Biochemical and Biophysical Research Communications xxx (2017) 1–9

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Decreased expression of orexin 1 receptor in adult mice testes during alloxan-induced diabetes mellitus perturbs testicular steroidogenesis and glucose homeostasis

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ARTICLE INFO

Article history:
Received 26 June 2017
Accepted 5 July 2017
Available online xxx

Keywords:
Diabetes
OX1R
Testis
Steroidogenesis
Glucose homeostasis

ABSTRACT

Diabetes mellitus (DM) affects male reproductive system and causes infertility. The male reproductive health is largely dependent upon uptake and proper utilization of glucose by testicular cells. Results show involvement of orexin A (OXA) and its receptor (OX1R) in regulation of steroidogenesis and glucose homeostasis in adult mice testis. However, the role of OX1R in regulation of testicular functions during hyperglycemia has not been investigated so far. The present study, therefore, examined the role of OX1R in regulation of steroidogenesis and glucose homeostasis in testis of adult mice during alloxan-induced type 1 DM. A significant decrease was noted in body weight and testis weight in alloxan-treated mice compared to controls. The blood glucose level, however, was markedly increased in treated animals than in controls. Further, serum and intratesticular level of testosterone, activities of testicular steroidogenic enzymes, and expressions of various steroidogenic markers, OX1R, glucose transporter 3 (GLUT3) and Wilms' tumor gene (WT1) were downregulated in treated mice. The level of glucose, activity of lactate dehydrogenase (LDH) and lactate concentration in the testes of diabetic mice were also decreased; a significant increase in the number of testicular apoptotic cells with concomitant increase in the expression of caspase-3 was noted in these mice. Furthermore, DM affected germ cell proliferation with decreased expression of proliferating cell nuclear antigen (PCNA). Results thus suggest that type 1 DM impairs testicular steroidogenesis and glucose homeostasis through inhibition of OXA/OX1R signaling cascade due to decreased OX1R expression in adult mice, thereby affecting germ cell survival and their proliferation in the testis.

1. Introduction

Diabetes mellitus (DM) is one of the most prominent public health threats in modern societies and its prevalence is rapidly increasing worldwide. DM is a metabolic disorder and is associated with falling birth rates and fertility [1,2]. DM may be classified into type 1 (T1D) and type 2 (T2D). T1D results from an absolute deficiency of insulin due to an autoimmune destruction of pancreatic β cells, while T2D is characterized by impaired insulin secretion and increased insulin resistance [3]. Both T1D and T2D are characterized by a hyperglycemic state and they are associated with male reproductive dysfunctions, resulting into poor sperm quality and reduced fertility [3]. Studies with T1D rodent male model indicate that the fertility is affected because of increased oxidative stress, altered steroidogenesis, and abnormal spermatogenesis in the testis [4,5]. However, the mechanism(s) by which T1D affects testicular functions remains poorly understood.

Orexins (OXs) are the hypothalamic neuropeptides consisting of orexin A (OXA) and orexin B (OXB) and they modulate their actions via two G-protein-coupled receptors, orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R), respectively [6,7]. OX1R is highly selective for OXA, while OX2R allows binding of both orexins with similar affinity [8]. Orexins and their receptors are found throughout the central nervous system [9,10]. They are also present in peripheral tissues [11]. Reports suggest that OXA participates in regulation of steroidogenesis in rat testis [12–14]. In our earlier study, we have reported the role of OXA and OX1R in testicular glucose metabolism with reference to GLUT3 and WT1 in neonatal mice [15]. Further, OXA-positive cells in lateral hypothalamus, gut and in pancreas are glucosensitive [16,17]. During hypoglycemic

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http://dx.doi.org/10.1016/j.bbrc.2017.07.026
0006-291X/

condition, OXA release is stimulated by the glucosensitive cells, while an increase in circulating level of glucose causes inhibition of OXA expression in these cells [18,19]. We have recently shown the presence of OXA and OX1R in mouse testis during different stages of postnatal development, with their maximal expression at adult stage [20]. Our unpublished results also demonstrate the involvement of OXA and OX1R in regulation of testicular steroidogenesis, glucose homeostasis and spermatogenesis in adult mouse testis. However, involvement of OX1R in regulation of testicular functions during hyperglycemic condition remains unexplored. Therefore, in the present study, the role of OX1R in regulation of steroidogenesis and glucose homeostasis in adult mouse testis during alloxan-induced T1D was investigated.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used in the present study were of analytical grade and purchased from HiMedia Laboratories and Merck India Ltd., Mumbai, unless otherwise mentioned.

