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Prepubertal onset of obesity negatively impacts on testicular steroidogenesis in rats

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ABSTRACT

Obesity is a global health problem and impacts negatively on levels of testosterone and quality of sperm production. At present little is known about mechanisms that attenuate testicular function in obese males. Our study characterized testicular steroidogenesis and explored levels of relevant paracrine and hormonal factors in rats with short- and long-term obesity.

We have found that obesity state increased serum levels of estradiol and leptin in both groups of obese rats and inhibited the expression of *StAR* and *Cyp11a1* associated with low levels of intratesticular testosterone in rats with long-term obesity. Further, long-term obesity reduced the number of Leydig cells, increased the testicular levels of the proinflammatory adipocytokine TNFα and the number of testicular macrophages.

All together, our data indicate that long-term obesity may cause chronic inflammation in the testis and negatively impacts on Leydig cell steroidogenesis.

1. Introduction

Obesity rates have nearly doubled since 1980 and over 10% of the worldwide population is obese (Finucane et al., 2011). Obesity is associated with a state of chronic inflammation and multiple and severe metabolic complications, mainly defined as the metabolic syndrome. Those are increasingly recognized also in children, predisposing them to early cardiovascular disease (Morrison et al., 2008a). During the last 50 years there has been a steady decline in fertility rates, which has occurred in parallel with the increasing incidence of obesity (Hammoud et al., 2006; Morrison et al., 2008b, Hammoud et al., 2012). Obese males have reduced levels of circulating testosterone associated with altered structure of the germ cells, which are clear signs of attenuated fertility (Du Plessis et al., 2010). Obesity may also affect male fertility by disturbing the functioning of the hypothalamic-pituitary-gonadal axis, thereby negatively influencing testicular steroidogenesis and spermatogenesis (Davidson et al., 2015). Several mechanisms might account for the effect of obesity on male infertility. One such mechanism might be the increased expression of aromatase (CYP19) in adipose tissue of obese individuals that can convert serum testosterone to estradiol, thereby interfering with androgen-dependent processes in obese persons (Hajshafiha et al., 2013) and thus inducing a negative feedback on LH secretion. In addition, obesity-associated inflammation may indirectly contribute to the development of hypogonadism through an elevated production of inflammatory cytokines such as TNFα and IL-1β by adipocytes, which can directly inhibit Leydig cell capacity to produce testosterone (Hong et al., 2004), which via the AR-dependent signaling pathway(s) supports normal function of Sertoli cells (Walker, 2010). Accordingly, endocrine changes associated with male obesity may develop a state of hypogonadotropic hyperestrogenic hypoandrogenaemia, which may adversely affect male reproductive function by affecting spermatogenesis and reducing sexual drive (Neves et al., 2012).

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Therefore, in the present study we explored hormonal status and steroidogenesis of rats in a model of short- and long-term obesity. We present novel mechanisms for the negative effect of diet-induced obesity on testicular steroidogenesis based on increased levels of TNFα by testicular macrophages with an associated suppression of the expression of steroidogenesis related genes and reduced number of Leydig cells in long-term obese rats.

2. Materials and methods

2.1. Anthropometric data

Animal studies were approved by the local authorities of the state of Saxony, Germany as recommended by the responsible local animal ethics review board (Landesdirektion Leipzig, T01/13, TVV60/13, TVV59/15, Germany). All LEW.1W (Lewis, F85) rats were housed in pathogen-free facilities in groups of three in Macrolon type III (rats) (Ehret GmbH, Emmendingen, Germany) at 22 ± 2 °C on a 12-h light/dark cycle. Animals were bred and kept in the Animal Laboratories at the University of Leipzig, and were fed a standard chow diet (Altromin GmbH, Lage, Germany) or high fat diet (60% energy fat; Sniff, Soest, Germany) and had ad libitum access to water and food at all times.

All animals were fully breastfeed until day 21. From day 21 six male rats were fed with standard chow (SC) and six ones with high fat diet (HFD). Before sacrificing animals underwent an EchoMRI700 (Echo Medical Systems, Houston, Texas) analysis to assay the amount of body fat, body fat content and the lean mass. Animals were sacrificed 3 (short-term) and 9 (long-term) months after the start of their diets (group 1 and 2, respectively). Blood was collected from the heart and centrifuged at 1000 g for 10 min to obtain serum. Serum samples and the testes were kept at −80 °C until they were used for hormonal measurements and steroidogenic genes analyses.

2.2. Morphological analysis of adipocytes

Adipose tissue from inguinal and epididymal depots was used to isolate preadipocytes and adipocytes with subsequent analysis of the adipocyte diameter as described previously (Kloting et al., 2010, Landgraf et al., 2015). The effective range of cell sizes analysed was 50–250 μm. For each sample, the average and the peak diameter of adipocytes (diameter at which frequency of adipocytes reaches maximum) was retrieved from the Multisizer graph as described previously (McLaughlin et al., 2007).

