5α-DIHYDROTESTOSTERONE TREATMENT INDUCES METABOLIC CHANGES ASSOCIATED WITH POLYCYSTIC OVARY SYNDROME WITHOUT INTERFERING WITH HYPOTHALAMIC LEPTIN AND GLUCOCORTICOID SIGNALING

Marina Nikolić1, Nataša Veličković1, Ana Djordjevic1, Biljana Bursać1, Djuro Macut2, Ivana Božić Antić2, Jelica Bjekić Macut3, Gordana Matić1 and Danijela Vojnović Milutinović1,*

1 Department of Biochemistry, Institute for Biological Research “Siniša Stanković”, University of Belgrade, 142 Despot Stefan Blvd., 11000 Belgrade, Serbia
2 Institute of Endocrinology, Diabetes and Metabolic Diseases, Clinical Center of Serbia and School of Medicine, University of Belgrade, Dr Subotića 13, 11000 Belgrade, Serbia
3 CHC Bežanijska kosa, Bežanijska kosa bb, 11080 Belgrade, Serbia

*Corresponding author: dvojnovic@ibiss.bg.ac.rs
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Abstract: Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age. It is a heterogenous disorder, with hyperandrogenism, chronic anovulation and polycystic ovaries as basic characteristics, and associated metabolic syndrome features. Increased secretion of leptin and leptin resistance are common consequences of obesity. Leptin is a hormone with anorexigenic effects in the hypothalamus. Its function in the regulation of energy intake and consumption is antagonized by glucocorticoids. By modulating leptin signaling and inflammatory processes in the hypothalamus, glucocorticoids can contribute to the development of metabolic disturbances associated with central energy disbalance. The aim of the study was to examine the relationship between hypothalamic leptin, glucocorticoid and inflammatory signaling in the development of metabolic disturbances associated with PCOS. The study was conducted on an animal model of PCOS generated by a continual, 90-day treatment of female rats with 5α-dihydrotestosterone (DHT). The model exhibited all key reproductive and metabolic features of the syndrome. mRNA and/or protein levels of the key components of hypothalamic glucocorticoid, leptin and inflammatory pathways, presumably contributing to energy disbalance in DHT-treated female rats, were measured. The results indicated that DHT treatment led to the development of hyperphagia and hyperleptinemia as metabolic features associated with PCOS. However, these metabolic disturbances could not be ascribed to changes in hypothalamic leptin, glucocorticoid or inflammatory signaling pathways in DHT-treated rats.

Key words: DHT; hypothalamus; leptin; glucocorticoids; inflammation

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common female endocrinopathy affecting 4-8% of women of reproductive age [1-3]. It is a heterogenous endocrinological disorder involving both reproductive and metabolic abnormalities, most importantly hyperandrogenemia, chronic anovulation and polycystic ovaries. Features of the metabolic syndrome, notably visceral obesity, dyslipidemia, low glucose tolerance and insulin resistance are frequently associated with PCOS [3,4]. It has been suggested that hyperandrogenemia, as one of the key features of PCOS, can affect food intake by increasing food craving, and thereby induce weight gain in women [5].

Obesity, most frequently the central type, is very common in PCOS, with hyperandrogenemia particularly stimulating the propagation of visceral adipose tissue [6,7]. The most important influences of obesity on the genesis and self-propagation of PCOS include the stimulation of hyperinsulinemia and insulin resistance, chronic low-grade inflammation and general lipotoxicity [8,9].
Adipose tissue influences central food intake and energy expenditure control through the secretion of adipokines, among which is leptin [9,10]. Leptin blood concentration is positively correlated with adipocyte size and general adiposity. Its secretion is stimulated by insulin and glucocorticoids, and inhibited by androgens [11,12]. Leptin passes the blood-brain barrier (BBB) and, after binding to the long form of leptin receptor (ObRb) in the hypothalamus, exerts its anorexigenic effects through the transcriptional activation/repression of a number of genes, including those coding for neuropeptide Y (NPY), Agouti-related peptide (AgRP) and proopiomelanocortin (POMC) [13].

