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Analysis of testosterone fatty acid esters in the digestive gland of mussels by liquid chromatography-high resolution mass spectrometry

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ABSTRACT

Several studies have indicated that up to 70% of the total steroids detected in molluscs are in the esterified form and that pollutants, by modifying the esterification of steroids with fatty acids, might act as endocrine disrupters. However, despite the strong physiological significance of this process, there is almost no information on which fatty acids form the steroid esters and how this process is modulated. This study (a) investigates the formation of fatty acid esters of testosterone in digestive gland microsomal fractions of the mussel *Mytilus galloprovincialis* incubated with either palmitoly-CoA or CoA and ATP, and (b) assesses whether the endocrine disruptor tributyltin (TBT) interferes with the esterification of testosterone. Analysis of testosterone esters was performed by liquid chromatography–high resolution mass spectrometry (UPLC-HRMS). When microsomal fractions were incubated with testosterone and palmitoly-CoA, the formation of testosterone palmitate was detected. However, when microsomes were incubated with CoA and ATP, and no exogenous activated fatty acid was added, the synthesis of 16:0, 16:1, 20:5 and 22:6 testosterone esters was observed. The presence of 100 μM TBT in the incubation mixture did not significantly alter the esterification of testosterone. These results evidence the conjugation of testosterone with the most abundant fatty acids in the digestive gland microsomal fraction of mussels.

1. Introduction

Fatty acid conjugation of steroids appears to be a well-conserved conjugation pathway during evolution; it is known to occur in both vertebrate and invertebrates. Fatty acid conjugation (or esterification) renders steroids to an apolar form, which is retained in the lipoidal matrices of the body, while reducing their activity, bioavailability, and susceptibility to elimination [1,2]. Steroid esters do not bind to receptors, but they can be hydrolyzed by esterases to liberate the active steroid [3].

Steroid esters have been reported in molluscs and some studies have suggested that esterification might play a key role in regulating levels of free steroids in these organisms. Thus, Gooding and LeBlanc [2] showed that the mud snail *Ilyanassa obsoleta*, primarily metabolized free testosterone to non-polar fatty acid ester conjugates via acyl-CoA:testosterone acyltransferase (ATAT), which is localized in the endoplasmic reticulum. The authors showed that esterification was the major biotransformation pathway for testosterone in snails, as exogenously provided testosterone was converted to fatty acid esters and retained in the organism. In addition, irrespective of the amount of testosterone administered to the snails, the amount of free testosterone measured in the tissues remained relatively constant and all excess of testosterone was converted to the fatty acid ester [4]. Similarly, exogenously administered testosterone and estradiol were extensively esterified by the mussel *Mytilus galloprovincialis*, whereas levels of unconjugated steroids remained almost unaltered [5,6].

On the other hand, few studies have investigated which are the specific fatty acid steroid conjugates formed by molluscs. Janer et al. [7] reported the esterification of estradiol in digestive gland microsomal fractions of *Crassostrea virginica* with a variety of saturated or unsaturated fatty acids (C16:0; C18:0; C16:1; C18:1; C18:2 and C20:4); however, identification was tentative. Labadie et al. [8] reported for the first time the identity of three estradiol fatty acid conjugates (C16:0; C16:1 and C16:2) in *Mytilus edulis* by using tandem mass spectrometry with direct probe insertion.

The organotin compound tributyltin (TBT) is a well-known endocrine disrupter in molluscs that it has been reported to interfere with the esterification of testosterone. Thus, Gooding et al. [9] showed that females of *Ilyanassa obsoleta* experimentally exposed to 10 ng/L TBT for 3 months had a lower ability to conjugate testosterone with fatty acid moieties; and females collected in an organotin-polluted site had lower levels of esterified testosterone than those collected in a clean site.

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Table 1
Testosterone esters and pantetheine thioesters detected in the digestive gland microsomal fraction of *M. galloprovincialis*. Testosterone undecanoate (T-11:0) and laurate (T-12:0) were used as internal standards.

Fatty Acid	Molecular ion $[M + H]^+$	Theoretical mass (Da)	Experimental mass (Da)	Mass difference (ppm)	Retention time (min)
Testosterone esters					
11:0	$C_{30}H_{49}O_3$	457.3682	457.3674	2.1	4.3
12:0	$C_{31}H_{51}O_3$	471.3838	471.3825	2.1	4.6
16:0	$C_{35}H_{59}O_3$	527.4459	527.4467	1.1	6.9
16:1	$C_{35}H_{57}O_3$	525.4303	525.4308	2.5	5.9
20:5	$C_{39}H_{57}O_3$	573.4305	573.4311	2.7	5.3
22:6	$C_{41}H_{59}O_3$	599.4464	599.4440	2.8	5.7
Acyl-S-pantetheine thioesters					
16:0	$C_{27}H_{53}N_2O_5S$	517.3675	517.3668	4.7	3.4
18:0	$C_{29}H_{57}N_2O_5S$	545.3988	545.3991	6.0	3.8

Table 2
Amounts of testosterone esters formed in digestive gland microsomal fractions co-incubated with 100 μM palmitoyl-CoA (n = 12) or 1 mM CoA and 2 mM ATP (n = 10). Values are pmol/h/mg protein. Results are expressed as mean ± SEM. Minimum and maximum values are shown in parenthesis.

