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Plasma protein binding of tetrodotoxin in the marine puffer fish *Takifugu rubripes*

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ABSTRACT

To elucidate the involvement of plasma protein binding in the disposition of tetrodotoxin (TTX) in puffer fish, we used equilibrium dialysis to measure protein binding of TTX in the plasma of the marine puffer fish *Takifugu rubripes* and the non-toxic greenling *Hexagrammos otakii*, and in solutions of bovine serum albumin (BSA) and bovine alpha-1-acid glycoprotein (AGP). TTX (100–1000 μg/mL) bound to protein in *T. rubripes* plasma with low affinity in a non-saturable manner. The amount of bound TTX increased linearly with the TTX concentration, reaching 3.92 ± 0.42 μg TTX/mg protein at 1000 μg TTX/mL. Approximately 80% of the TTX in the plasma of *T. rubripes* was unbound in the concentration range of TTX examined, indicating that TTX exists predominantly in the unbound form in the circulating blood of *T. rubripes* at a wide range of TTX concentrations. TTX also bound non-specifically to *H. otakii* plasma proteins, BSA, and bovine AGP. The amount of the bound TTX in the plasma of *H. otakii* and BSA, respectively, was 1.86 ± 0.36 and 4.65 ± 0.70 μg TTX/mg protein at 1000 μg TTX/mL, and that in the bovine AGP was 8.78 ± 0.25 μg TTX/mg protein at 200 μg TTX/mL.

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1. Introduction

Marine puffer fish, the family Tetraodontidae, generally contain a potent neurotoxin, tetrodotoxin (TTX), which is a voltage-gated sodium channel blocker, in specific tissues such as liver, ovary, and skin (Halstead, 1988; Soong and Venkatesh, 2006; Lee and Ruben, 2008). There is somewhat general agreement that marine puffer fish accumulate TTX by bioaccumulation via the marine food chain (Noguchi and Arakawa, 2008), based on the findings that cultured marine puffer fish are not toxic (Matsui et al., 1981, 1982; Lin et al., 1998; Noguchi et al., 2006) and non-toxic cultured puffer fish become toxic by feeding on artificial TTX-containing diets (Matsui et al., 1981; Yamamori et al., 2004; Honda et al., 2005; Kono et al., 2008). Marine puffer fish seem to have a unique competence to accumulate TTX in the body. We previously demonstrated in the marine puffer fish *Takifugu rubripes* that TTX is well absorbed into the systemic circulation from the gastrointestinal tract and accumulates in the liver after a single administration (Matsumoto et al., 2008a). Pharmacokinetic studies of TTX in *T. rubripes* have led to the classification of three groups of tissues: (1) the central compartment, including kidney and spleen, in which the TTX concentration is instantaneously proportional to the blood concentration; (2) the peripheral compartment, including the liver, in which the TTX concentration increases even as the blood concentration decreases; and (3) the peripheral compartment, including muscle and skin, in which the TTX concentration is independent of the blood concentration (Matsumoto et al., 2008b). TTX transport via the circulating blood is deeply involved in the disposition of TTX in the puffer fish body.

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Recent progress in studies of the plasma protein binding of TTX in marine puffer fish has revealed some interesting findings. The puffer fish saxitoxin (STX) and TTX-binding protein (PSTBP) was first purified from the plasma of the marine puffer fish *Takifugu niphobles* as a possible TTX-carrier protein (Matsui et al., 2000). Yotsu-Yamashita et al. (2001) also purified PSTBP from the plasma of other species of the marine puffer fish *Takifugu pardalis* and examined its binding property. The TTX affinity to *T. pardalis* PSTBP is much lower than that of STX, because the IC_{50} value of tritium-labeled STX binding to *T. pardalis* PSTBP for TTX is 1400 times larger than that for STX, although TTX and STX share the same binding site on *T. pardalis* PSTBP (Yotsu-Yamashita et al., 2001, 2002). These findings suggest that PSTBP has an important role in the plasma protein binding of TTX and STX in the Tetraodontidae family of marine puffer fish. The actual abundance of PSTBP in the puffer fish plasma, however, is still unknown, and it is unclear whether other plasma proteins affect the protein binding of TTX in puffer fish plasma.