Leptin resistance is a state in which leptin is not able to perform its functions, in spite of its high levels in the circulation [14]. It is frequently a consequence of visceral obesity [14], and can induce food consumption abnormalities, which intensify the effects of obesity on the pathophysiology of PCOS [15]. Leptin resistance can arise at different stages of the leptin transport through the BBB, or the hypothalamic ObRb signaling: most importantly the stimulated downregulation of ObRb [14,16,17] and/or expression of the suppressor of the cytokine signaling 3 (SOCS-3) gene, which leads to the increased negative feedback regulation of the leptin signal transduction [14,18,19]. Furthermore, an important role in the genesis of leptin resistance can be attributed to the local inflammatory mediator tumor necrosis factor α (TNFa), interleukin 6 (IL-6), IkB kinase (IKKβ)/nuclear factor kB (NFkB) and the proinflammatory actions of free fatty acids (FFA) [13,14,18,19].

Glucocorticoid hormones can modulate the leptin signaling pathway at the level of signal transducer and activator of transcription 3 (STAT3) and SOCS-3 gene expressions [20]. The levels of bioactive forms of glucocorticoids can be regulated locally through the action of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1)/hexosyl-6-phosphate dehydrogenase (H6PDH) enzymatic system, which converts the inactive forms of these hormones to the active ones [21,22]. Increased activation of glucocorticoids is considered important for the genesis of metabolic disorders, including those linked to PCOS [21-24]. Glucocorticoids are also well-known as anti-inflammatory molecules generally suppressing the expression and modifying the activities of proinflammatory cytokines, such as IL-6 and TNF-α, and transcription regulators, such as NFkB [25,26], aforementioned as possible mediators of leptin resistance in the hypothalamus.

Our previous work has shown the possibility of involvement of glucocorticoid genomic effects, generally exerted after binding to and activating the glucocorticoid receptor (GR), in metabolic disturbances in the rat model of PCOS generated by 5α-dihydrotestosterone (DHT) treatment [27]. More specifically, we have found changes in visceral adipose tissue lipid metabolism leading to hypertrophic visceral obesity [27]. Considering the positive correlation of obesity and adipocyte size with leptin secretion, the importance of leptin in the hypothalamic regulation of energy intake and its aforementioned interactions with glucocorticoid signaling in the hypothalamus, we investigated the link between hypothalamic leptin, glucocorticoid and inflammatory signaling changes, and energy intake disturbances in female rats subjected to long-term DHT treatment.

MATERIALS AND METHODS

Animals and treatment

On the 21st day after birth, female Wistar rat pups were separated from lactating dames and randomly divided into two groups. The first group was implanted with 90-day-continuous-release pellets containing 7.5 mg of DHT (daily dose, 83 μg; DHT group), and the second was treated with pellets lacking the bioactive component (Placebo group). DHT and placebo pellets were purchased from Innovative Research of America (Sarasota, FL, USA). The dose of DHT was chosen to induce the hyperandrogenic state corresponding to that seen in women with PCOS [28,29]. Each experimental group was comprised of 12 animals (n=12), which were housed three per cage, kept
in a space with controlled temperature (22±2°C) and constant humidity, and under a standard 12 h/12 h light/dark cycle. All animals had ad libitum access to commercial chow and tap water. During the 90-day treatment, food intake was measured daily and body mass weekly. Energy intake was calculated as daily calories ingested through food (food mass (g) × 11 kJ). At the end of the treatment, rats were killed by decapitation in the diestrus phase of the estrous cycle. The stage of cyclicity was determined by microscopic analysis of the predominant cell type in vaginal smears obtained daily from each animal from the 10th week to the end of the treatment. All protocols were compliant with the European Communities Council Directive (86/609/EEC) for the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research “Siniša Stanković”, University of Belgrade (No 2-20/10), according to the guidelines of the EU-registered Serbian Laboratory Animal Science Association (SLASA).

Tissue and blood sample collection and determination of plasma parameters

Immediately after the experimental animals were killed by rapid decapitation, visceral adipose tissue and hypothalami were isolated, weighed and frozen in liquid nitrogen for storage until further use. Trunk blood was collected at decapitation in EDTA-containing tubes and the blood triglyceride concentration was measured on site by MultiCare strips (Biochemical Systems International, Arezzo, Italy). Plasma was isolated by centrifugation at 1600 × g for 15 min at room temperature, and then stored at -70°C. The plasma level of FFA was determined using a modified version of Duncombe’s method [30]. Total plasma leptin concentrations were measured by the Rat Leptin ELISA Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. Absorbance at 450 nm (reference 590 nm) was read using a plate reader (Multiskan Spectrum, Thermo Electron Corporation, Waltham, MA, USA, and plasma leptin concentrations, determined by 4PL curve fitting analysis (ReaderFit Software, MiraiBio Group of Hitachi Solutions America, Ltd., San Bruno, CA, USA), were presented in ng/mL. The intra-assay coefficient of variation (CV) was 5.9%, while inter-assay CV was 8.9%.

