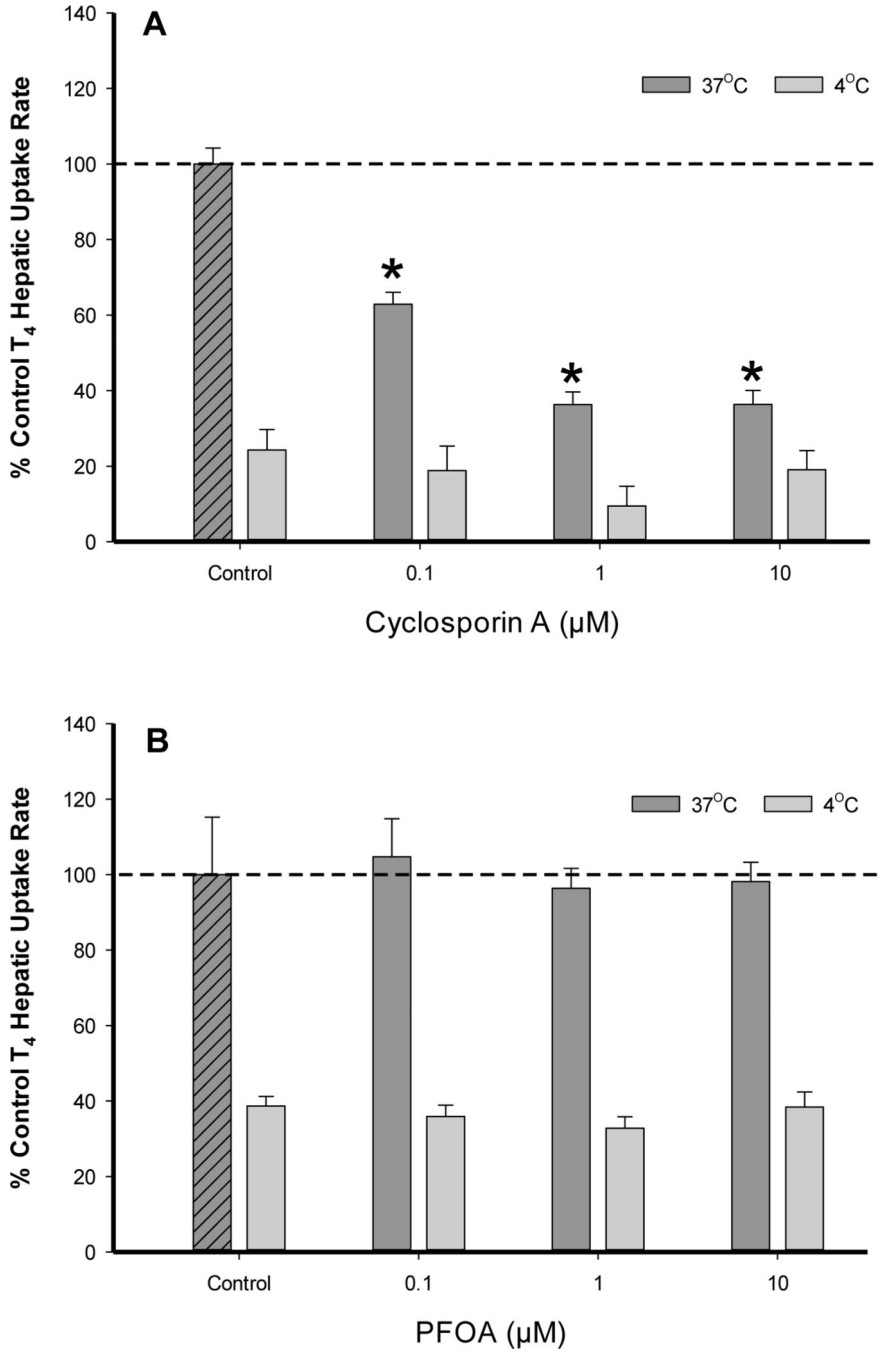

FIG. 4.

Percent difference from 37°C control of T_4 (1.0 μM) uptake in cryopreserved rat hepatocyte suspensions treated with varying concentrations of the transport inhibitor (A) cyclosporin A and (B) PFOA. Data represent the linear rate of T_4 hepatic uptake (pmoles/10^6 cells/sec) determined from individual timelines conducted in triplicate within a single experiment. Error bars represent ± SD, asterisks denote statistically significant differences versus temperature control regression slopes (*p<0.05).

