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Characterization of plasma triiodophenol binding proteins in vertebrates and tissue distribution of triiodophenol in *Rana catesbeiana* tadpoles

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ABSTRACT

We investigated the interaction of 2,4,6-triiodophenol (TIP), a potent thyroid hormone disrupting chemical, with serum proteins from rainbow trout (*Onchorhynchus mykiss*), bullfrog (*Rana catesbeiana*), chicken (*Gallus gallus*), pig (*Sus scrofa domesticus*), and rat (*Rattus norvegicus*) using a [^{125}I]TIP binding assay, gel filtration chromatography, and native polyacrylamide gel electrophoresis. [^{125}I]TIP bound non-specifically to proteins in trout serum, specifically but weakly to proteins in bullfrog serum, and specifically and strongly to proteins in chicken, pig, and rat serum samples. Candidate TIP-binding proteins included lipoproteins (220–320 kDa) in trout, albumin in bullfrog, albumin and transthyretin (TTR) in chicken and pig, and TTR in rat. TTR in the chicken, pig, and rat serum samples was responsible for the high-affinity, low-capacity binding sites for TIP (dissociation constant $2.2–3.5\times10^{-10}$ M). In contrast, a weak interaction of [^{125}I]TIP with tadpole serum proteins accelerated [^{125}I]TIP cellular uptake in vitro. Intraperitoneal injection of [^{125}I]TIP in tadpoles revealed that the radioactivity was predominantly accumulated in the gallbladder and the kidney. The differences in the molecular and binding properties of TIP binding proteins among vertebrates would affect in part the cellular availability, tissue distribution and clearance of TIP.

1. Introduction

An increasing number of chemicals released into the environment has been detected in the air, soil, sediment, and water of, and in organisms living in, contaminated habitats. Although the concentrations of most chemicals found in contaminated habitats are not apparently toxic, such chemicals accumulate in organisms at concentrations several orders of magnitude higher than those measured in the environment (Vallack et al., 1998). The accumulation of such chemicals is due to their lipophilic nature and to their movement through food chain. Consequently, the bioaccumulation of environmental chemicals can interfere with the endocrine system through many mechanisms of action (Boas et al., 2006).

The bioavailability and bioactivity of the environmental chemicals in vertebrates are affected by how these chemicals are transported in the bloodstream (Nagel et al., 1997). Plasma proteins that have significant binding affinity for environmental chemicals include the thyroid hormone (TH)-binding proteins (THBPs). For example, highly lipophilic polyhalogenated aromatic hydrocarbons, such as dioxins, benzo[*a*]pyrene, and polychlorinated biphenyls, bind non-specifically (i.e., non-saturable and non-displaceable) to lipoproteins, and specifically, but with low affinity in vivo and in vitro, to albumin (Borlakoglu et al., 1990; Monteverdi and Di Giulio, 2000), whereas relatively lipophilic chemicals with polar groups (e.g., hydroxyl groups) bind specifically to albumin (Sheehan and Young, 1979), and transthyretin (TTR) (Van den Berg, 1990; Lans et al., 1993; Ishihara et al., 2003).

2,4,6-Triiodophenol (TIP), a halogenated phenol, is an environmental chemical with the potential for bioactivity in vertebrates. Either released into the environment from industrial, medical and agricultural runoffs or formed in the environment when iodine reacts with phenol to produce iodophenols or converts chlorophenols to iodophenols (Patnaik and Khoury, 2003), TIP has been detected at concentrations of 0.53 μg/L or less in the surface water of rivers in the USA (Kannamkumarath et al., 2004). Previously, we demonstrated that TIP interfered with TH-binding to TTR and disrupted thyroid hormone activity in both *Xenopus laevis* cell culture system and tadpoles (Kudo and Yamauchi, 2005). In other studies, the binding affinity of TIP for human TTR was the highest of the halogenated phenols tested (McKinney et al., 1985; Van den Berg, 1990) and was 3.8 times greater than that for TH (McKinney et al., 1985).