Preparation of hypothalamic whole cell extracts

Hypothalami were homogenized in 4 vol. (w/v) of radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet NP40, 0.1% SDS, 2 mM DTT, 1 mM EDTA-Na2, 0.15 mM spermine, 0.15 mM spermidine, protease and phosphatase inhibitors) using a glass/teflon (Potter-Elvehjem) homogenizer. The homogenates were sonicated on ice (3 x 10 s at 10 MHz, Hielshcer Ultrasun Processor, Hielshcer Ultronics GmbH, Teltow, Germany) and incubated for 60 min at 0°C prior to 20-min centrifugation at 14000 x g. The resulting supernatants were stored at -70°C. Protein content was determined according to Spector [31].

SDS-PAGE and immunoblotting

Proteins were resolved on 7.5% SDS-polyacrylamide gels using Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA, USA). Transfer of proteins from acrylamide gels to PVDF membranes (Immobilon-FL, Millipore Billerica, MA, USA) was performed in 25 mM Tris buffer, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol, at 135 mA overnight in Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked by phosphate-buffered saline (PBS, 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 2.7 mM KCl, 0.14 M NaCl, pH 7.2) containing 3% non-fat dry milk for 90 min at room temperature. After extensive washing (PBS containing 0.1% Tween20), membranes were incubated overnight at 4°C with respective primary antibodies: rabbit polyclonal anti-leptin receptor (ab5593, Abcam, Cambridge, UK), rabbit polyclonal anti-GR (PA1-511, Thermo Scientific, Waltham, MA, USA), rabbit polyclonal anti-11β-HSD1 (ab109554, Abcam, Cambridge, UK), rabbit polyclonal anti-H6PD (sc-67394, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-NFκB.
and mouse monoclonal anti-β-actin (AC-15, Sigma-Aldrich, Saint Louis, MO, USA), which was used as an equal loading control. After thorough washing, all membranes were incubated with alkaline phosphatase conjugated secondary antibodies (Amersham Pharmacia Biotech, Little Chalfont, UK; 1:20000). The immunoreactive proteins were visualized by an enhanced chemiluminescence method (ECF, Amersham Pharmacia Biotech, Little Chalfont, UK) and quantitative analysis was performed by Image-Quant software (GE Healthcare, Little Chalfont, UK).

RNA isolation and reverse transcription

Total hypothalamic RNA was isolated using TRI Reagent® (AmBion, Waltham, MA, USA). RNA was dissolved in RNase-DNase free water (Eppendorf, Hamburg, Germany) and its concentration and purity were tested spectrophotometrically (OD 260/280>1.8 was considered satisfactory). RNA integrity was confirmed by 2% agarose gel electrophoresis. RNase inhibitor (Applied Biosystems, Foster City, CA, USA) was added and the samples were frozen at -70°C until use. Prior to cDNA synthesis, DNA contamination was removed by DNase I treatment (Fermentas, Waltham, MA, USA). cDNA was synthesized from 2 μg of RNA. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions, and cDNA was stored at -70°C until use.

Quantitative real-time PCR

Quantification of ObRb, GR, NPY, SOCS-3, TNFα and IL-6 mRNA levels in the hypothalamus was performed by TaqMan® Real Time PCR. Primers and probes for GR, ObRb, NPY, SOCS-3, TNFα and IL-6 (Rn00567167_m1, Rn00561369_m1, Rn01410145_m1 and Rn00585674_s1, Rn01525859_g1, Rn01410330_m1, respectively) were obtained from Applied Biosystems Assay-on-Demand Gene Expression Products. HPRT1 (Rn01527840_m1) was used as a previously validated endogenous control. Quantitative real time PCR (qPCR) was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City CA, USA) in a total volume of 25 μL containing 1 × TaqMan® Universal Master Mix with AmpErase UNG, 1 × Assay Mix (Applied Biosystems, Foster City CA, USA) and the cDNA template (20 ng of RNA converted to cDNA) as follows: at 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 90 s. No template control was used in any run. All reactions were run in triplicate. Relative quantification of target genes was performed using the comparative 2−ΔΔCt method (Livak and Schmittgen, 2001). The obtained results were analyzed by Sequence Detection Software version 1.2.3 for 7000 System SDS Software RQ Study Application (Applied Biosystems, Foster City CA, USA) with a confidence level of 95% (p≤0.05).

