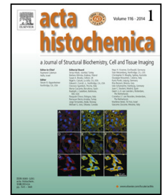
Acta Histochemica 116 (2014) 1462–1468

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis



Expression of visfatin in alloxan-induced diabetic rat testis

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ARTICLE INFO

Article history:
Received 13 August 2014
Received in revised form
28 September 2014
Accepted 29 September 2014

Keywords:
Visfatin
Diabetes
Testis
Testosterone
Rat

ABSTRACT

Diabetes mellitus is a potential epidemic all over the world and causes dysfunction of reproductive activity. Visfatin, one of the adipokines, is present in various tissues including the testis. Our hypothesis was the level of testicular visfatin is affected in diabetic condition. The aim of the present study was to investigate the expression and localization of visfatin in the diabetic rat testis. No similar studies have been performed in diabetic rat testis with reference to visfatin. Overnight fasted adult male Wistar rats were made diabetic by the administration of alloxan (150 mg/kg i.p., in 0.9% saline). Blood glucose levels were tested on five days after alloxan treatment, rats with high blood glucose levels (>250 mg/dL) were considered as diabetic. Immunolocalization and Western blotting analysis of visfatin were performed. Correlation of visfatin expression was made in relation to body weight, testis weight, glucose concentration and serum testosterone level. Expression of visfatin was observed in Leydig cells, spermatocytes and sperm in control as well as in the diabetic group. Mild immunostaining of visfatin was observed in affected seminiferous tubules of alloxan-induced diabetic rat testis. Western blot analysis showed decreased expression of testicular visfatin in diabetic rats. The expression of visfatin showed a positive correlation with serum testosterone levels, body and testis weight, while a negative correlation was observed with blood glucose levels. This study showed involvement of visfatin in diabetic associated impairment of testicular activity.

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Introduction

Diabetes mellitus (DM), a group of metabolic diseases, is a very common disease worldwide and poses a major problem in terms of various pathophysiological conditions (American Diabetes Association, 2013). Although the etiology of this disease is still not well defined, viral infection, autoimmune disorder and environmental factors have been implicated (Weeratunga et al., 2014). Changing lifestyles, reduced physical activity and increased obesity further augment the severity of DM (Shaw et al., 2010). DM is fast gaining the status as a potential epidemic in India with more than 62 million diabetic individuals currently diagnosed with the disease (Kumar et al., 2013). Alloxan and streptozotocin are the main chemical agents which induce DM in experimental animals. These pathophysiological develop owing to the destruction of pancreatic β-cells by reactive oxygen species and lead to hyperglycemic conditions. DM is the cause of several malfunctions and complications in several other organs including the liver, kidney, eye and reproductive organs. Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important role in the pathogenesis of chronic DM (Baynes and Thorpe, 1999). In experimental animals and humans, DM causes impairment of reproductive activity and leads to infertility (Calvo et al., 1984; Ficher et al., 1984; Steger et al., 1989; Ricci et al., 2009). Diabetic rats have been shown to possess low serum testosterone with decreased testicular weight, sperm count and sperm motility (Seethalakshmi et al., 1987; Cai et al., 2000; Scarano et al., 2006; Murray et al., 1983; Kuhn-Velten et al., 1984).

Adipokines are cytokines secreted by adipose tissue and are important owing to their crucial mediator roles and active participation in various metabolic functions. Adipose tissue secretes many biologically active adipokines with diverse functions (Wiecek et al., 2002). The role of adipokines such as leptin, adiponectin and resistin in reproductive physiology has been well documented (Tena-Sempere et al., 2002; Caminos et al., 2008; Nogueiras et al., 2004). Visfatin is an adipokine which is predominantly produced and secreted by visceral fat (Fukuhara et al., 2005). Visfatin (nicotinamide phosphoribosyltransferase; NAMPT) is an adipocytokine

Abbreviations: DM, *Diabetes mellitus*; MZUAEC, Mizoram University Animal Ethics Committee; NAD, nicotinamide adenine dinucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NMN, nicotinamide mononucleotide; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TMB, tetramethyl benzidine; TNF-α, tumor necrosis factor-α.

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http://dx.doi.org/10.1016/j.acthis.2014.09.011
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shown to play an important role in obesity, dyslipidemia, type II DM and immune disorders (Chen et al., 2006; Haider et al., 2006; Brentano et al., 2007; Varma et al., 2007; Adeghate, 2008). It was found to act as an intracellular enzyme that catalyzes the rate-limiting conversion of nicotinamide to nicotinamide mononucleotide (NMN), a substrate in the biosynthesis of NAD. Visfatin has been shown to be expressed in various tissues including testes (Rongvaux et al., 2002; Revollo et al., 2007; Ocon-Grove et al., 2010), though the possible contribution of testicular NAMPT has not yet been explored in the diabetic testis. The biological role of visfatin is still not entirely understood, but several studies indicated glucose lowering and insulin-mimicking or sensitizing effects of Visfatin. There are conflicting data on visfatin circulating levels in obese humans with studies showing that the synthesis and secretion of visfatin is modulated by glucocorticoids, tumor necrosis factor-α (TNF α), Interluekin-6 and growth factors (AL-Suhaimi and Shehzad, 2013).

