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Kinetic characterization of the inhibition of acyl coenzyme A: Steroid acyltransferases by tributyltin in the eastern mud snail (*Ilyanassa obsoleta*)

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Abstract

Exposure to tributyltin (TBT) has been causally associated with the global occurrence of a pseudohermaphroditic condition called imposex in neogastropod species. TBT elevates free testosterone levels in these organisms, and this upsurge in testosterone may be involved in the development of imposex. We investigated the ability of TBT to inhibit acyl coenzyme A:testosterone acyltransferase (ATAT) activity as well as microsomal acyl-coenzyme A:17β-estradiol acyltransferase (AEAT) in a neogastropod, the eastern mud snail *Ilyanassa obsoleta* as a mechanism by which TBT elevates free testosterone. TBT significantly inhibited both ATAT and AEAT activities in vitro at toxicologically relevant in vivo concentrations. Kinetic analyses revealed that TBT is a competitive inhibitor of ATAT ($K_i = \sim 9$ μM) and is a weaker, noncompetitive inhibitor of AEAT ($K_i = \sim 31$ μM). ATAT and AEAT activities associated with different microsome preparations were significantly correlated, and 17β-estradiol competitively inhibited the fatty acid esterification of testosterone suggesting that one enzyme is responsible for biotransforming both testosterone and 17β-estradiol to their corresponding fatty acid esters. Overall, the results of this study supply the much-needed mechanistic support for the hypothesis that TBT elevates free testosterone in neogastropods by inhibiting their major regulatory process for maintaining free testosterone homeostasis—the fatty acid esterification of testosterone.

Keywords: Tributyltin; Imposex; Acyl coenzyme A:steroid acyltransferase; Testosterone; 17β-Estradiol; Endocrine; Snail

1. Introduction

Tributyltin (TBT) has been used ubiquitously as a biocide in antifouling paints for ships, boats, and offshore installations. Exposure to TBT has been causally linked with a pseudohermaphroditic condition known as imposex (Smith, 1971; Jenner, 1979). Imposex is the superimposition of male sex characteristics, such as a penis, vas deferens, and semeniferous tubules, on female neogastropods. Imposex has been documented in over 150 species of marine neogastropods worldwide (Fioroni et al., 1991; Horiguchi et al., 1997). Early stages of imposex decrease the reproductive capacity of females, whereas later stages result in the sterilization of females and even death (Oehlmann et al., 1996). Thus, TBT pollution has caused serious effects at the population level (Oehlmann et al., 1996), especially for those species of neogastropods that lack a planktonic larval stage as part of their life history making the recolonization of severely impacted areas difficult.

Previous studies have associated TBT exposure with increased testosterone titers in imposexed females (Spooner et al., 1991; Bettin et al., 1996; Gooding et al., 2003). At least two hypotheses have been proposed for the mechanism by which TBT causes imposex and the related observation that imposexed females have elevated testosterone levels. First, TBT could act as a neurotoxicant by altering the secretion of neurohormones (e.g., penis morphogenic factor [PMF]) that contribute to sexual differentiation in neogastropods (Feral and LeGall, 1983). Pursuing this hypothesis, Oberdorster and McClellan-Green (2000, 2002) showed that the neuropeptide APGWamide significantly induces imposex in the eastern mud snail *Ilyanassa obsoleta* and implicated APGWamide as a candidate for the PMF in this species. Consequently, the authors proposed that TBT stimulates the release of the PMF in female neogastropods leading to the development of androgen-producing male accessory sex

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organs. Nevertheless, a causal relationship among TBT exposure, abnormal APGWamide release, and imposex has yet to be established.

Alternatively, TBT could elevate testosterone levels by inhibiting its enzymatic conversion to various metabolites; this upsurge in testosterone could then initiate a cascade of biochemical events resulting in male reproductive organ development in females. A caveat to this hypothesis is that imposex was evident prior to the detection of an increase in testosterone titers in one laboratory experiment (Bettin et al., 1996). Even so, the means by which TBT inhibits the enzymatic conversion of testosterone to various metabolites has been addressed in several studies. Sulfotransferase (Ronis and Mason, 1996), cytochrome P450 aromatase (Spooner et al., 1991; Bettin et al., 1996), and acyl coenzyme A:steroid acyltransferase (Gooding et al., 2003; Janer et al., 2005a) are three classes of enzymes that have been considered as targets for inhibition by TBT.

