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Dihydrotestosterone deteriorates cardiac insulin signaling and glucose transport in the rat model of polycystic ovary syndrome

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ABSTRACT

It is supposed that women with polycystic ovary syndrome (PCOS) are prone to develop cardiovascular disease as a consequence of multiple risk factors that are mostly related to the state of insulin resistance and consequent hyperinsulinemia. In the present study, we evaluated insulin signaling and glucose transporters (GLUT) in cardiac cells of dihydrotestosterone (DHT) treated female rats as an animal model of PCOS. Expression of proteins involved in cardiac insulin signaling pathways and glucose transporters, as well as their phosphorylation or intracellular localization were studied by Western blot analysis in DHT-treated and control rats. Treatment with DHT resulted in increased body mass, absolute mass of the heart, elevated plasma insulin concentration, dyslipidemia and insulin resistance. At the molecular level, DHT treatment did not change protein expression of cardiac insulin receptor and insulin receptor substrate 1, while phosphorylation of the substrate at serine 307 was increased. Unexpectedly, although expression of downstream Akt kinase and its phosphorylation at threonine 308 were not altered, phosphorylation of Akt at serine 473 was increased in the heart of DHT-treated rats. In contrast, expression and phosphorylation of extracellular signal regulated kinases 1/2 were decreased. Plasma membrane contents of GLUT1 and GLUT4 were decreased, as well as the expression of GLUT4 in cardiac cells at the end of androgen treatment. The obtained results provide evidence for alterations in expression and especially in functional characteristics of insulin signaling molecules and glucose transporters in the heart of DHT-treated rats with PCOS, indicating impaired cardiac insulin action.

1. Introduction

Polycystic ovary syndrome (PCOS) is a common reproductive disorder frequently associated with various metabolic abnormalities, and substantially leading to the increased risk for type 2 diabetes [1]. According to the Androgen Excess and PCOS (AE-PCOS) Society, PCOS is defined by the obligatory presence of hyperandrogenism, combined with ovarian dysfunction indicated by oligo-ovulation and/or polycystic ovaries, after exclusion of other related disorders [2].

According to the literature data PCOS may have its onset before or during puberty and is associated with excessive androgen production during early puberty [3–5]. Since PCOS is primary hyperandrogenic state, several androgens have been used to induce PCOS conditions in rats. In this study, we used 5α-dihydrotestosterone (DHT) to induce a state of androgen excess in prepubertal age of rats. DHT is non-aromatizable androgen with

Abbreviations: PCOS, polycystic ovary syndrome; DHT, dihydrotestosterone; GLUT, glucose transporter; IR, insulin receptor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; Akt, protein kinase B; ERK1/2, extracellular signal regulated kinases 1/2; BCA, bicinchoninic acid; HOMA, homeostasis model assessment; PM, plasma membrane; LDM, low density microsomes; TG, triglycerides; NEFA, nonesterified fatty acids.

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high affinity for the androgen receptor. The chosen dose of DHT was used to induce a hyperandrogenic state mimicking that is seen in women with PCOS, whose plasma DHT levels are 1.7 times higher than those of healthy controls [6,7]. As Manneras et al. [8] have demonstrated, treatment of juvenile rats with DHT induces both reproductive and metabolic abnormalities similar to those seen in women with PCOS. They have shown that DHT treated rats had irregular estrous cycles and other ovarian features similar to human PCOS, including increased numbers of large atretic follicles and follicular cysts with diminished granulosa layer.

Insulin resistance and hyperinsulinemia appear to be major contributors to the pathophysiology of PCOS. There is a general agreement that obese women with PCOS are insulin resistant, while some groups of lean affected women may have normal insulin sensitivity [9].

Insulin is a hormone involved in regulation of cardiac metabolism, contractility, protein synthesis, growth, apoptosis and survival of cardiomyocytes, and myocardial blood perfusion [10]. When insulin binds to insulin receptor (IR), it induces the phosphorylation of IR substrates (IRSs) at tyrosine residues, thereby activating a complex signal transduction network. The phosphatidylinositol 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways are two main pathways of that network. PI3K is considered to be the main player of the metabolic action of insulin, whereas the MAPK pathway is principally involved in cell growth and differentiation. A key effector of the PI3K pathway is the protein kinase termed protein kinase B (Akt). Akt phosphorylates a variety of intracellular substrates, regulating cell growth, metabolism, and survival [11]. Insulin stimulates glucose uptake by increasing the translocation of the insulin-responsive glucose transporters (GLUT). The most abundant GLUT in the heart is GLUT4, which is insulin-regulated *via* PI3K/Akt pathway, while GLUT1 is primarily responsible for basal glucose transport [12].