	T-16:0	T-16:1	T-20:5	T-22:6
Palmitoyl-CoA	24.8 ± 5.3 (7–72)	–	–	–
CoA + ATP	42.6 ± 12.0 (n.d.–76)	32.8 ± 8.8 (n.d.–58)	15.5 ± 4.8 (0.3–47)	23.2 ± 3.0 (n.d.–33)

Similarly, Janer et al. [10] reported a 60–85% decrease in esterified testosterone in females of *Marisa cornuarietis* exposed to TBT; however, the decrease could not be directly linked with a decrease in microsomal acyl-CoA:testosterone acyltransferase (ATAT) activity, the enzyme that catalyzes the conjugation of steroids with different fatty acids.

Recent advances in analytical techniques have allowed the sensitive analysis of a wide range of lipids, including lipoidal derivatives of steroids, by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) [11,12]. Different mass spectrometers, including accurate mass Time-of-Flight (TOF) and Orbitrap have been evaluated for the analysis of short chain steroid esters (2 to 11 carbons) [13]. The use of these analyzers would be the method of choice for the detection of target analytes. For a non-target analysis, a mass resolving power high enough to separate analyte ions from isobaric co-eluting ions is necessary to reduce misidentification.

This study investigates the use of ultra-high performance liquid chromatography–high resolution mass spectrometry (UPLC-HRMS) to assess the formation of fatty acid esters of testosterone in digestive gland microsomal fractions of the mussel *Mytilus galloprovincialis* incubated with either palmitoly-CoA or CoA and ATP. The study also assess whether the endocrine disruptor tributyltin (TBT) interferes with the esterification of testosterone.

2. Experimental

2.1. Reagents

Palmitoyl-CoA lithium salt, coenzyme A trilithium salt, adenosine 5′-triphosphate disodium salt hydrate, tributyltin chloride and testosterone were purchased from Sigma Aldrich (Steinheim, Germany). All solvents were of analytical grade from Merck (Darmstadt, Germany). Testosterone laurate (T-12:0) and undecanoate (T-11:0) were from Steraloids (Wilton, NH, USA).

2.2. Tissue preparation

Mussels, *Mytilus galloprovincialis* (3 to 5 cm) were collected from the bivalve farms located in the Ebro Delta (NE Spain) at different times of the year. Mussels were carried to the laboratory, the digestive glands immediately dissected, frozen in liquid nitrogen and stored at −80 °C. Subcellular fractions were prepared as described in Fernandes et al. [6]. Digestive glands (each sample a pool of 4 digestive glands) were homogenized in ice-cold 100 mM phosphate buffer pH 7.4, containing 150 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.1 mM phenylmetylsulfonyl fluoride (PMSF). Homogenates were centrifuged at 500-*g* for 15 min at 4 °C. The supernatant was collected and centrifuged at 12,000-*g* for 45 min and further ultracentrifuged at 100,000-*g* for 90 min. The resulting pellet was resuspended in homogenization buffer and further centrifuged at 100,000-*g* for 30 min. Microsomal pellets were then resuspended in a small volume of 100 mM phosphate buffer pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 20% w/v glycerol. Protein concentrations were determined by the method of Bradford [14], using bovine serum albumin as a standard.

2.3. Enzyme assays

Digestive gland microsomal proteins (400–500 μg) were incubated in 0.1 M sodium acetate buffer pH 6.0 with 2 μM testosterone, 100 μM palmitoyl-CoA and 5 mM $MgCl_2$ in a final volume of 500 μL. Endogenous esterification was assayed in the presence of 1 mM CoA and 2 mM ATP instead of 100 μM palmitoyl-CoA. Reactions were initiated by the addition of palmitoyl-CoA or CoA, and stopped after 90 min incubation at 30 °C by the addition of 1 mL of ethyl acetate. Blank reactions, which consisted of microsomal fractions incubated in the absence of co-factors and/or testosterone, were included in every run.

The interaction of TBT with the esterification reaction was investigated by incubating the microsomal fraction with testosterone, the corresponding co-factors and 100 μM TBT. TBT was delivered in absolute methanol and the solvent removed from the assay by evaporation under a gentle nitrogen stream, prior to the addition of microsomes.

2.4. Analysis of testosterone esters

Testosterone esters were extracted with 1 mL of ethyl acetate (x3), the extracts collected and evaporated to dryness under a gentle nitrogen stream and reconstituted in methanol. Separation and identification of the esterified metabolites was achieved in an Acquity UPLC system (Waters, USA) connected to a Time-of-Flight Detector (LCT Premier XE) with an Acquity UPLC BEH C_8 column (1.7 μm particle size, 100 mm × 2.1 mm, Waters, Ireland) at a flow rate of 0.3 mL/min and column temperature of 30 °C. The mobile phases were (A) methanol