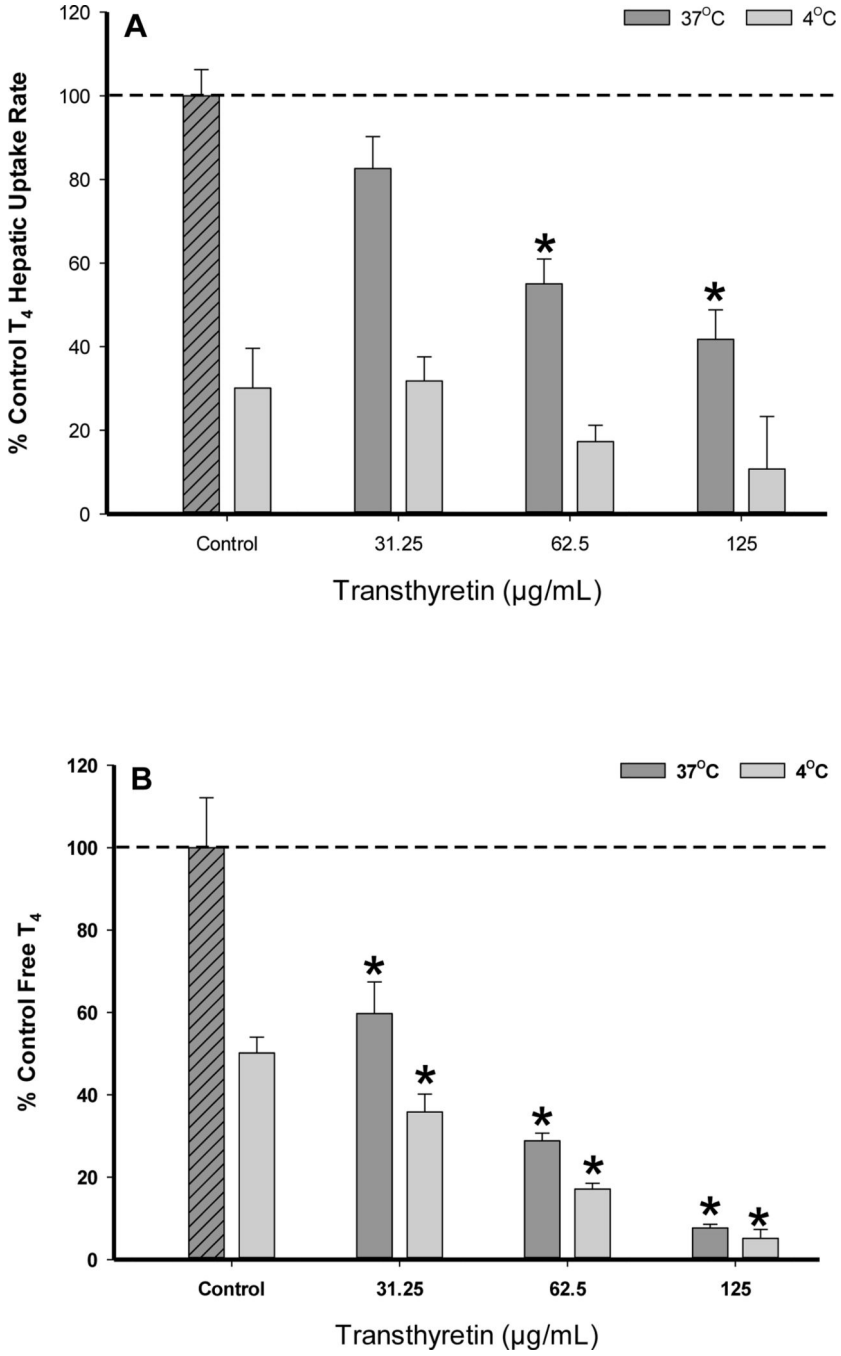

FIG. 5.

(A) Percent difference from 37°C control of T_4 (1.0 µM) uptake in cryopreserved rat hepatocyte suspensions treated with varying concentrations of the serum protein transthyretin. Data represent the linear rate of T_4 hepatic uptake (pmoles/10^6 cells/sec) determined from individual timelines conducted in triplicate within a single experiment. Error bars represent ± SD, asterisks denote statistically significant differences versus the temperature control regression slopes (*p<0.05). (B) Percent difference from 37°C control of free T_4 (1.0 µM) levels determined by 10kDa filtration of running buffer solutions with

varying transthyretin concentration levels (no hepatocytes). Error bars represent ± SD, asterisks denote statistically significant differences versus the temperature control (*p<0.05).

