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SHORT COMMUNICATION

STEROID PROFILES OF BROWN ADIPOSE TISSUE

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Summary—In brown adipose tissue of alp-marmot (*Marmota marmota*), badger (*Meles meles*) and Wistar rats steroids of C_{21}- and C_{19}-type are identified and quantified. The detection of 3α-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5β-pregnan-20-one, 3α,21-dihydroxy-5α-pregnan-20-one, 3α,21-dihydroxy-5β-pregnan-20-one and 3β,21-dihydroxy-5α-pregnan-20-one is of special interest since sleep-inducing properties have been described with these steroids.

INTRODUCTION

Brown adipose tissue (BAT) was first described by Gessner [1] in the alp-marmot formerly known as the alp-mouse. Presently the function of BAT found in various animals including new born humans, is not fully understood. BAT is clearly distinct from other adipose tissue due to rich vascularization and innervation, the presence of intracellular multilocular lipid droplets and a large number of densely packed mitochondria [2, 3]. These unusual properties suggest the possibility of endocrinological and immunomodulating functions of BAT [4–6].

Since the quantity of BAT is significantly increased in hibernating animals, a functional relationship to hibernation is very likely. While this function remains speculative the involvement of BAT in nonshivering thermogenesis is unequivocally established [7].

One approach to explain the function of BAT was undertaken by Ptak [8–10], who chemically assayed adipose tissues of mice, syrian hamsters and rats and detected the steroid hormones H, C, CC, 11-DOC and DOH. We became interested in performing a more thorough chemical analysis of BAT since it is known that fat derived from the alp-marmot (*Marmota marmota*), badger (*Meles meles*) and mink (*Mustela vison*) is used in traditional medicine for the treatment of various skin diseases such as neurodermitis and psoriasis. As a result of our work in this area numerous steroids in the fat of the alp-marmot and badger were identified and quantified [11]. These findings prompted us to systematically search for steroids in BAT of hibernating animals (marmot, badger) and Wistar rats. This search was also designed to include steroids with sleep-inducing activity [12, 13].

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Abbreviations: H, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione (hydrocortisone); C, 17α,21-dihydroxy-4-pregnene-3,11,20-trione (cortisone); CC, 11β,21-dihydroxy-4-pregnene-3,20-dione (corticosterone); 11-DOC, 17α,21-dihydroxy-4-pregnene-3,20-dione (11-deoxycorticosterone); DOH, 21-hydroxy-4-pregnene-3,20-dione (17-hydroxycortexone); P, 4-pregnene-3,20-dione (progesterone); 20β-DIOH-P, 17α,20β-dihydroxy-4-pregnen-3-one; 5α-P, 3α-hydroxy-5α-pregnan-20-one; 5β-P, 3α-hydroxy-5β-pregnan-20-one; 5α-P-3α, 3α,21-dihydroxy-5α-pregnan-20-one; 5β-P-3α, 3α,21-dihydroxy-5β-pregnan-20-one; 5α-P-3β, 3β,21-dihydroxy-5α-pregnan-20-one; A, 3α-hydroxy-5α-androstan-17-one (androsterone).

EXPERIMENTAL

Origin of the tissues and general methods

Samples designated as MF1–4 were obtained from a male alp-marmot (*Marmota marmota*), body weight 3.5 kg, that was shot in Tirol (Austria) in the Autumn of 1989. In alp-marmot, BAT is mainly dissectable in the axillary region and the posterior mediastinum [3]. MF1 is retroperitoneal white adipose tissue, MF2 is subcutaneous adipose tissue, MF3 is BAT from the posterior mediastinum and MF4 is interscapular BAT. Sample BF1 was obtained from a young female german badger, and sample RF1 was obtained from a male Wistar rat, body weight 360 g, according to Ref. [14]. The identity of the tissues was confirmed by electron microscopy. Reference steroids were obtained from Sigma, München and their identity was assessed by mass (MS) and infrared (IR) spectrometry in comparison with reference data.

Extraction of adipose tissues

Tissue samples were frozen in liquid nitrogen and powdered. The powder from 100 to 500 mg of tissue was extracted twice with 10 ml of ethanol, the ethanol extract was filtered and evaporated to dryness. The residue was subjected to a solvent–solvent extraction step using a dichloromethane–water mixture (1:1, 10 ml, 3 times). The water fraction was subsequently treated with ethylacetate–buffer (2 g of sodium chloride per 10 ml water adjusted to pH 1 with 2 M sulfuric acid; equal volumes of ethylacetate and buffer, total volume about 30 ml) at 37°C for 72 h for the solvolysis of hydrophilic conjugates. The ethylacetate-phase (about 15 ml) was evaporated to dryness and the residue was dissolved in 80% aqueous acetonitrile (5 ml). The acetonitrile solution was eluted through two SPE (solid phase extraction) columns connected in series, the first packed with 1 g of RP18 derivatized silica gel (HPLC grade, obtained from Serva, Heidelberg), and the second packed with 1 g of amino derivatized silica gel (obtained from Millipore, Eschborn). Elution was performed with two

