Molecular and Cellular Endocrinology 434 (2016) 199–209

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

# Ghrelin acts as energy status sensor of male reproduction by modulating Sertoli cells glycolytic metabolism and mitochondrial bioenergetics

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A R T I C L E   I N F O

*Article history:*
Received 11 February 2016
Received in revised form
4 July 2016
Accepted 4 July 2016
Available online 5 July 2016

*Keywords:*
Ghrelin
Sertoli cells
Growth hormone secretagogue receptor
Spermatogenesis
Obesity

A B S T R A C T

Ghrelin is a growth hormone-releasing peptide that has been suggested to interfere with spermatogenesis, though the underling mechanisms remain unknown. We studied the effect of ghrelin in human Sertoli cells (hSCs) metabolic phenotype. For that, hSCs were exposed to increasing concentrations of ghrelin (20, 100 and 500 pM) mimicking the levels reported in obese, normal weight, and severely undernourished individuals. The metabolite production/consumption was determined. The protein levels of key glycolysis-related transporters and enzymes were assessed. The lactate dehydrogenase (LDH) activity was measured. Mitochondrial complexes protein levels and mitochondria membrane potential were also measured. We showed that hSCs express the growth hormone secretagogue receptor. At the concentration present in the plasma of normal weight men, ghrelin caused a decrease of glucose consumption and mitochondrial membrane potential in hSCs, though LDH activity and lactate production remained unchanged, illustrating an alteration of glycolytic flux efficiency. Exposure of hSCs to levels of ghrelin found in the plasma of severely undernourished individuals decreased pyruvate consumption and mitochondrial complex III protein expression. All concentrations of ghrelin decreased alanine and acetate production by hSCs. Notably, the effects of ghrelin levels found in severely undernourished individuals were more pronounced in hSCs metabolic phenotype highlighting the importance of a proper eating behavior to maintain male reproductive potential. In conclusion, ghrelin acts as an energy status sensor for hSCs in a dose-dependent manner, showing an inverse association with the production of lactate, thus controlling the nutritional support of spermatogenesis.



## 1. Introduction

Regulation of whole body energy homeostasis is essential for a proper functioning of male reproductive function. This event involves a fine coordination between central regulators and peripheral nutrient-sensing molecules. In fact, an inverse U-shaped association has been established between body mass index (BMI) and male fertility, in which both obese and underweight men are less likely to have children than normal weight individuals (Jokela et al., 2008). It has also become evident, by the data available in the literature, that starvation and malnutrition can impair fertility.

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http://dx.doi.org/10.1016/j.mce.2016.07.008
0303-7207/

**Abbreviations**

| | |
|---|---|
| BMI | body mass index |
| cDNA | complementary DNA |
| FBS | fetal bovine serum |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GHS-R | growth hormone secretagogue receptor |
| GLUT | glucose transporter |
| HBSS | Hanks Balanced Salt Solution |
| HADHB | mitochondrial trifunctional protein subunit beta |
| hSCs | human Sertoli cells |
| ITS | insulin-transferrin-sodium selenite |
| JC-1 | 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide |
| LDH | lactate dehydrogenase |
| MCT4 | monocarboxylate transporter 4 |
| NMR | nuclear magnetic resonance |
| PDH | pyruvate dehydrogenase |
| PFK-1 | phosphofructokinase-1 |
| PKM1/2 | pyruvate kinase M1/2 isoform |
| qPCR, | quantitative polymerase chain reaction |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| SCs | Sertoli cells |
| SRB | Sulforhodamine B |
| TBS | Tris-buffered saline solution |
| TESE | testicular sperm extraction |
| tRNA | total RNA |
| $UCP2^{-/-}$ | uncoupling protein 2 knockout mice. |

Diets poor in nutrients and rich in fats and sugars are responsible for the increased incidence of obesity and associated metabolic diseases (e.g. diabetes mellitus or metabolic syndrome). As individuals consume less healthier diets, a concomitant and rapid increase in the number of children, adolescents and young adults diagnosed with metabolic diseases is observed, raising the concerns over the fertility status and reproductive health of male population (for review (Rato et al., 2014)).

The leptin-ghrelin axis mediates the hormonal control of food intake and energy homeostasis. In particular, ghrelin controls energy metabolism in such a way that it mirrors the energy status of the individual (Broglio et al., 2001, 2004). In fact, negative energy balance (body stores) translates into increased circulating levels of ghrelin, while a surplus energy balance (obesity) is reflected in a decrease in ghrelin levels (Rosicka et al., 2003; Shiiya et al., 2002). In what concerns the male reproductive system, it has been reported that gut hormones may cooperate with other regulatory signals in the integrated control of energy homeostasis and reproduction (Alves et al., 2016; Barreiro and Tena-Sempere, 2004). Indeed, ghrelin inhibits the proliferative activity of immature Leydig cells *in vivo* (Barreiro et al., 2004) and, more recently, it was proposed that it acts as a modulator of spermatogenesis avoiding excess of build-up of germ cells (Kheradmand et al., 2012). Ghrelin outcomes in tubular stem cell factor expression was also reported (Barreiro et al., 2004), which has been described as crucial for spermatogonia survival (Hakovirta et al., 1999). As for the effect of this hormonal axis on Sertoli cells (SCs), while it was recently shown that leptin directly modulates glucose metabolism in human SCs (hSCs) (Martins et al., 2015), the effects of ghrelin remain unknown. So far, only the presence of its receptor, the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999) has been reported in rat and human SCs, by means of the immunohistochemistry technique (Barreiro and Tena-Sempere, 2004; Gaytan et al., 2004).

The efficiency of spermatogenesis is intrinsically associated with SCs population and function (Petersen and Soder, 2006), and particularly with its metabolism (Rato et al., 2012b). Notably, there is a relationship between the number of germ cells supported by each SC and sperm production (Weber et al., 1983). The SCs are known as “nurse cells” since they are responsible for the physical and nutritional support of developing germ cells (Rato et al., 2012b) and for maintaining the ionic homeostasis of the tubular fluid (Oliveira et al., 2009a; Rato et al., 2010). In these cells, membrane glucose transporters (GLUTs) are responsible for the uptake of glucose (Alves et al., 2013), which is primarily metabolized to pyruvate and then converted to lactate by lactate dehydrogenase (LDH) (Alves et al., 2013). This metabolite is then exported by monocarboxylate transporter 4 (MCT4) (Rato et al., 2012a) to be used by germ cells for the production of energy, regulating their metabolic activity and survival (Jutte et al., 1982). Besides lactate, SCs also produce other metabolic cofactors and substrates, such as acetate, which is suggested to be crucial for the high rate of lipid synthesis by germ cells (Alves et al., 2012). A normal spermatogenesis is highly dependent on the metabolic cooperation established between SCs and germ cells, a process known to be sensitive to hormonal regulation (Alves et al., 2013; Oliveira et al., 2012).

Although there is strong evidence that ghrelin has a central role in male reproduction, the molecular mechanisms by which this hormone controls spermatogenesis remain unknown. We hypothesize that ghrelin can affect spermatogenesis through a direct action on SC function, modulating its bioenergetic status, in a manner that may be linked to the nutritional status of the individual. To test our hypothesis, we evaluated the glycolytic and bioenergetic profile of hSCs in the presence of levels of ghrelin found in obese, normal weight, and severely undernourished individuals in order to mimic *in vitro* the situations found in the clinical setting.

## 2. Materials and methods

### 2.1. Chemicals

HEPES was purchased from Acros Organics (Geel, Belgium). Gentamycin was purchased from Lonza (Basel, Switzerland). Heat inactivated fetal bovine serum (FBS) and Trypsin–EDTA solution were purchased from Biochrom AG (Berlin, Germany). Ghrelin was purchased from Bachem (Weil am Rhein, Germany). Primers were purchased from STABVIDA (Oeiras, Portugal). Agarose and DNA ladder were purchased from NZYTech (Lisbon, Portugal). Sulforhodamine B (SRB) was purchased from Biotium (Hayward, CA, USA). ECF detection system was purchased from GE, Healthcare (Weßling, Germany). Human liver total RNA (tRNA) was purchased from AMS Biotechnology (Abingdon, UK). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

### 2.2. Patient's selection and testicular tissue preparations

The patient's clinical study and testicular tissue handling was performed at the Centre for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) in accordance with the Guidelines of Local (P.N. 12/12CES), National and European Ethics Committees. The studies were also performed according to the Declaration of