Species-specific differences in TIP binding to plasma proteins, and the subsequent cellular uptake, of and cellular response to TIP, are possible (Morgado et al., 2007) as the THBPs and their binding affinities and capacities for THs differ among vertebrates. More than half of THs are bound to lipoproteins, predominantly high-density

Abbreviations: HDL, high-density lipoprotein; IC_{50}, 50% inhibitory concentration; i.p., intraperitoneally; K_d, dissociation constant; T_3, 3,3′,5-triiodo-L-thyronine; T_4, L-thyroxine; TBG, thyroxine-binding globulin; TH, thyroid hormone; THBP, TH-binding protein; TIP, 2,4,6-triiodophenol; TTR, transthyretin; PAGE, polyacrylamide gel electrophoresis; SEM, standard error of the mean.

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lipoprotein (HDL) in rainbow trout (*Oncorhynchus mykiss*) (Babin, 1992). In adult bullfrogs (*Rana catesbeiana*), major THBP is albumin, whereas in tadpole bullfrogs, TTR also participates in TH binding as high-affinity binding sites (Yamauchi et al., 1993). Albumin and TTR are the major THBPs in birds and rodents (Schreiber and Richardson, 1997), whereas albumin, TTR and thyroxine-binding globulin (TBG) are the major THBPs in large eutherians. In humans, 70–80% of protein-bound THs are transported by TBG, due to its high affinity for THs (Robbins, 1996).

The tendency of environmental chemicals to accumulate primarily in aquatic ecosystems from industrial residues, medical and agricultural runoffs pose a threat to vertebrates living in riparian habitats. In this study, we investigated which plasma proteins interact with, and their affinity for, TIP in serum from two ectotherms (rainbow trout and bullfrog), and three endotherms (chicken, pig, and rat), the effect of bullfrog tadpole and rat serum proteins on the uptake of TIP into in vitro, and the tissue distribution of TIP after administration to tadpoles.

2. Materials and methods

2.1. Reagents

Radiolabeled iodine ($Na^{125}I$; 629 GBq/mg as iodine) was purchased from PerkinElmer (Waltham, MA, USA). L-Thyroxine (T_4; ≥98% purity), 3,3′,5-triiodo-L-thyronine (T_3; ~98% purity), all-*trans*-retinoic acid (≥98% purity), and pentachlorophenol (99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ioxynil (3,5-diiodo-4-hydroxybenzonitrile, analytical standard, 99% purity) was purchased from Riedel-de Haën Fine Chemicals (Seelze, Germany) and TIP (98% purity) was from Wako Pure Chemical Industries (Tokyo, Japan). Diethylstilbestrol (>98% purity) and tetrabromobisphenol A (>98% purity) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Sodium *p*-toluenesulfonchloramide trihydrate (chloramine-T; >98% purity) and chromatography-grade methanol were obtained from Kanto Chemical (Tokyo, Japan).

All chemicals tested as binding competitors were dissolved in dimethyl sulfoxide to concentrations of 2–10 mM, except for retinoic acid, which was dissolved in phosphate-buffered saline to a concentration of 10 mM. All chemicals were diluted with an appropriate buffer to give less than 0.4% (v/v) solvent. A control assay without the test chemicals was performed in the presence of the solvent at a concentration of less than 0.4% (v/v). The solvent did not affect the competitive [^{125}I]TIP binding assays.

2.2. Radiolabeling

2,4,6-Triiodophenol was radiolabeled by iodine exchange reaction using the chloramine-T method (Greenwood et al., 1963), with some modifications. An aliquot of the diluted TIP solution (660–1320 pmol) was mixed with $Na^{125}I$ (7.4 MBq), 280–560 pmol of unlabeled NaI, and 525 nmol of chloramine-T in 53 μL of 20 mM sodium phosphate, pH 7.2. After incubating the mixture for 10 min, the radiolabeling was stopped by the addition of 16 μL of 4 mM sodium disulfite. The reaction mixture was extracted with 300 μL of chloroform:acetic acid (99:1). The organic phase was collected and then evaporated with a test tube evaporator (type TVE-1000, EYELA, Tokyo, Japan). The residue was reconstituted in an appropriate volume of methanol:distilled water:acetic acid (74:25:1), and purified by high-performance liquid chromatography on a reverse-phase C_{18} analytical column (Mightysil RP-18 GP, 250 mm × 4.6 mm, 5 μm particle diameter; Kanto Chemical), with an isocratic mobile phase (methanol:distilled water:acetic acid, 74:25:1) at a flow rate of 1.0 mL/min. The concentration of chemical was monitored by absorbance at 254 nm. Both radiolabeled and unlabeled TIPs were eluted at 17.4 min. The respective fractions were collected and radioactivity was measured in a γ-counter (Auto Well Gamma System ARC-380CL, Aloka; Tokyo, Japan). The specific activity of TIP was estimated from the amount of and the radioactivity of the purified TIP, which was 3.4–8.9 TBq/mmol (0.043–0.11 radioactive atom/TIP molecule), with a yield of approximately 40%. The purity of the isolated TIP was assessed by thin-layer chromatography. Aliquots of the radiolabeled TIP were loaded on a thin-layer chromatography plate (PE SIL G/UV, Whatman, Maidstone, Kent, England, UK) and run for 1.0–1.5 h in chloroform/acetic acid (99/1) solvent. The amount of purified TIP was estimated from a calibration line of standard TIP at defined amounts. The purified TIP was stored in dimethyl sulfoxide at 4 °C.