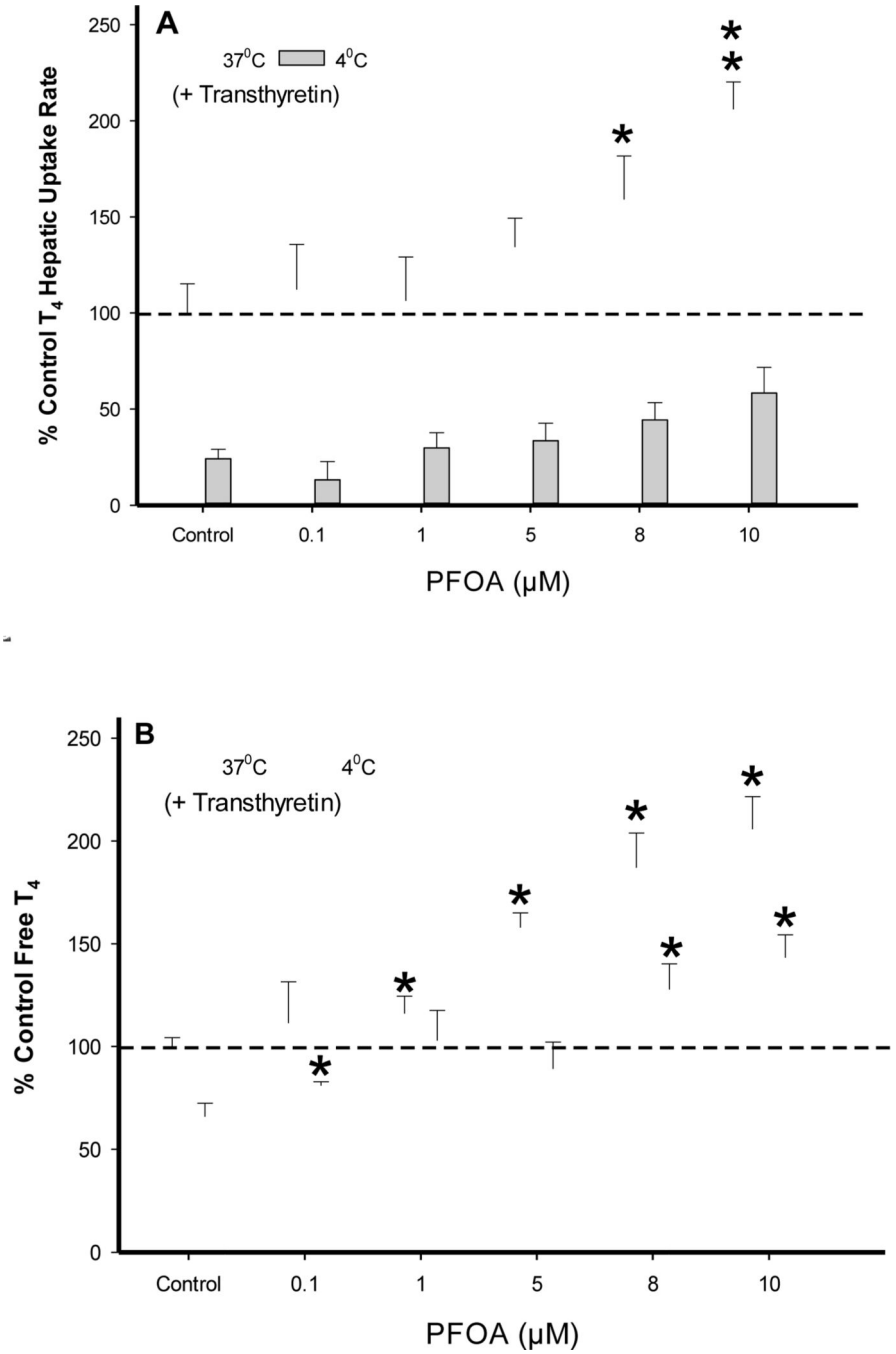

FIG. 6.

(A) Percent difference from 37°C control of T_4 (1.0 μM) uptake in cryopreserved rat hepatocyte suspension treated with varying PFOA concentrations containing the serum protein transthyretin (62.5 μg/mL). Data represent the linear rate of T_4 hepatic uptake (pmoles/10^6 cells/sec) determined from individual timelines conducted in triplicate within a single experiment. Error bars represent ± SD, asterisks denote statistically significant differences versus temperature control regression slopes (*p<0.05, **p<0.01). (B) Percent difference from 37°C control of free T_4 (1.0 μM) levels in the presence of (62.5 μg/mL)

transthyretin determined by 10kDa filtration of running buffer solutions (no hepatocytes). Error bars represent ± SD, asterisks denote statistically significant differences versus the temperature control (*p<0.05).