Statistical analyses

Statistical analyses were performed using Prism software 5.00 (GraphPad, San Diego, CA, USA). The results are expressed as means±SD for biochemical and hormonal parameters, and as means±SEM for data from Western blot analysis and qPCR. Values were considered statistically significant when the p value was less than 0.05.

RESULTS

Morphological and basic metabolic parameters of DHT-treated female rats

The energy intake of DHT-treated rats was significantly increased when compared placebo-treated rats and was accompanied by an increase in visceral adipose tissue mass (Table 1, p<0.05).

Blood triglyceride, FFA and leptin levels

Statistically significant increases in blood triglyceride level (***, p<0.001), as well as in the plasma levels of FFA (*, p<0.05) and leptin (**, p<0.01) were observed
after DHT-treatment in comparison to the Placebo group (Figs. 1 and 2).

**Leptin signaling alterations in DHT-treated animals**

The effects of DHT treatment on the expression of hypothalamic ObRb, SOCS-3 and NPY were examined by qPCR and Western blot. Subsequent Student t-test analyses showed the lack of significant changes in both relative protein and mRNA levels of ObRb, as well as in the relative amounts of SOCS-3 and NPY mRNAs in the hypothalami of DHT-treated rats (Fig. 3).

**Glucocorticoid signaling in the hypothalami of DHT-treated rats**

Changes in glucocorticoid signaling in the hypothalami of female rats after DHT treatment were examined by semiquantitative Western blot analyses of glucocorticoid prereceptor metabolism, qPCR analysis of relative GR mRNA levels and Western blot analysis of relative GR protein levels in the hypothalamic whole cell extracts. The results obtained are shown in Fig. 4, and imply an unchanged glucocorticoid regeneration and signaling in the hypothalamus of the DHT-treated rats.

**Local inflammation in the hypothalami of DHT-treated rats**

The relative local expression of several cytokines potentially involved in the changes of hypothalamic leptin signaling was also studied by qPCR and Western blot analyses. Succeeding statistical evaluations showed that neither IL-6 and TNFα mRNA, nor relative NFκB protein levels were significantly influenced by DHT treatment (Table 2, Fig. 5).

**DISCUSSION**

This study was performed on a hyperandrogenemic rat model of PCOS, obtained by the continual subcutaneous administration of a nonaromatizable form of testosterone, DHT, from the beginning of puberty up to adulthood. The model exhibited the main reproductive and metabolic features of PCOS [27]. The obtained results confirmed the existence of visceral adiposity, dyslipidemia and hyperphagia with increased energy intake in DHT-treated female rats (Table 1, Fig. 1). Significant hypertrophy of visceral fat adipocytes was also previously observed in the same animals [27]. Taking this fact and the noticed metabolic changes into account, the presence of blood hyperleptinemia and possible hypothalamic leptin resistance seems to be a reasonable assumption [11,12,14]. A significant eleva-

| Table 1. Energy intake and visceral adipose tissue mass of DHT-treated and placebo rats. |
|------------------------------------------|---------|---------|
|                                         | Placebo | DHT     |
| Energy intake (kJ/day/rat)               | 177.20±30.10 | 191.30±34.90 ** |
| Visceral adipose tissue mass (g)         | 9.34±2.29     | 13.02±2.48 ** |

Data were analyzed by Student t-test and represent the mean values ± SD of 12 animals per group. A value of p<0.05 was considered statistically significant (**, p<0.01).
tion in blood leptin concentration was indeed observed in the DHT-treated females (Fig. 2). Namely, hyperleptinemia is often linked with hypothalamic leptin resistance, especially in obese animals. The state of leptin resistance can include signal transduction impediments after hormone binding to ObRb [17], involving the increased expression of the SOCS-3 gene in response to leptin and/or increased negative regulation of ObRb receptor gene expression [14,18,19]. However, in spite of the observed hyperleptinemia, the results of qPCR and Western blot analyses did not indicate these changes in the leptin signaling pathway (Fig. 3A, B and C).

