Biochimica et Biophysica Acta xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Leptin modulates human Sertoli cells acetate production and glycolytic profile: A novel mechanism of obesity-induced male infertility?

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ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form 20 May 2015
Accepted 8 June 2015
Available online xxxx

Keywords:
Sertoli cells
Leptin
Obesity
Spermatogenesis
Male fertility

ABSTRACT

Human feeding behavior and lifestyle are gradually being altered, favoring the development of metabolic diseases, particularly type 2 diabetes and obesity. Leptin is produced by the adipose tissue acting as a satiety signal. Its levels have been positively correlated with fat mass and hyperleptinemia has been proposed to negatively affect male reproductive function. Nevertheless, the molecular mechanisms by which this hormone affects male fertility remain unknown. Herein, we hypothesize that leptin acts on human Sertoli cells (SCs), the "nurse cells" of spermatogenesis, altering their metabolism. To test our hypothesis, hSCs were cultured without or with leptin (5, 25 and 50 ng/mL). Leptin receptor was identified by qPCR and Western blot. Protein levels of glucose transporters (GLUT1, GLUT2 and GLUT3), phosphofructokinase, lactate dehydrogenase (LDH) and monocarboxylate transporter 4 (MCT4) were determined by Western Blot. LDH activity was assessed and metabolite production/consumption determined by proton nuclear magnetic resonance. Oxidative damage was evaluated by assessing lipid peroxidation, protein carbonilation and nitration. Our data shows that leptin receptor is expressed in hSCs. The concentration of leptin found in lean, healthy patients, upregulated GLUT2 protein levels and concentrations of leptin found in lean and obese patients increased LDH activity. Of note, all leptin concentrations decreased hSCs acetate production illustrating a novel mechanism for this hormone action. Moreover, our data shows that leptin does not induce or protect hSCs from oxidative damage. We report that this hormone modulates the nutritional support of spermatogenesis, illustrating a novel mechanism that may be linked to obesity-induced male infertility.

1. Introduction

Human eating habits and lifestyle have been dramatically changing. Increased consumption of energy together with a sedentary lifestyle has led to a positive energy balance. These changes contribute to an increased incidence of obesity and associated metabolic diseases. Until recently, these chronic metabolic diseases were only associated with aging. However, this paradigm is being shifted and a growing number of children, adolescents and young adults in reproductive age are affected by these pathological conditions [1].

Leptin, is a peptide hormone mainly produced in adipose tissue stores [2]. It was initially called "satiety hormone", since it was thought to be solely produced by adipocytes of white adipose tissue, to control energy homeostasis and decrease food intake [3]. More recently, leptin has been reported to be also produced in other tissues [4]. The plasma concentration of leptin tends to be increased in most obese individuals and positively correlated with total body fat [5,6], with the exception of the rare individuals with congenital leptin deficiency [7]. In fact, leptin possesses anti-obesity functions, based on its ability to suppress appetite and decrease body weight and adiposity [2]. Notably, leptin is now a FDA approved [8] therapeutic for several medical conditions.

Abbreviations: 4-HNE, 4-hydroxynonenal; BTB, blood–testis barrier; cDNA, complementary deoxyribonucleic acid; DNP, Dinitrophenyl; FBS, fetal bovine serum; GLUT1, glucose transporter 1; GLUT2, glucose transporter 2; GLUT3, glucose transporter 3; hL, human liver; hSC, human Sertoli cell; ITS, insulin–transferrin–sodium selenite; LDH, lactate dehydrogenase; MCT4, monocarboxylate transporter 4; NT, nitro-tyrosine; Ob-R, leptin (or obesity) receptor; OS, oxidative stress; PFK, phosphofructokinase; ROS, reactive oxygen species; SC, Sertoli cell; SRB, sulforodamine B.

