General and Comparative Endocrinology 174 (2011) 225–231

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

# Competitive binding to plasma thyroid hormone transport proteins and thyroid disruption by phenylbutazone used as a probe

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ARTICLE INFO

*Article history:*
Received 17 May 2011
Revised 31 August 2011
Accepted 2 September 2011
Available online 10 September 2011

*Keywords:*
Thyroid hormone
Competitive binding
Homeostasis

ABSTRACT

A model of thyroidectomized sheep intravenously supplemented with thyroid hormone (TH) was developed to mimic endogenous TH exposure and to analyze the impact on plasma TH homeostasis of xenobiotic interference with TH binding to plasma proteins. TH was displaced from plasma protein binding sites by using phenylbutazone (PBZ) as a test xenobiotic, to compare the effect of PBZ on steady state free and total plasma TH concentrations between the *in vivo* situation and an *in vitro* system. While PBZ increased free TH *in vitro*, PBZ administration *in vivo* produced an immediate reduction in both total and free plasma TH. The decrease in the total TH was consistent with a PBZ-induced displacement of TH from its plasma binding proteins, leading to an increase in total TH plasma clearance. However, this reduction in total TH was not expected to be accompanied by a parallel decrease in free plasma TH since the free TH is determined by the clearance of the free plasma TH. This suggested that PBZ may also have interfered with the clearance mechanisms of free TH.

It can be concluded that our thyroidectomized sheep model enables a dual action of a xenobiotic on plasma TH to be distinguished, namely a displacement of TH from its binding proteins leading to a decrease in the total plasma concentration, which is not relevant to thyroid function *versus* an interference with the intrinsic TH clearance leading to a change in the free plasma TH, which has a major impact in terms of thyroid disruption.

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## 1. Introduction

There is growing evidence that the thyroid is vulnerable to endocrine-disrupting effects of environmental chemicals. Due to their structural resemblance to thyroxine (T4) or tri-iodothyronine (T3), several chemicals are well-known to compete for binding to serum thyroid hormone (TH) transport proteins, for example polychlorinated biphenyls [4] and polybrominated diphenyl ethers [11]. Chemical interference with TH binding to transport proteins is suspected to be a potential disrupting mechanism of thyroid homeostasis [3]. Hence, it is often claimed that the *in vivo* competitive binding of chemicals to TH transport proteins, thereby displacing the natural ligands (TH), is followed by an increase in bioavailable free TH [14] with subsequent increased effects of TH.

The importance of plasma protein-binding displacement as a clinically important endocrine disruption mechanism needs to be evaluated in pharmacology. For drug–drug interactions at plasma protein binding sites, it has been wrongly assumed for a long time that when a displacing agent interacts with a primary drug, the result is an increase in the free concentration of the displaced drug in the plasma [7]. It is now well established that an *in vivo* displacement of drug molecules from plasma proteins leads to a decrease in the total drug concentration in the plasma while the concentration of free drug remains unchanged. Since the biological activity, such as the *in vivo* efficacy of a drug is determined by the free drug concentration at the site of action, drug–drug interaction at plasma protein binding sites has no clinical consequences since any increase in the pharmacological effects may be transient and cannot be sustained (for an explanation, see [2] for review).

In endocrinology, transposing these general concepts to TH and potential endocrine disrupters, it can be postulated that the total plasma TH concentration could be decreased due to some competition at the hormone sites binding while the free plasma TH steady state may remain unchanged. In this situation, no increase in the effects of TH should be expected since any increase in the free plasma TH concentration will be buffered by TH redistribution. To test this hypothesis *in vivo* is complicated by the fact that a potential change in the plasma TH concentration due to competitive binding can be masked by compensatory mechanisms at the level of the hypothalamo–pituitary–thyroid axis, leading to a modification in

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doi:10.1016/j.ygcen.2011.09.001

TH secretion. Species differences in circulatory transport proteins also complicate extrapolations of experimental data from animals to humans [13]. In mammals, the plasma binding proteins for TH are thyroid-binding globulin (TBG), transthyretin (TTR) and albumin [15]. Transthyretin (TTR) is the major plasma carrier of TH in rodents [9] while TBG is the major TH transport protein in human plasma, carrying about 68% and 80% of T4 and T3, with the remaining TH fractions being mainly bound to TTR and albumin [16]. Thyroidectomised sheep supplemented with TH by continuous intravenous infusion represent a model that overcomes the confounding effects of displacers on TH secretion by controlling the entry rate of TH into the plasma. Furthermore, sheep serum proteins have human-like TH-binding properties [29], which makes the sheep a relevant animal model to study the impact of competitors of TH binding to human plasma proteins.

Using this sheep model, the aim of the present study was to compare the influence of TH displacement from the plasma binding proteins by an exogenous substance, phenylbutazone (PBZ), *in vivo versus in vitro*. PBZ was selected as a probe since it represents a so-called displacer drug, i.e. a drug having a high affinity for albumin [5] and for which the therapeutic plasma molar concentrations (320 μM) [28] are not far from the molar concentration of albumin (600 μM). In addition, despite the fact that the affinity of phenylbutazone for TBG binding sites is several orders of magnitude less than that of T4, it may cause significant displacement of T4 at relevant therapeutic phenylbutazone concentrations and represent an important competitor for T4 binding to plasma proteins [28]. Finally, although PBZ is no longer used therapeutically, its interaction with other drugs is well documented and it constitutes a relevant model [25] that can be transposed to the question of TH and endocrine disrupters (see Section 4).