One of the most important effector molecules in leptin control of energy balance is NPY [13], whose expression is influenced by glucocorticoids and insulin [32]. The effect of glucocorticoids on the NPY secretion in the hypothalamus is notably opposite to that of leptin and insulin [33] and involves the NPY gene transcriptional control [32,34]. Therefore, in this study the changes in the corresponding hypothalamic mRNA levels were analyzed, but our results did not show a statistically significant increase in NPY gene expression after DHT treatment (Fig. 3D).

The analyses of local glucocorticoid signaling were also performed, taking into account the aforementioned crosstalk between glucocorticoid and leptin signaling at the levels of NPY, ObRb and SOCS-3 gene expressions in central energy-intake regulation [20,32,33]. Analyses of the levels of 11β-HSD1 and

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**Fig. 2.** Plasma leptin levels. Data represent the mean values ± SD of 12 animals per each group, DHT-treated (DHT) and Placebo. Group comparisons were done by Student t-test. A value of p<0.05 was considered statistically significant (**, p<0.01).

**Fig. 3.** The levels of ObRb, SOCS-3 and NPY gene expressions. Representative Western blot and relative levels of ObRb protein in the hypothalamic whole cell extract of placebo (P) and DHT-treated rats (DHT) (A). Respective relative hypothalamic levels of ObRb (B), SOCS-3 (C) and NPY (D) mRNA in placebo and DHT-treated rats. Blots were probed with β-actin antibody as an equal loading control. Data are presented as mean ± SEM and were analyzed by Student t-test.
H6PDH enzymes, the hypothalamic proteins involved in the regeneration of biologically active glucocorticoids [34], revealed that glucocorticoid prereceptor metabolism, as well as hypothalamic GR mRNA and protein levels were unaltered by DHT treatment (Fig. 4). Together, the observed results imply an unaffected glucocorticoid signaling, and therefore the absence of the potential effects of glucocorticoids on the leptin-signaling components and NPY expression in the hypothalamus of the chosen PCOS model.

An important role in the metabolic disturbances linked to the energy intake disbalance can be assigned to local inflammatory processes in the hypothalamus. Local inflammatory consequences can arise due to the overactivity of the IKKβ/NFkB system, or the increase in TNFα and IL-6 proinflammatory mediators [13,14,18,19]. However, the analyses performed in this study did not show changes in TNFα and IL-6 mRNA levels (Table 2), which is in accordance with the unaltered NFkB protein level in the hypothalamic whole-cell extract of DHT-treated rats (Fig. 5).

### Table 2. IL-6 and TNFα mRNA levels in the hypothalamus of DHT-treated and placebo rats.

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<th>Placebo</th>
<th>DHT</th>
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<tr>
<td>TNFα</td>
<td>1.00±0.06</td>
<td>1.16±0.09</td>
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<tr>
<td>IL-6</td>
<td>1.00±0.14</td>
<td>0.78±0.10</td>
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Data were analyzed by Student t-test and represent the mean values ± SD of 12 animals per group. A value of p<0.05 was considered statistically significant.

### Fig. 5. NFkB protein level. A representative Western blot and relative level of NFkB protein in the hypothalamic whole cell extract of placebo (P) and DHT-treated rats (DHT). Blots were probed with β-actin antibody as an equal loading control. Data are presented as mean ± SEM and were analyzed by Student t-test.
The results of the present study point to a disturbed energy balance after DHT treatment, as illustrated by visceral adiposity, dyslipidemia and an increased energy intake, with adjoining hyperleptinemia. At the same time, the performed molecular analyses did not confirm the relation of the listed metabolic changes with either hypothalamic leptin, glucocorticoid or inflammatory signaling changes in the chosen PCOS model. Therefore, some additional aspects of leptin resistance, such as FFA-influenced STAT3 regulation, or the hyperleptinemia-stimulated inhibitory phosphorylations of ObRb, should be analyzed as potential markers of disturbed energy balance control in DHT-treated rats.

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Conflict of interest disclosure: The authors declare no conflicts of interest.

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