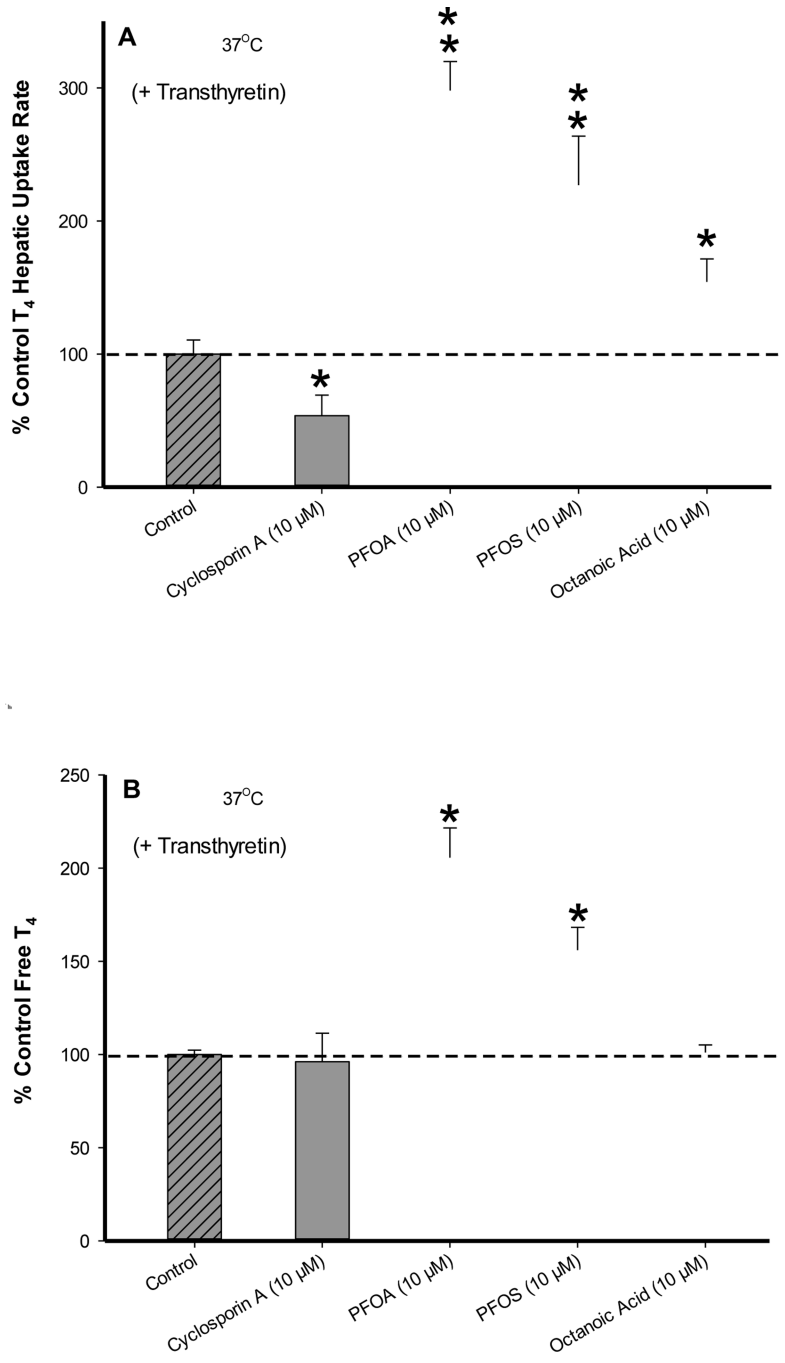

FIG. 7.

(A) Percent difference from 37°C control of T_4 (1.0 μM) uptake in cryopreserved rat hepatocyte suspensions treated with CsA, PFOA, PFOS and Octanoic Acid containing the serum protein transthyretin (62.5 μg/mL). Data represent the linear rate of T_4 hepatic uptake (pmoles/10^6 cells/sec) determined from individual timelines conducted in triplicate within a single experiment. Error bars represent ± SD, asterisks denote statistically significant differences versus temperature control regression slopes (*p<0.05, **p<0.01). (B) Percent difference from 37°C control of free T_4 (1.0 μM) levels in the presence of (62.5 μg/mL)

transthyretin determined by 10kDa filtration of running buffer solutions (no hepatocytes). Error bars represent ± SD, asterisks denote statistically significant differences versus the temperature control (*p<0.05).

Table 1

Summary of experimental design with pooled cryopreserved rat hepatocyte lots.

Experiment[a]		Number of individual experiments by lot			Number of individual experiments	Number of samples for timeline analysis	TTR treatment % control
		SNQ (donors = 24)	CZX (donors = 6)	ZHN (donors = 45)			
Control	No TTR	2	2		4	48	
	TTR	1	1	1	3	36	
PFOA (10 μM)	No TTR	1			1	12	
	TTR	1		1	*2*	*24*	*206–298*
PFOS (10 μM)	No TTR	1			1	12	
	TTR			1	1	*12*	*226*
Octanoic Acid (10 μM)	No TTR						
	TTR			1	1	12	154
Cyclosporin A (10 μM)	No TTR	1			1	12	
	TTR			1	1	12	53
Number of experiments		7	3	5	15		
Number of samples for timeline analysis		84	36	60		180	