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Hyperglycemia is associated with reduced testicular function and activin dysregulation in the *Ins2*$^{Akita+/-}$ mouse model of type 1 diabetes

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ABSTRACT

Type 1 diabetes (T1D) is associated with subfertility in men. We hypothesised that this results from inhibitory effects of chronic hyperglycemia on testicular function and used the *Ins2* $^{Akita+/-}$ mouse model to investigate this. Diabetic mice exhibited progressive testicular dysfunction, with a 30% reduction in testis weight at 24 weeks of age. Diabetic mice showed significantly reduced seminiferous tubule diameters and increased spermatogenic disruption, although testes morphology appeared grossly normal. Unexpectedly, serum LH and intra-testicular testosterone were similar in all groups. *Ins2*$^{Akita+/-}$ mice displayed elevation of the testicular inflammatory cytokines activin A and IL-6. Intratesticular activin B was downregulated, while the activin regulatory proteins, follistatin and inhibin, were unchanged. Activin signalling, measured by pSmad3 and Smad4 production, was enhanced in diabetic mice only. These results suggest that prolonged exposure to hyperglycemia in the *Ins2*$^{Akita+/-}$ mice leads to progressive testicular disruption mediated by testicular activin activity, rather than hormonal dysregulation.

1. Introduction

Type 1 diabetes mellitus (T1D) is a chronic, lifelong condition with worldwide increasing incidence. It affects a growing number of men of reproductive age since 90% of these patients are diagnosed before the age of 30 years (Silink, 2002; Wild et al., 2004; Agbaje et al., 2007). Studies in T1D diabetic men have reported reduced fertility associated with sperm DNA damage, erectile dysfunction and hypogonadism (Alves et al., 2013a, 2013b; Ballester et al., 2004; Jangir and Jain, 2014; Mallidis et al., 2009; Maneesh et al., 2006). Animal experiments have reported testicular oxidative mitochondrial DNA damage accompanied by impaired sperm function, altered glucose metabolism in Sertoli cells (Alves et al., 2013a; Kim and Moley, 2008; Rato et al., 2015), and disruption of the hypothalamic-pituitary-gonadal (HPG) axis (Ballester et al., 2004; Schoeller et al., 2014). Late-stage T1D was also characterized by oxidative stress in different tissues and subsequent secondary effects such as accelerated atherosclerosis were associated with a number of pro-inflammatory molecules, including C-reactive protein, endothelin and interleukin-6 (Heier et al., 2015; Kiec-Wilk et al., 2016). This led to our hypothesis that these changes were linked to elevated levels of activin A which was recently shown to facilitate the release of pro-inflammatory cytokines (Jones et al., 2007).

The activin proteins A and B, are homodimers of the inhibin β subunits β_A and β_B and act via a typical TGF-β family signalling pathway. Their activity is regulated by the endogenous inhibitors inhibin and follistatin. Activins bind to activin receptor type 2A (Acvr2a) or 2B (Acvr2b) enabling the phosphorylation of intracellular Smads 2 and 3. A complex formed with the common Smad4 facilitates their entry into the nucleus enabling changes in gene transcription (Young et al., 2015; Heldin and Moustakas, 2012). The major sites of activin production during testicular development and spermatogenesis are the Sertoli, Leydig, peritubular cells and

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testicular macrophages. In particular, activin A is elevated both locally in the testis as well as systemically in disorders of the reproductive system (de Kretser et al., 2004; Hedger and Winnall, 2012).

Recent evidence suggests that activin A is linked to the complications of diabetes. Activin A levels were correlated with blood glucose concentrations and cardiovascular events (Andersen et al., 2011; Ofstad et al., 2013; Wu et al., 2013). Moreover, activins A and B, as well as their binding protein follistatin have been shown to modulate insulin secretion (Andersen et al., 2011; Ofstad et al., 2013; Wu et al., 2013; Chen et al., 2013; Szabat et al., 2010; Hashimoto and Funaba, 2011).

In the majority of animal studies, diabetes was induced using the cellular toxin streptozotocin, an agent known to inhibit poly(-ADP ribose) polymerase and thereby inducing testicular DNA damage in addition to its effect on hyperglycemia (Kilarkaje et al., 2014). In order to study hyperglycemia without the DNA damage elicited by streptozotocin, we utilized the Akita transgenic mouse model. Studies in Akita mice, homozygous for a constitutive missense mutation in the insulin 2 gene (*Ins2*), indicated that abnormal testicular function was inhibited due to a disruption in the HPG axis as early as 3 weeks of age. This trait could be rescued by exogenous insulin and leptin, which normalized levels of LH and testosterone (Schoeller et al., 2012, 2014).

Our hypothesis is that long-term hyperglycemia *per se* causes male subfertility by testicular dysfunction. Given the previously reported association of high blood glucose and activin A concentrations we suggest that the secretory patterns and actions of the activin family of proteins in the testis exposed to high glucose levels are dysregulated. To address this hypothesis, we used male heterozygous *Ins2*$^{Akita+/-}$ mice, which have a diabetes prevalence of 50%. These mice display rapid and severe onset of diabetes by the age of 4 weeks, but can be maintained for more than 24 weeks. This allowed us to directly investigate the impact of long-term hyperglycemia on testicular function.