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http://dx.doi.org/10.1016/j.bbadis.2015.06.005
0925-4439/

The biological actions of leptin are carried out through interaction with the specific membrane-spanning leptin (or obesity) receptor (Ob-R) [9]. This hormone also mediates metabolic signals to the reproductive system, denoting when sufficient fat stores are available to meet the caloric demands of the reproductive events [10]. It has been suggested that the hypothalamus may be the primary target for most of leptin's actions on the reproductive axis [11]. However, based on the characterization of leptin receptor distribution and the effects of leptin on in vitro systems, direct action sites for this hormone have been suggested both in female [12] and male reproductive tissues [13–15].

Several studies using a leptin deficient rodent model emphasized the role of leptin in male reproductive function. *Ob*/*ob* mice present an autosomal-recessive mutation on chromosome 6, promoting a profound decrease in circulating leptin levels. Among other characteristics, these mice are obese and infertile [10,16]. Of note, a low-dosage leptin treatment restored fertility in *ob*/*ob* male mice [17]. In fact, those leptin-treated mice presented increased testicular and seminal vesicle weight [10,16,17] and elevated sperm counts [10]. In humans, congenital leptin deficiency is associated with hypogonadotropic hypogonadism, which may be reverted upon recombinant leptin treatment [18]. Yet, although it is known that leptin crosses the Sertoli–Sertoli cell barrier (BTB) [19] and is present in the seminal plasma [14], the molecular mechanisms by which it regulates male reproductive function remain unknown. The Sertoli cell (SC), which is the somatic component of BTB, plays an essential role in spermatogenesis. These cells are responsible for the physical and nutritional support of the developing germ cells [20]. The preferential substrate of developing germ cells is lactate, which is produced by the SC from several metabolic sources, particularly glucose [21]. The metabolic cooperation established between SCs and developing germ cells is essential for the occurrence of spermatogenesis [20,22]. The SC metabolism is sensitive to hormonal fluctuations and presents an enormous metabolic plasticity [23]. Therefore, we hypothesized that exposure to leptin can affect spermatogenesis by modulating SC metabolism. To test our hypothesis, we firstly evaluated the expression of the Ob-R on human SCs (hSCs). Then, hSCs were exposed to three distinct concentrations of leptin (the physiological concentration found in lean, healthy patients and in seminal plasma; a concentration usually detected in obese patients and a concentration found in morbidly obese individuals). The effects on metabolite production/consumption and protein levels and/or activity of key glucose and monocarboxylate transporters and metabolic enzymes were determined. Finally, since leptin has been suggested to alter oxidative equilibrium in cells, oxidative damage in exposed hSCs was evaluated by assessing lipid peroxidation, protein carbonilation and nitration.

2. Material and methods

2.1. Chemicals

NZY M-MuLV Reverse Transcriptase, random hexamer primers, dNTPs, NZTaq 2× Green Master Mix, agarose and DNA ladder were obtained from NZYTech (Lisboa, Portugal). Leptin was obtained from Bachem (Bubendorf, Switzerland). Primers were obtained from STABVIDA (Oeiras, Portugal). All other chemicals were purchased from Sigma-Aldrich (St. Louis, USA), unless stated otherwise.

2.2. Sertoli cells primary culture

Clonetics™ human SCs (MM-HSE-2305) were purchased from Lonza (Walkersville, USA). The hSCs were thawed following the manufacturer protocol optimized by our group. In brief, the vial with frozen cells was thawed at 33 °C and cells were placed in culture flask with Sertoli culture medium (1:1 mixture of DMEM-Ham's F12, pH 7.4) supplemented with 15 mM HEPES, 50 U/mL penicillin, 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone, 50 μg/mL gentamicin and 10% heat inactivated fetal bovine serum (FBS). Cells were incubated at 33 °C in an atmosphere of 6% CO_2. Sertoli cells cultured in the presence of 10% FBS in F12:DMEM remain mitotically active as described [24]. The cells used for all experiments were obtained between the third and eighth passage to ensure reproducibility. Each "n" corresponds to a cell passage and all experiments were performed in triplicate. After 96 h, cultures were examined by phase contrast microscopy and hSCs culture purity was determined as described [25].