2.2. Animal care and maintenance

Both maintenance of mice and experiments were carried out in accordance with the guidelines of Banaras Hindu University Animal Ethics Committee (No. Dean/13–14/CAEC/340; dated, 27-07-2013) as per approval of committee for the purpose for control and supervision of experimental animals (CPCSEA), Government of India (No. 1802/G0/Re/S/15/CPCSEA). Adult (age 12–14 weeks) male Parkes (P) strain mice, weighing 32–36 g, were used in the present investigation. The details of animal care and maintenance are described elsewhere [20].

2.3. Induction of T1D in adult mice using alloxan monohydrate

Healthy adult male mice were weighed and randomly allocated to two (I–II) groups, each comprising fifteen individuals ($n = 15$). Diabetes was induced by a single intraperitoneal injection of freshly prepared alloxan monohydrate (Sigma–Aldrich, St. Louis, MO, USA) (175 mg/kg BW) dissolved in 0.9% NaCl. After overnight fasting, animals of group I (control) were intraperitoneally (i.p.) injected with 0.9% NaCl, while animals in group II (diabetic) were administered alloxan monohydrate (175 mg/kg BW) in a similar way. Diabetes was diagnosed on the third day after alloxan administration by measuring glucose level in the blood (diabetic mice had glucose level > 250 mg/dL), sampled from the tail vein. Thirty days after alloxan injection, animals were weighed and sacrificed under mild ether anaesthesia by decapitation. Blood from trunk region was collected, and serum was separated and stored at −20 °C until further use. Testes were randomly excised from either the left or the right side of five mice for immunohistochemistry and TUNEL assay; testes from the contralateral sides of the above mice and both testes from the remaining mice were excised, blotted free of blood, weighed and kept frozen at −80 °C until processed for RT-PCR, immunoblot and biochemical analyses.

2.4. Estimation of glucose

The level of glucose in the serum and in the testis was estimated with commercial kit (Span Diagnostics Ltd, Mumbai, India) according to manufacturer's instructions, with minor modifications [15,21].

2.5. Determination of LDH activity

Detailed procedure for determination of LDH activity in the testis of control and diabetic mice has been described in our earlier publication [21].

2.6. Estimation of lactate concentration in testis

A fluorometric estimation of testicular L-lactate concentration was determined using a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA) following manufacturer's instruction, with minor modifications [21].

2.7. Testosterone assay

Serum and intratesticular levels of testosterone (T) in control and diabetic mice were measured by ELISA as per manufacturer's instruction (DiaMetra, Segrate, Italy), with minor modifications [22].

2.8. Steroidogenic enzymes activities

Activities of 3β- and 17β-HSD in the testis of control and diabetic mice were measured as described previously [23].

2.9. RT-PCR analyses

Expressions of SF1, StAR, P450scc, 17β-HSD, P450arom, WT1, GLUT3 and OX1R were evaluated in the testis of control and diabetic mice at mRNA level. Procedure of total RNA isolation and RT-PCR analysis is described in our earlier study [20]. The primer sequence and PCR conditions are shown in Table 1.

2.10. Immunoblot analyses

A detailed description of protein extraction and immunoblot analysis is described elsewhere [21]. Details of the antibodies used in the present study are summarized in Table 2.

2.11. TUNEL-assay

TUNEL-assay was performed in testicular sections using an apoptosis detection kit (FITC-labeled POD, catalog no-L00299) according to manufacturer's instruction (GenScript, Piscataway, NJ, USA), with minor modifications [24]. TUNEL-positive cells stained brown and were counted according to Verma and Singh [24].

2.12. Immunohistochemistry of PCNA

Immunohistochemical analysis of PCNA was performed to assess proliferation of testicular cells in accordance with the protocol of Sarkar and Singh [21]. PCNA-positive cells in the sections were counted in 10 tubular cross sections per testis from each animal. The proliferation index was calculated using the formula: (PCNA-positive cells/total number of germ cells in a tubule) X 100.

2.13. Statistical analysis

Data (mean ± SEM) were analyzed by Student's *t*-test using SPSS 16.0 software. Differences were considered significant at $p < 0.05$.

Please cite this article in press as: D. Joshi, et al., Decreased expression of orexin 1 receptor in adult mice testes during alloxan-induced diabetes mellitus perturbs testicular steroidogenesis and glucose homeostasis, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.07.026