2. Research design and methods

2.1. Animals and reagents

12- and 24-week-old *Ins2*$^{Akita+/-}$ mice on C57Bl6/J background were used. Mice were maintained following approval from the Animal Ethics Committee, in accordance with German Animal Welfare Act for the care and use of laboratory animals and the rules of the regulatory authorities in Baden-Württemberg (Reg. präsidium Karlsruhe, Germany). Blood glucose was monitored consecutively over the study period. At 8 weeks of age, approximately 50% of these mice had permanent blood glucose levels above 14.0 mmol/l and were classified as severely hyperglycemic, insulin-dependent diabetes according to criteria for diabetic ketoacidosis (Gosmanov et al., 2014). Further, this cut off corresponds to values greater than the 95th percentile of glucose distribution of non-diabetic C57Bl/6 J mice population in our animal facility. Insulin was occasionally administered to prevent critical weight loss. Non-diabetic *Ins2*$^{Akita+/-}$ mice were included in all experiments as phenotypic controls. 12- and 24-week-old male C57Bl/6 J mice were obtained from Charles River Laboratories (Sulzfeld, Germany), as wild-type controls. All animals were housed in groups of 3–4 animals at a temperature of 21 ± 1 °C with a 12 h light–dark cycle. At the end of experimental period, animals were killed by cervical dislocation under intraperitoneal ketamine/xylazine anaesthesia. 200–500 μl non-fasting blood was collected via the retroorbital venous plexus from all mice and glycated hemoglobin (HbA1c) was determined as described previously (Pfister et al., 2008). After clotting, the serum was separated by whole-blood centrifugation at 1300 rpm for 10 min. Unless stated otherwise, all reagents were obtained from Sigma-Aldrich Chemie GmbH (Seelze, Germany).

2.2. Immunohistochemistry

Whole-mouse testes were fixed in Bouin's fluid and then dehydrated in a graded series of ethanol and embedded in paraffin. 5 μm thick sections were processed for staining. Sections were deparaffinised and rehydrated prior to antigen retrieval. Localization of the Sertoli cell marker protein SOX-9 was performed using a polyclonal ChIP Grade antibody (diluted 1:300, ab3697, Abcam, Cambridge, U.K.). The sections were counterstained with Harris' haematoxylin and mounted under DPX (BDH Laboratory Supply, Poole, U.K.). Apoptotic events were analysed using TUNEL assay (Millipore, Merck KGaA, Darmstadt, Germany). Specific signal was visualized by treatment with sheep peroxidase-conjugated anti-digoxigenin antibodies (Vectastain® Elite ABC Kit, Vector Laboratories, CA, USA) followed by diaminobenzidine (DAB) development. Both the microscopic assessment of spermatogenesis and the measurement of seminiferous tubule diameter were undertaken using ImageJ software. Seminiferous tubules were evaluated for spermatogenesis index (SI) by the Johnsen's score (Johnsen, 1970). A Score from 10 (complete spermatogenesis) to 1 (no cells present) was given to each tubule within one cross-section.

2.3. Sperm analysis

In order to perform a morphological assessment of sperm fresh epididymides from C57Bl7/6 J or *Ins2*$^{Akita+/-}$ mice were placed in Petri dishes containing 1 ml of high saline bicarbonate (HSB) buffer and milked using forceps to release stored sperm. Sperm were then incubated at 37 °C until assessment of sperm count and sperm morphology according to WHO criteria (World Health Organization, 2010), which followed shortly after excision.

2.4. Protein measurement

Tissue samples were homogenised in 1 mL ice-cold phosphate-buffered saline (PBS, buffered to pH 7.4) for 30 s and the insoluble fragments removed by centrifugation at 12 000 × g for 30 min at 4 °C. Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Carlsbad, CA, USA) was used to measure total protein concentrations according to the manufacturer's instructions for the microplate procedure. Samples were read at 562 nm on a Labsystems Multiscan RC plate reader (LabX, Midland, ON, Canada).

2.5. ELISA and RIA

Total activin A was measured using a specific ELISA (Knight et al., 1996) (Oxford Bio-Innovations, Oxfordshire, U.K.) with some modifications as described previously (O'Connor et al., 1999). The limit of detection was 14.1 pg/ml with an average intra-assay CV of 4.7% and an inter-assay CV of 9.6%.

Activin B was measured against a human activin B standard using reagents supplied by Oxford Brookes University with previously described methodology (Ludlow et al., 2009). The limit of detection was 23.0 pg/ml and the average intra-assay CV was 4.1%.

The absorbance values of each well were measured using an automated microplate reader (Benchmark microplate reader, Bio-Rad Laboratories, CA, USA) with compatible software (Microplate Manager 5.2, Bio-Rad Laboratories), using a measurement wavelength of 450 nm and a reference wavelength of 620 nm.

Total follistatin concentrations in mouse tissue homogenates were measured using a discontinuous RIA, as described previously