## 2. Materials and methods

### 2.1. Animals and surgery

Six female Lacaune sheep (10 months old), weighing 43.7 ± 2.7 kg were used. The sheep were aseptically thyroidectomized under general anaesthesia as previously described [17]. All the animal procedures were conducted in accordance with accepted standards of humane animal care under agreement number 31–247 for animal experimentation from the French Ministry of Agriculture.

### 2.2. Design

#### 2.2.1. In vitro binding experiments

Equilibrium dialysis was used to establish the conditions of PBZ exposure required to fulfil the criteria of a TH displacer by first evaluating *in vitro* the effect of increasing plasma PBZ concentrations on free tri-iodothyronine (T3) concentrations (Exp 1). Then, T3 plasma protein binding was determined with and without PBZ at a concentration shown from Exp1 to lead to an increase in plasma free T3 (1621 μM, Exp 2). The endogenous TH were removed from the plasma obtained from three adult female sheep by mixing 1 g of charcoal and 100 mg of dextran 70 with 10 ml of plasma for 1 h at room temperature. The charcoal was then removed by centrifugation (4 × 10 min at 3000*g*). For Exp 1, sheep plasma free of TH was used to prepare plasma containing T3 at 5 ng/ml (7.7 nM) by dilution of a concentrated T3 solution in ethanol (0.5 μg/ml). PBZ was added to aliquots of the T3 plasma solutions to obtain final PBZ concentrations ranging from 0.5 (1.6 μM) to 1000 μg/ml (3242.5 μM) by dilution of concentrated PBZ solutions in DMSO (0.05–100 mg/ml). For Exp 2, plasma free of TH was used to prepare plasma containing T3 ranging from 0.5 to 10,000 ng/ml (0.77nM–15.4 μM) from concentrated solutions of T3 in ethanol (0.1–1000 μg/ml). PBZ was added to the T3 plasma solutions to give a final concentration of 500 μg/ml (1621 μM). These plasma fractions were then rapidly frozen at −20 °C and kept at this temperature until protein binding studies.

#### 2.2.2. In vivo displacement experiment

The experiment was designed to evaluate the *in vivo* effects of the inhibition of TH binding to plasma proteins by PBZ on the free and total TH, and TSH plasma concentrations in the model of thyroidectomized sheep supplemented with T3 (or thyroxine, T4) via a continuous T3 (or T4) intravenous infusion. Preliminary measurements of basal total and free plasma TH concentrations were carried out to characterize the parameters of thyroid function in intact sheep. This was done by collecting five-ml blood samples at 2-h intervals for 24 h before the sheep were thyroidectomised. The *in vivo* displacement experiment took place about 2 months after thyroidectomy and included two periods separated by 25–27 days. The appropriate dosage regimen (loading dose and infusion rate) that reproduced the physiological steady state TH plasma concentrations in sheep was determined from the TH pharmacokinetic parameters previously described [10]. Hence, during the first period, the sheep were given an iv bolus of T3 at a dose of 1.32 μg/kg followed by a continuous infusion of T3 at 4.15 μg/h for 8 days. During the second period, sheep received 2.8 μg/kg of T4 followed by a continuous infusion of T4 at 3.20 μg/h for 8 days. PBZ was administered intravenously three days after the beginning of the T3 (or T4) infusion.

Five-ml blood samples were collected every hour for the 4 h following the T3 (T4) bolus loading , and then 2–4 times per day during the following 48 h. Blood samples were collected at 20 min intervals during the 4 h preceding PBZ administration to characterize steady state plasma TH concentrations. The effect of PBZ on plasma T3 (T4) levels was assessed from blood samples collected at 10-min intervals for 1 h, then every h for 6 h and finally once a day during the 5 days following PBZ administration.

### 2.3. Protein binding

*In vitro* protein binding of T3 was measured by equilibrium dialysis using a Dianorm$^{R}$ system (CH8135, Langenau, Zurich, Switzerland). One compartment contained plasma containing T3 with or without PBZ (0.9 ml) and the other, 0.9 ml of 0.1 mM phosphate buffer (pH = 7.4). Ten microliters of a 10 pM $^{125}$I-labeled T3 solution (3076 μCi/μg, Amersham BiosciencesR, Buckinghamshire, England) in ethanol:water (3:1) was added to each 1 ml plasma solution as a tracer (200,000 dpm per cell). Radiochemical purity of the $^{125}$I-labeled T3 solution was greater than 90%. After dialysis for 1 h at 37 °C, the dialyzed plasma and buffer were removed from the cells.

Since even minimal free iodine contamination of the iodinated T3 tracer leads to major over-estimates of the free T3 fraction, free T3 in the dialyzate buffer was precipitated with magnesium using a method adapted from Sterling and Brenner, [27]. Briefly, 340 μl of a 1 mg/ml carrier thyroxine solution in 0.033 N NaOH were added to 500μl of dialyzed buffer. After mixing, upon adding 165 μl of a magnesium chloride precipitating solution (10% $MgCl_2$, 6H2O in Tris-sodium chloride, pH 9.3), a dense white precipitate was seen. The tube was again agitated and centrifuged for 30 min at 20000*g*. After decantation of the supernatant, the precipitate was washed three times with 670 μl of the same magnesium chloride solution at pH 8.7. The radioactivity of the precipitate and dialyzed plasma was measured using a gamma scintillation counter. The yield of free T3 precipitation was determined in duplicate through the evaluation of the ratio between the radioactivity of the precipitate and the initial radioactivity of two $^{125}$I-T3 buffer solutions at

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