Gooding and LeBlanc (2001, 2004) found the fatty acid esterification of testosterone by microsomal acyl coenzyme A:testosterone acyltransferase (ATAT) to be a major regulatory process for maintaining free testosterone homeostasis in *I. obsoleta*. In laboratory and field studies, these investigators observed that TBT suppresses testosterone esterification and elevates free testosterone in this species (Gooding et al., 2003). In spite of this association, ATAT activity in microsomes isolated from TBT-exposed *I. obsoleta* was not altered regardless of imposex condition implying that TBT does not suppress ATAT protein expression; concentrations of TBT as high as 10 μg/L (0.03 μM) TBT did not directly inhibit in vitro ATAT activity (Gooding et al., 2003). However, snails collected from a contaminated site where the incidence of imposex was 100% contained whole body TBT concentrations (based upon tin analyses) of 700 μg/kg (∼5.9 μM) (Gooding et al., 2003) which is greater than the range of TBT concentrations tested in the in vitro ATAT assays. Therefore, a primary objective of this study was to determine if in vitro ATAT activity of *I. obsoleta* is directly inhibited by TBT at toxicologically relevant in vivo concentrations. Any observed inhibitory activity would then be subjected to kinetic analyses to characterize the means by which TBT inhibits ATAT.

Clearly, much of the research regarding TBT's mechanism of action has focused on the association of TBT exposure with increased testosterone titers in imposexed females presupposing that testosterone is a functional androgen in gastropods (see above and Matthiessen and Gibbs, 1998). However, compelling molecular and cellular substantiation for the existence of an androgen receptor in molluscs is lacking, and phylogenetic analyses suggest that the androgen receptor evolved after the emergence of jawless fish (Thornton, 2001). On the other hand, mounting molecular and cellular evidence points to the existence of an estrogen receptor in molluscs (Stefano et al., 2003; Thornton et al., 2003; Canesi et al., 2004). Free 17β-estradiol homeostasis in molluscs appears to be regulated in a manner similar to that of testosterone (Janer et al., 2005b). In *I. obsoleta*, 17β-estradiol is maintained primarily in the fatty acid ester conjugate form (personal observation). Thus, the additional objectives of this study were to determine if the fatty acid esterification of 17β-estradiol may be susceptible to inhibition by TBT as well as establish whether the enzyme that biotransforms testosterone to a fatty acid ester is the same enzyme that biotransforms 17β-estradiol to its fatty acid ester counterpart.

2. Materials and methods

2.1. Chemicals

Testosterone, 17β-estradiol, and palmitoyl-coenzyme A (palmitoyl-CoA) lithium salt (approximately 90%) were procured from Sigma–Aldrich (St. Louis, MO). [4-^{14}C]testosterone (50 mCi/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). [4-^{14}C]17β-estradiol (54 mCi/mmol) and [1-^{14}C]palmitoyl-CoA (55 mCi/mmol) were acquired from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Tributyltin chloride (TBTCl) (96%) was obtained from Sigma–Aldrich (St. Louis, MO). All solvents were of HPLC grade. Stock solutions for the steroids and palmitoyl-CoA (both unlabeled and radiolabeled) were prepared in 100% ethanol and 100 mM potassium phosphate buffer (pH 7.4), respectively. The stock solution for TBT was prepared by adding 100% ethanol to a weighted amount of TBTCl.

2.2. Snail collection

Snails used for microsome preparation were collected from a field population on Oak Island, NC. Imposex females have never been detected in this field population. Snails in a randomly chosen section of the site were collected, rinsed with seawater to remove mud and sand, and transported back to the laboratory in a container filled with ambient seawater from the site. In the laboratory, groups of approximately 75 snails were kept in 5 gal aquaria filled with 1 L reconstituted seawater (30 ± 1 ppt) (Instant Ocean: Aquarium Systems, Mentor, OH). These aquaria were housed in incubators programmed to maintain environmental conditions (i.e., a temperature and photoperiod appropriate for the time of year). The temperature and photoperiod were adjusted on a weekly basis to conform to changing environmental conditions. Aquaria water was gently aerated, and medium was replaced daily. Snails were fed TetraMin® Tropical Flakes (Tetra Holding, Inc., Blacksburg, VA) ad libitum. Snails used for correlating ATAT and AEAT activities were transported back to the laboratory on ice and held at 4 °C until dissection within 24 h of collection.

2.3. Microsome preparation

The gonad–viscera complex was isolated from individual snails, snap frozen in liquid nitrogen, and stored at −80 °C until homogenization. Tissue from three to eight snails was homogenized in 100 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1 mM phenanthroline, and 0.5 mg/mL trypsin inhibitor (Gooding et al., 2003) using a glass homogenizer. Microsomes were prepared by differential centrifugation (van der Hoeven and Coon, 1974). The resulting microsomal pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA