Biochemical and Biophysical Research Communications xxx (2017) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Regulation of testicular steroidogenesis by Foxa3 via transcriptional modulation of ERα signaling in type 2 diabetes mellitus (T2DM)

Yong Zhao [a, 1], Hong-xin Li [b, 1], Ke Wang [a], Bin-yuan Yan [c], Wei Li [d, *]

[a] Reproductive Medical Center, Navy General Hospital, Beijing 100048, China
[b] Department of Dermatology, Capital Institute of Pediatrics Affiliated Children's Hospital, Beijing 100022, China
[c] Department of Epidemiology, School of Public Health, Fourth Military Medical University, Xi'an 710032 China
[d] Department of Histology and Embryology, Fourth Military Medical University, Xi'an 710032 China

ARTICLE INFO

Article history:
Received 15 June 2017
Accepted 19 June 2017
Available online xxx

Keywords:
Diabetes
Foxa3
Testosterone
Leydig cells
ERα

ABSTRACT

Although both insulin and estrogen receptor α (ERα) are known to exert inhibitory effects on testicular steroidogenesis, it remains unknown whether these pathways regulate testosterone (T) production under certain pathological conditions [*e.g.,* type 2 diabetes mellitus (T2DM)] in a coordinated manner. Here, we found that the expression of forkhead box protein A3 (Foxa3), an essential transcriptional regulator engaged in adipogenesis and energy metabolism, was significantly down-regulated in the Leydig cells (LCs) from T-deficient T2DM mice. Functionally, upon hCG stimulation, Foxa3 recruits to the *Esr1* promoter and suppresses the transactivation of *Esr1* gene. Disruption of this recruitment by T2DM-elicited hyperinsulinemia led to abnormal activation of ERα pathway, inhibited steroidogenic enzyme genes expression, and thus caused inadequate T production. Therapeutically, insulin-impaired and Foxa3 ablation-compromised steroidogenesis were effectively rescued by a pharmacological inhibitor of the ERα pathway. These findings reveal an obligatory coregulatory role of Foxa3 in the regulation of ERα expression and of the Foxa3/ERα cascade, at least in part, in the pathogenesis of androgen deficiency caused by T2DM.

1. Introduction

The prevalence of type 2 diabetes mellitus (T2DM) increases with age but with a worrying trend of increasingly young age of diagnosis. In this regard, disruption of male reproductive function by T2DM has recently received much attention [1]. Low T levels are frequently associated with male T2DM patients. Up to one-third of men with T2DM have significantly subnormal levels of both free and total testosterone (T) [2]. In male mice with nicotinamide/streptozotocin-induced T2DM, serum luteinizing hormone (LH), T levels, and sperm count and viability were all substantially decreased [3]. On the other hand, low T levels have also been identified as reliable predictors of insulin resistance and the likelihood of developing T2DM in the future [4]. T supplementation successfully enhanced insulin sensitivity and improved the metabolic symptoms in T2DM mice [5]. Therefore, the complicated interaction between androgen and insulin pathways appears to be reciprocal. Multiple etiologies, including interference with hypothalamic-pituitary-testicular axis [6], deregulation of critical transcriptional regulators and repression of steroidogenic genes expression [7] may all account for the T2DM-impaired T production at different levels. Despite all the advances, however, most mechanisms still remain obscure.

The forkhead box (FOX) proteins are members of a family of transcriptional regulators that are derived from adipocyte and bind DNA through a conserved winged-helix binding motif. Recent advance in this filed has shown that Foxa3 plays an essential role in the regulation of energy expenditure and age-associated metabolic disorders through transcriptional modulation of brown fat gene programs [8]. While all three *Foxa* mRNAs are present in liver and pancreas, only Foxa3 is expressed in the testis, with the predominant localization in postmeiotic germ and interstitial Leydig cells (LCs). *Foxa3*$^{-/-}$ mice suffers loss of germ cells and has reduced fertility. The authors therefore conclude that Foxa3 may function as a pivotal transcriptional regulator with a dominant phenotype in

* Corresponding author. Department of Histology and Embryology, Fourth Military Medical University, No. 169 Changle West Road, Xi'an, 710032 Shaanxi Province, China.
E-mail address: liweipepeyato@163.com (W. Li).
[1] These authors contribute equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2017.06.118
0006-291X/

germ cell maintenance [9]. However, the physiological relevance of Foxa3 signaling in steroidogenic LCs remains poorly understood.

Emerging data evidence a close association between adiposity and impairment of reproductive function. Thus, subnormal plasma T concentrations and reduced sex hormone binding globulin (SHBG) levels are detected in obese men. The precise etiology of these alterations is unclear, although they are reversed after weight loss, thus suggesting a link to adipocyte cell dysfunction [10]. On the above basis, we aim to assess the potential role of Foxa3 in LCs function. To this end, quantitative approaches were undertaken to evaluate the expression of Foxa3 in the LCs isolated from murine diabetic testis. As our initial evidence demonstrated a tight association between Foxa3 deregulation and T2DM-elicited T deficiency, different experimental settings were used to define the functional role and corresponding molecular mechanisms of this molecule in the direct control of LCs testosterone secretion.