2.3. Immunohistochemical staining for testicular macrophages and TNFα

The samples were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, dehydrated with gradually increasing concentrations of aqueous ethanol, embedded in paraffin (P3808, Sigma Aldrich) and cut into 4–5 μm-thick sections and placed on glass slides (J1800AMNZ, Gerhard Menzel, Germany) for morphological analysis and staining for macrophages, TNFα and Leydig cells.

Samples for immunofluorescence (IF) staining were first subjected to antigen retrieval using citrate buffer (pH 6.0) in a water bath at 95 °C for 30 min and cooled down for another 30 min. Afterwards slides were incubated for 30 min in 10% donkey serum (017-000-001, Jackson Immunoresearch, USA) diluted in 1% BSA-1 × Tris-buffered saline (TBS). To explore number of testicular macrophages, we used a marker CD68, which is commonly used to identify macrophages (Goluza et al., 2014). After incubation with a primary anti-mouse CD68 antibody (1:100) (Santa Cruz, USA) or anti-rabbit TNFα (1:50) (Novus Biology, USA) as well as unspecific IgGs (used as negative controls at the same concentration) dissolved in 1% BSA-TBS overnight at 4 °C, the slides were washed three times for 5 min each with TBS. The specificity of TNFα antibody was tested by pre-absorption with the antigen as described earlier (Schafers et al., 2003). Anti-rabbit TNFα antibody was incubated with 10-fold excess of recombinant rat TNFα (Sigma Aldrich, USA) at 4 °C overnight.

Slides were then incubated either with a secondary antibody conjugated to Cy3 or with a secondary antibody conjugated to HRP depending on the type of staining. The secondary antibodies were diluted in blocking serum and the incubation was done for 30 min at RT. After washing, the samples were incubated with TSA-Plus Fluorescein (NEL741001KT, Perkin Elmer, USA) or TSA-Plus Cy3 (NEL744001KT, Perkin Elmer) according to the manufacturer's protocol. After washing, the samples were mounted in Vectashield mounting medium with Dapi (H-1500, Vectro, USA). All stained sections were photographed using a microscope (Eclipse E800; Nikon; Japan) with a 12.5 million-pixel, cooled digital colour camera (Olympus DP70, Japan). Calculation of CD68-positive testicular macrophages was carried out blinded by counting 15 fields per slide in 5 animals per each group and the average was calculated.

2.4. Leydig cell count and morphometric measurements

Testicular sections were immunostained for CYP11 (1:100) (Santa Cruz Biotechnology, Dallas, TX, USA) and counterstained with hematoxylin. Two stereological methods (point counting and nucleator method) were used to count Leydig cell number per testis from control and HFD rats (Sharpe et al., 2000, Tan et al., 2005), both utilizing 63× objective fitted to Zeiss AxioScope A1 Microscope equipped by digital camera AxioCam ERC 5s-Zeiss and Software program Image Pro Plus. The volume of CYP11-positive cells per testis was determined by point counting method using a graticule with 121 points fitted in eyepiece. In brief, testicular cross sections from each of four animals per group were examined. Applying a systematic sampling pattern from a random starting point, 50 fields (6050 points) were counted. Points falling over CYP11 positive nuclei were scored separately and expressed as relative (%) volume per testis. These data were converted to absolute nuclear volume (ANV) per testis by multiplying by testis weight (= volume), because shrinkage was minimal. Separately, an area of interest was created by drawing around the Leydig cell nucleus, within which the computer program then determined the area and the mean diameter of Leydig cell nuclei. The measurement was done for a minimum of 100 Leydig cell nuclei per testis and mean nuclear volume (MNV) then was obtained using the equation.

$$MNV = \frac{4\pi}{3} \times r^3$$

where $r = d/2$, (Wreford, 1995)

The latter was used to convert data for Leydig cell absolute nuclear volume (ANV) per testis to Leydig cell numbers per testis. Cell nuclear volume can be equated to numbers of cells per testis, assuming no change in nuclear diameter of the target cell in the different experimental groups (Tan et al., 2005).

2.5. Hormone levels assay

Serum levels of rat LH were measured by specific ELISA (Cloud-Clone Inc., USA). Serum levels of testosterone and 17β-estradiol were quantified employing relevant ELISA's from DRG Diagnostics (DRG Diagnostics, N.J., USA). Serum levels of leptin was analysed by ELISA from Crystal Chem (Crystal Chem. Inc., USA). Intraassay and interassay coefficients for testosterone were 6.4 and 4%,