2.3. Biological material

Serum samples were collected from rainbow trout (*O. mykiss*; males $n=10$, females $n=10$), bullfrogs (*R. catesbeiana*; adult males $n=3$, adult females $n=3$, tadpoles $n=50$), chickens (*Gallus gallus*; adult males $n=3$, adult females $n=3$, juveniles $n=5$), pigs (*Sus scrofa domesticus*, adult male $n=3$), Sprague–Dawley rats (*Rattus norvegicus*; adult male $n=4$), and C3H/HeJ mice (*Mus musculus*; adult male $n=4$). Blood was collected from sexually mature trout at the Fuji Trout Hatchery in the Shizuoka Prefectural Research Institute of Fishery, Fujinomiya, Shizuoka. Tadpoles and adult bullfrogs were obtained from Saitama Amphibian Institute, Saitama, Japan. Tadpoles (8–18 g body mass) were anesthetized by immersion in 0.2% (w/v) ethyl 3-aminobenzoate methanesulfonic acid (Sigma-Aldrich), whereas adult bullfrogs (170–200 g body mass) were anesthetized by injecting ethyl 3-aminobenzoate methanesulfonic acid then pithed. Tadpole and adult bullfrog blood were collected as described previously (Yamauchi et al., 1993). Tadpole red blood cells were prepared as described previously (Yamauchi et al., 1989). Chicken and pig blood were collected from mature animals at the Swine and Poultry Research Center in the Shizuoka Prefectural Research Institute of Animal Industry, Kikugawa, Shizuoka, Japan. Blood was also collected from 60-day-old chickens at a local abattoir in Shizuoka, Japan. Rat and mouse blood were kindly provided by Dr T. Koike, Department of Biological Science, Faculty of Science, Shizuoka University. Serum was separated from blood cells by centrifugation at 400 *g* for 15 min at 4 °C. Serum samples were used immediately or stored at −20 °C.

The care and treatment of animals used in this study were in accordance with the Guidelines for Proper Conduct of Animal Experiments, Japan.

2.4. Binding assay

Serum or TTR, purified as described previously (Eguchi et al., 2008), was incubated with 0.5 nM [^{125}I]TIP (6×10^4 dpm) in 250 μL of buffer containing 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM $NaHCO_3$, and 20 mM HEPES, pH 7.5 (Buffer A) in the presence or absence of 5 μM unlabeled TIP for 1 h at 4 °C. Competitive [^{125}I]TIP binding was performed with solvent only or increasing concentrations of unlabeled competitors. Protein-bound [^{125}I]TIP was separated from free [^{125}I]TIP by the Dowex method (Lennon, 1992). Radioactivity was measured in a γ-counter. The amount of non-specifically bound [^{125}I]TIP (in the presence of 5 μM unlabeled TIP) was subtracted from that of total bound [^{125}I]TIP (in the absence of 5 μM unlabeled TIP) to give the value of specifically bound [^{125}I]TIP. Background radioactivity, which was estimated using tubes containing no serum proteins, was also subtracted. The K_d for TIP binding to proteins was determined from curvilinear Scatchard plots (Rosenthal, 1967).

2.5. Gel filtration column chromatography

After incubating serum with 0.7 nM [^{125}I]TIP (7×10^4 dpm) for 1 h at 4 °C, 0.5 mL of each sample was applied to a Cellulofine GCL 1000-sf