Post-binding defect in IR signaling has been detected in PCOS, likely due to increased receptor and IRS-1 serine phosphorylation that selectively affects metabolic, but not mitogenic pathway in classic insulin target tissues and in the ovary [9]. On the other hand, constitutive activation of serine kinases in the MAPK-extracellular signal regulated kinases 1/2 (ERK1/2) pathway may contribute to resistance to insulin metabolic actions in skeletal muscle. These findings have been directly translated into PCOS therapy with insulin-sensitizing drugs [9,13]. In addition, a growing body of evidence supports a notion that women with PCOS display an increased prevalence of cardiovascular disease risk factors, most of which are etiologically correlated with insulin resistance and a consequent hyperinsulinemia [14].

Although insulin resistance was already studied in skeletal muscles [15–20], adipose tissue [20–23], fibroblasts [24,25] or endometria [26,27] of women with PCOS and in skeletal muscles of experimental animals with PCOS [28], there are no data regarding insulin signaling and action in the heart of women with PCOS or animal models of the syndrome. To shed new light on the issue of possible PCOS-accompanied cardiac insulin resistance, female Wistar rats were exposed to a 90-days long treatment with subcutaneous DHT pellets to obtain an animal model of PCOS [8]. At the end of the treatment, the protein level of insulin signaling molecules (IR, IRS-1, Akt and ERK1/2) and glucose transporters GLUT1 and GLUT4, as well as their activating/inhibiting phosphorylation or subcellular localization were analyzed in the whole heart.

2. Materials and methods

2.1. Materials

Anti-phospho-IRS-1 (Ser^{307}) antibody (07–247) was a product of Merck Millipore (Billerica, MA, USA). Anti-Akt 1/2/3 antibody (#9272), anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}) antibody (#9101) and anti-ERK1/2 antibody (#9102) were products of Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-IRβ (sc-711), anti-IRS-1 (sc-8038), anti-phospho Akt 1/2/3 (Ser^{473}) (sc-7985-R), anti-phospho Akt 1/2/3 (Thr^{308}) (sc-16646-R), anti-GLUT4 antibody (sc-7938), anti-GLUT1 (sc-7903), and anti-actin antibody (sc-1616-R) were products of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). RIA kit for insulin was a product of INEP (Zemun, Serbia). Reagents for the bicinchoninic acid (BCA) assay were purchased from Pierce (Rockford, IL, USA). DHT and placebo pellets were purchased from Innovative Research of America (Sarasota, FL). Electrophoretic reagents were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA).

2.2. Animals and treatment

At 21-days of age female Wistar rats were separated from lactating dams and randomly divided into two groups ($n = 12$ per group). At that age rats were implanted subcutaneously on the back of the neck with 90-days continuous-release pellets containing 7.5 mg DHT (daily dose, 83 μg, DHT group) or 7.5 mg placebo pellets (control group).

The animals were housed three per cage and kept in a temperature-controlled room ($22 \pm 2\,^{\circ}C$) with a 12 h light/dark cycle (lights on at 07:00 h) and constant humidity. Rats were fed with commercial chow and drinking water available *ad libitum*. At the end of the experimental period rats were killed by decapitation in the diestrus phase of estrous cycle. All animal procedures were in compliance with the EEC Directive (86/609/EEC) on 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).

2.3. Glucose and insulin concentration determination and HOMA index calculation

For measurement of glucose and insulin concentration, animals were fasted overnight before collection of blood samples to avoid changes of insulin and glucose induced by food intake. The glucose concentration was measured in whole blood using Accutrend glucometer (Roche Diagnostics GmbH, Mannheim, Germany). For the determination of plasma insulin concentration blood was collected at decapitation in EDTA-pretreated tubes and centrifuged at 3000 rpm for 10 min to obtain plasma samples. The plasma insulin level was determined by the RIA method, using rat insulin standards. The reference level of rat fasting insulin was 12.06–48.26 mIU/l. Assay sensitivity was 0.6 mIU/l and an intra-assay coefficient of variation was 5.24%.

Homeostasis model assessment (HOMA) index, as an indicator of insulin resistance, was calculated from fasted plasma insulin and glucose concentration using the formula described by Matthews et al. [29]: insulin (mU/l) × [glucose (mmol/l)/22.5].

2.4. Measurement of circulating triacylglycerols and non-esterified fatty acids

The TG level was measured using a Multicare analyzer (Biochemical Systems International, Arezzo, Italy) and the plasma non-esterified fatty acid (NEFA) level was determined using a colorimetric method [30].

2.5. Preparation of cardiac cell lysate

Cardiac tissue samples were homogenized at 4 °C with an Ultra-Turrax homogenizer (3× 30 s) in four volumes (m:V) of modified