We previously demonstrated plasma protein binding of TTX in the marine puffer fish *T. rubripes* and revealed that the percentage of the unbound form against the total TTX concentration in the plasma was approximately 60% at concentrations ranging from 1.4 to 22.5 μg TTX/mL (Matsumoto et al., 2008a). Based on these findings, we predict that TTX has a low affinity to the total plasma protein of *T. rubripes*. In the present study, to elucidate the involvement of plasma protein binding in the disposition of TTX in puffer fish, we used equilibrium dialysis to measure protein binding of TTX in the plasma of the marine puffer fish *T. rubripes* and the non-toxic marine greenling *Hexagrammos otakii*, and in solutions of bovine serum albumin (BSA) and bovine alpha-1-acid glycoprotein (AGP).

2. Materials and methods

2.1. Materials

Marine puffer fish *T. rubripes* specimens ($n = 6$, 720–1210 g body weight) and the non-toxic marine greenling *H. otakii* specimens ($n = 5$, 1060–1290 g body weight) were obtained live from a Tokyo central wholesale market and transported to our laboratory. TTX used in the protein binding assay was purified from the ovaries of *T. pardalis* by a combination of ultrafiltration and a series of column chromatographic separations, as reported previously (Matsumoto et al., 2007). Crystalline TTX (Wako Pure Chemical Industries, Osaka, Japan) was used as a standard for the liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) analysis. BSA and bovine AGP (Sigma Aldrich, St. Louis, MO, USA) were used in the protein binding assay. BSA was used as a standard for the protein determination. All other chemicals were reagent grade.

2.2. Equilibrium dialysis

Protein binding of TTX was determined using an equilibrium dialysis unit (Sanplatec Co., Tokyo, Japan), as described previously (Matsumoto et al., 2008a). Approximately 25 mL blood/kg body weight was collected from *T. rubripes* or *H. otakii* using a heparinized disposable syringe. The blood was centrifuged at 1200 × *g* for 10 min at 4 °C and the plasma was collected and stored on ice until use. The dialysis membrane (MWCO 3500, Spectrum Laboratories, Compton, CA, USA) was rinsed twice in milliQ water, soaked in modified Hank's balanced salt solution (pH 7.4) for 30 min, and placed into the equilibrium dialysis unit between the two compartments of the dialysis chamber. A 950 μL aliquot of fish plasma was then mixed with a 50 μL aliquot of TTX solution (0–1000 μg TTX/50 μL), and a 950 μL aliquot of this mixture (1000 μL) was introduced into one side of the chamber (termed the plasma sample). Then, a 950 μL aliquot of modified Hank's balanced salt solution (pH 7.4) was placed into the counter side (termed the buffer sample) of the dialysis chamber. Plasma protein binding of TTX was assessed after incubation at 20 °C for 48 h. Plasma and buffer samples were withdrawn from each side of the chamber. Another series of experiments were performed using solutions of BSA and bovine AGP, in which BSA and bovine AGP were dissolved in modified Hank's balanced salt solution (pH 7.4) at a concentration of 60 mg BSA/mL and 4 mg AGP/mL, respectively.

2.3. Sample preparation and TTX determination

After the sampling, the plasma sample was mixed with a methanol/acetic acid solution, and purified by ultrafiltration (MWCO 5000). The filtrate was lyophilized, and dissolved in the mobile phase of LC/ESI-MS. The amount of TTX in the sample solution was determined by the LC/ESI-MS analysis, as described previously (Matsumoto et al., 2008a).

2.4. Determination of protein amount

Protein determination was performed by the method of Lowry et al. (1951) with BSA as the standard.

2.5. Statistics

The determination was performed in triplicate in individual experiments. Data are expressed as mean ± standard error (SE). Tukey's test and Student's *t*-test were used to analyze the significance of differences among means at the 5% significance level in the examination of the time to achieve equilibrium.

3. Results

The plasma from the marine puffer fish *T. rubripes* and the non-toxic marine fish *H. otakii* used in this study initially did not contain detectable amounts of TTX (<10 ng TTX/mL plasma).

3.1. The time to equilibrium

The time to achieve equilibrium was examined with the plasma of marine puffer fish *T. rubripes*. The total protein concentration in the plasma was 49.1 ± 9.5 mg protein/mL ($n = 3$). The mixture of *T. rubripes* plasma and the TTX solution was incubated against an equal volume of the

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