2. Materials and methods

2.1. Animal model

Male 10-week-old BALB/c mice, purchased from the Animal Research Center of university, were fed *ad libitum*. We used two methods, one single intraperitional injection of streptozotocin (STZ, Sigma-Aldrich, Beijing, China) or high-fat feeding (HFD) using Rodent Diet with 60 kcal% fat (Research Diets Inc., Shanghai, China) along with STZ administration, to induce T1DM and T2DM, respectively [11]. Mice with blood glucose level ≧250 mg/dl, as detected using a blood glucose monitor (Bioland Technology Ltd., Hong Kong, China) two weeks after STZ injection, were considered as T1DM. The HFD/STZ mice with distinct hyperglycemia concomitant with insulin resistance [as determined using an enzymatic assay kits (Crystal Chem, Downers Grove, IL, USA), beyond 1–2 S.E.M. values from the normal mice mean] were considered as T2DM. The local ethical committee approved all the animal procedures.

2.2. Cells treatment

LCs were isolated from control and diabetic mice, and their purity was determined using immunohistochemistry and quantitative RT-PCR (qRT-PCR) as described in detail in our previous work [12].

MA-10 LCs were obtained from ATCC (Rockville, MD, USA) and was recently authenticated in April 2014 by the short tandem repeat analysis method using Promega Power-Plex1.2 analysis system (Genewiz, Inc). Cells used for the current study had less than 20 passages. MA-10 was cultured in DMEM/F-12 medium, supplemented with 10% FBS, 2.2 mg/mL sodium bicarbonate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin and incubated at 37 °C with 5% CO_2. To study the effect of insulin on *Foxa3* expression, primary LCs and MA-10 were treated different concentrations of insulin (Sigma-Aldrich) for 2 h or treated with 40 nM insulin for different durations, followed by qRT-PCR analysis as described below. Specific knockdown of *Foxa3* was achieved by stably transfecting MA-10 with shRNA against *Foxa3* or with a control shRNA (Sigma-Aldrich). 48 h after transfection, cells were subjected to other experiments. MA-10/hFOXA3 cells were generated by transfection of pCMV3-FOXA3 (Sino Biological Inc., Beijing, China) using Lipofectamine 2000 (Invitrogen), followed by selection with 50 μg/mL kanamycin (Sino Biological). To block the ERα pathway, MA-10 was pretreated with 50 nmol/L AZD9496 (MedChem Express, Shanghai, China) for 12 h before being subjected to other assay.

2.3. Hormone assays

Animal blood, collected from the orbital sinus after animals had been anesthetized, was obtained between 9 and 10 a.m. Pooled serum samples from each group were measured for testosterone (T) concentration by RIA. For *in vitro* assay, MA-10 cells were seeded at the density of 1.2×10^5 cells/well in 24-well plates. When they reached 5×10^5 cells/well, cells were washed with 1-mL warm serum-free medium supplemented with 1-mg/mL BSA and then stimulated with 1 ng/mL hCG (Sino Biological) for 5 h, followed by the measurement of progesterone content in the culture media using RIA. Samples were assayed in duplicate, and each data point consisted of 3–6 samples. Intra- and inter-assay coefficients of variation were approximately 8.6% and 7.8%, respectively, for testosterone and 8.2% and 9.5% for progesterone [13].

2.4. RT-qPCR

RT-qPCR was carried out according to our previous work [14]. The primers used were listed in Supplementary Table 1. PCR products were then quantified by SYBR green intercalation using the MiniOpticon™ system (Bio-Rad, Hercules, CA, USA). The relative abundance of target transcript was quantified using the comparative Ct method, with *Gapdh* as an internal control.

2.5. Immunoblotting

Immunoblotting was carried out as described in our previous work [15]. Membranes were incubated with different primary antibodies as indicated in Supplementary Table 2 in blocking solution overnight at 4 °C. Final signals were detected using an ECL kit (Amersham Biosciences, Shanghai, China).

2.6. Luciferase reporter assays

pLightSwitch-*ESR1* and pLightSwitch-Blank vector were obtained from SwitchGear Genomics (Shanghai, China). 3T3 cells were transfected with 0.5 μg reporter plasmid in the presence or absence of pCMV3 vector or pCMV3-FOXA3 by using FuGENE®6 (Promega, Madison, WI, USA) according to the manufacturer's instructions. At 2 days after transfection, the cells were subjected to insulin incubation (40 nM) for 1 h, followed by luciferase activity measurements using LightSwitch Assay Reagents.

2.7. Statistical analysis

Results are presented as mean ± S.E.M. from at least 3 independent experiments. Data normality was determined using normal probability plots and compared using *Student's t*-test or ANOVA for normally distributed data or 2-sample rank testing for data not distributed normally. Correlation between relative *Esr1* and *Foxa3* mRNA levels in primary LCs was determined based on Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Foxa3 down-regulation in LCs is correlated to T deficiency in T2DM

To study the effects of diabetes on Foxa3 expression, we firstly used STZ or HFD/STZ to induce two types of diabetes (Supplementary Fig. 1). Among 40 mice with T2DM, about 17 mice exhibited a significant reduction in plasma testosterone concentration 3 months after T2DM induction, whereas control group and

Please cite this article in press as: Y. Zhao, et al., Regulation of testicular steroidogenesis by Foxa3 via transcriptional modulation of ERα signaling in type 2 diabetes mellitus (T2DM), Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.06.118