Table 1. Steroid contents (μg/g tissue) of adipose tissues from the alp-marmot

	MF1	MF2	MF3		MF4	
	free	free	free	bound	free	bound
H	ND	ND	ND	ND	21.1 ± 1.76	30.5 ± 2.37
C	ND	ND	ND	ND	ND	ND
CC	ND	ND	1.5 ± 0.12	ND	ND	ND
DOH	ND	ND	1.0 ± 0.11	ND	ND	ND
11-DOC	ND	ND	1.7 ± 0.18	ND	ND	ND
P	ND	ND	ND	ND	ND	ND
5α-P	ND	ND	1.9 ± 0.17	ND	14.7 ± 1.52	ND
5β-P	ND	ND	1.4 ± 0.07	ND	ND	ND
5α-P-3α	ND	ND	ND	3.8 ± 0.28	ND	ND
5β-P-3α	ND	ND	ND	3.4 ± 0.34	ND	ND
5α-P-3β	ND	ND	1.5 ± 0.09	ND	ND	ND
A	ND	ND	ND	4.6 ± 0.30	ND	ND

ND, not detectable; free, determination without solvolysis; and bound, determination after solvolysis.

column-volumes of 80% aqueous acetonitrile. The eluate was collected, and evaporated to dryness, and the residue was dissolved in acetonitrile (0.2 ml). The dichloromethane extract was evaporated to dryness, and the residue was treated by a solvent–solvent extraction step with *n*-hexane acetonitrile (10 ml). The acetonitrile phase was diluted with water (to an acetonitrile concentration of 80%), and this extract was subjected to SPE-column chromatography as described above. Aliquots of the acetonitrile solutions (0.02 ml) were directly analysed by HPLC, the conditions were as follows: HP 1090 liquid chromatograph with diode-array-detector; HP chemstation pascal; column: Hibar LiChrospher 100 RP18 250-4 (Merck), and Solvent: A 10% acetonitrile (1.5 μmol phosphoric acid), B acetonitrile, linear gradient 20 to 70% B over 1 h.

For GC-MS, aliquots of the acetonitrile solution containing the samples were evaporated to dryness under a gentle stream of nitrogen, and MO-TMS derivatives were prepared [15]. The GC conditions were as follows: gas-chromatograph HP 5890; detector HP MSD 5970 mass selective detector, HP Chemstation Pascal; Column, Chrompack CPSIL5CB, length 25 m, inside dia 0.25 mm, film-thickness 0.4 μm and the temperatures were: injector 270°C, purgeoff time 2 min; transfer line 300°C; oven initial 120°C for 3 min to 240°C with 6°C/min to 300°C with 3°C/min, hold for 7 min.

Quantitative estimation was done by adding known amounts of reference substances to tissue samples before the freezing step. For steroids in the fraction designated as "free" each value in the table is the mean of three pairs of determinations (each with and without added reference substances). For H in the fractions designated as bound we added H sulphate as reference. For other steroids in the bound fraction we made a correction based on recovery calculated for H sulphate, because in these fractions we found no H.

Table 2. Steroid contents (μg/g tissue) of adipose tissues from the Wistar rat and badger

	RF1	BF1	
	free	free	bound
H	1.1 ± 0.11	80.4 ± 7.29	58.7 ± 5.09
C	1.9 ± 0.17	ND	ND
CC	0.7 ± 0.05	ND	ND
DOH	ND	ND	ND
11-DOC	0.5 ± 0.08	ND	ND
P	0.3 ± 0..01	ND	ND
5α-P	ND	3.1 ± 0.30	ND
5β-P	ND	2.7 ± 0.14	ND
5α-P-3α	ND	ND	ND
5β-P-3α	ND	ND	ND
5α-P-3β	ND	ND	ND
A	ND	1.9 ± 0.13	ND

ND, not detectable; free, determination without solvolysis; and bound, determination after solvolysis.

The identification procedure was based on four items. First relative retention time (RRT) relative to 20β-DIOH-P in HPLC. Second u.v. spectra taken by a diode array detector on line. Third RRT relative to 20β-DIOH-P in capillary GC. Fourth mass spectra of MO-TMS derivatives taken on line. For identitication to be taken as positive all data have to fit data obtained with reference substances.

RESULTS AND DISCUSSION

The analysis revealed the steroid pattern summarized in Table 1 and 2. Whereas no glucocorticoids could be detected in white adipose tissue of the alp-marmot, H, C, CC, 11-DOC and DOH were identified in BAT of the alp-marmot, badger and rat, as previously reported by Ptak [8–10]. In addition P, 5α-P, 5β-P, 5α-P-3α, 5β-P-3β, and A were identified in BAT for the first time.

Our results indicate two clearly distinct patterns of steroid content. The first pattern demonstrated in the interscapular BAT of the alp-marmot and badger, is represented by high amounts of H, in both free and conjugated forms. The second pattern demonstrated in the mediastinal BAT of the alp-marmot and the interscapular BAT of the rat, is represented by lower amonts of C, CC, 11-DOC, 5α-P, 5β-P and 5α-P-3β in the mediastinal BAT of the alp-marmot, and by lower amounts of C, CC, 11-DOC, DOH and P in the interscapular BAT of the rat.

The detection of 5α-P, 5β-P, 5α-P-3α, 5β-P-3α and 5α-P-3β is of special interest since sleep-inducing properties have been described in these steroids (see for example [16, 17]). These steroids and also A were only detected in BAT of the hibernating species. Various groups detected structures in cells of BAT by electron microscopy, that point towards production and secretion of steroids (so called "endoplasmatic lipid granule secretion" [18].) This modified apocrine secretion in the cells of BAT is similar to secretion in cells of adrenal glands. Our data and these aspects of BAT morphology lead us to propose that BAT is a site of steroidogenesis. Further investigations are required to test our hypothesis that steroids with sleep-inducing properties are involved in regulating the hibernating state.

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