Recent studies have elucidated the role of testicular visfatin in spermatogenesis and steroidogenesis (Hameed et al., 2012; Ocon-Grove et al., 2010). However to the best of our knowledge, there are no reports describing the localization of visfatin in diabetic testis and this is the first study to elucidate the role. The objectives of the current study were to characterize testicular visfatin protein localization and to determine a correlation of testicular visfatin expression with serum testosterone levels, glucose levels, body weight and testis weight in an alloxan induced diabetic rat model. We provide novel evidence that visfatin expression is significantly decreased in the alloxan induced diabetic testis compared with the non-diabetic testis.

Materials and methods

Experimental animals

All the experiments were conducted on Adult male Wistar rats (weighing 183 ± 2.25 g). Rats were housed in polypropylene cages (12:12-h day light/darkness cycle) and had access to water and food *ad libitum*. Experiments were approved (MZU/IAEC/14-15/08) and performed according to the guidelines of the Mizoram University Animal Ethics Committee (MZUAEC), Mizoram.

Induction of diabetes

Rats were randomized into two groups of seven rats each as follows: (i) control group; and (ii) diabetic group. Adult male Wistar rats were made diabetic by intraperitoneal administration of alloxan (cat# 52011; SD Fine Chemicals, Kolkata, West Bengal, India) in 0.9% saline in a single dose of 150 mg/kg of body weight (n = 7). Control rats (n = 7) were injected with saline (0.9%) only. Prior to these injections, rats were fasted for 12 h. Five days after alloxan injection, blood glucose levels were measured by the strip method using One Touch Select Simple Kit (Life Scan Johnson and Johnson, Mumbai, Maharashtra, India and if greater than 250 mg/dL were considered diabetic and included in the experiment (n = 7). The blood glucose levels were measured after 7, 14 and 21 days to monitor the stability of the DM condition. Twenty one days after diabetes induction, rats were sacrificed by decapitation under a mild dose of ether anesthetic. Serum was collected from blood and kept at −20 °C for further assay. The testis of one side of each animal was kept at −20 °C for immunoblot and the other testis was fixed in Bouin's fluid for immunohistochemistry.

Immunohistochemistry

After sacrifice of the rats, testes were collected and cleaned. Testes were fixed in Bouin's fixative for 24 h. Then Bouin's fixative was removed with lithium carbonate in 70% ethanol. Tissue was dehydrated with a graded series of ethanol (70%, 80%, 90% and 100%) for about 1 h each for two times followed by xylene treatment and embedding in paraffin wax. Immunohistochemistry was performed according to the method described earlier by Roy and Krishna (2013). In brief, embedded testes in paraffin wax were sectioned at 5 μm. Deparaffinization of sections was done in xylene followed by graded alcohol series treatment and sections were treated for 10 min with 3% of H_2O_2 in methanol for blocking of endogenous peroxidases. Sections were incubated with blocking goat serum (Lot#H0113; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, followed by incubation with the primary antibody (visfatin against anti-human rabbit IgG; Cat#V9139; 1:500, Sigma-Aldrich, St. Louis, MO, USA). The sections were then washed and incubated with the biotinylated goat anti-rabbit IgG secondary antibody (Lot#H0113; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature, followed by another 30 min with horseradish avidin-peroxidase conjugated (Lot#E3013;Santa Cruz Biotechnology, USA). These steps were performed according to the instructions accompanying immunoCruz™ rabbit ABC staining kit (Lot#sc-2018; Santa Cruz Biotechnology, USA). After incubation with horseradish avidin-peroxidase conjugate, sections were washed, and incubated again with the chromagen substrate (0.1% 3 3′ diaminobenzidine tetra hydrochloride (DAB) in 0.5 M Tris 7.6 and 0.01% H_2O_2) for 10 min and counterstained with hematoxylin for 30 s. Slides were analyzed under a light microscope (Leica DM 2500) with a digital camera (model-DFC 450C) (Leica Microsystems, Wetzlar, Germany) and photographed. To test the specificity of the immunoreactivity in the negative control section, the primary antiserum was replaced with 1% normal rabbit serum.

Western blotting

The collected rat testis were pooled and homogenized in suspension buffer (0.01 M Tris pH 7.6) 0.001 M EDTA disodium salt dehydrate (cat# E5134; Sigma-Aldrich, St. Louis, MO, USA) pH = 8.0, 0.1 M NaCl, 1 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF) in a glass homogenizer to produce 10% (w/v) homogenate. Protein extraction and immunoblot were performed as described previously (Roy and Krishna, 2013). Equal amount of protein (40 μg) as determined by Lowry's method (Lowry et al., 1951) was loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE – 10% gel) (Himedia, Mumbai, India) for electrophoresis. Then, proteins were transferred electrophoretically to polyvinylidene fluoride membrane (Millipore India Pvt. Ltd., Bangalore, India) overnight at 4 °C. Membranes were blocked for 60 min with Tris-buffered saline (TBS; Tris 50 mM (pH 7.5), NaCl 150 mM, 0.02% Tween 20) containing 5% fat-free dry milk and incubated with the primary antibody visfatin (1:1000) for 1 h at room temperature. Membranes were then washed with three changes of TBS over 10 min. Immunoreactive bands were detected by incubating the membranes with horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (at a dilution of 1:2000; Merck Specialties Pvt. Ltd, Mumbai, India) for 2 h. Finally, the blot was washed three times with phosphate buffered saline (PBS) and developed with an enhanced chemiluminescence (ECL) detection system (cat#-K820; Biovision, Milpitas, CA, USA). Blot was repeated three times. The densitometric analysis of the blots was performed by scanning and quantifying the bands for density value using computer assisted

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