2.3. Experimental groups

Cells were allowed to grow until reach 80–85% of confluence and serum-starved before treatment. The culture medium was then replaced by serum-free medium (DMEM: F12 1:1, pH 7.4) supplemented with insulin–transferrin–sodium selenite (ITS medium; final concentration of 10 mg/L; 5.5 mg/L; 6.7 μg/L, respectively). To evaluate the effect of leptin on the glycolytic profile of hSCs we defined a control group with ITS medium without leptin and three groups supplemented leptin (5 ng/mL, 25 ng/mL and 50 ng/mL). The concentration of 5 ng/mL was chosen agreeing with the physiological levels found in lean, healthy patients [26] and the concentration found in seminal plasma [14]. The concentration of 25 ng/mL was chosen based on the levels reported in the literature for obese patients [26]. We also found relevant to evaluate the effects of a concentration reported in morbidly obese men (50 ng/mL) [27]. After 24 h of treatment, culture medium was collected. Cells were detached, counted with a Neubauer chamber and collected. Viability was evaluated by the Trypan Blue Exclusion test.

2.4. Cytotoxicity assay

The cytotoxicity of hSCs to leptin was determined by the colorimetric sulforodamine B (SRB) assay [28]. In brief, cells were seeded and treated with selected concentrations of leptin. After treatment, cells were washed twice in phosphate buffered saline solution and fixed overnight in 1% acetic acid in methanol. Cells were then incubated with 0.5% (w/v) SRB in 1% of acetic acid for 1 h at 37 °C. The unbound dye was removed by washing with 1% acetic acid solution. Dye bound to cell proteins was extracted with 10 mM Tris solution (pH 10) and the optical densities of the resulting media were determined at 540 nm. No cytotoxicity was observed for the doses of leptin used in this work (data not shown).

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

The extraction of total RNA (tRNA) from hSCs was performed using the E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, USA) as indicated by the manufacturer. tRNA concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (Nanophotometer™, Implen, München, Germany). Human liver (hL) tRNA was purchased from AMS Biotechnology (Abingdon, UK). tRNA from hSCs and hL was reversely transcribed as described [29]. The resulting complementary deoxyribonucleic acid (cDNA) was used with exon-exon spanning primers set designed to amplify Ob-R (Forward primer: TCTGGACTGCTCACGGTC AT; Reverse primer: ACCCAGCATTTTCACGGTTTG), Sox-9 (Forward primer: AGGAAGTCGGTGAAGAACGG; Reverse primer: AAGTCGATAGGGGG CTGTCT) and GATA-4 (Forward primer: CTAGCAGCTTCTGCGCCTGT; Reverse primer: GTGGTTCCGGAAGCTGATGTA). PCR were carried out as described [30]. Primers' optimal annealing temperature was set to 62 °C to Ob-R, 56 °C to Sox-9 and 58 °C to GATA-4. 35 cycles were required for the exponential amplification phase of fragments (180 bp to Ob-R and GATA-4 and 275 bp to Sox-9). hL was used as positive control for Ob-R experiments and cDNA-free sample was used as negative control. Samples were run in 1.5% agarose gel electrophoresis (120 mV, 40 min) and visualized using software Molecular Imager FX Pro Plus MultiImager (BioRad, Hercules, USA) coupled to an image acquisition system (Vilber Lourmat, Marne-la-Vallée, France). The size of the expected products was compared to a DNA ladder.

Please cite this article as: A.D. Martins, et al., Leptin modulates human Sertoli cells acetate production and glycolytic profile: A novel mechanism of obesity-induced male infertil..., Biochim. Biophys. Acta (2015), http://dx.doi.org/10.1016/j.bbadis.2015.06.005