Expression of Steroidogenic Acute Regulatory (StAR) Protein is Associated with Blood Glucose Levels in Streptozotocin (STZ)-induced Diabetic Mice

การแสดงออกของโปรตีน StAR สัมพันธ์กับระดับน้ำตาลในเลือดในหนูไมซ์เบาหวานที่ถูกเหนี่ยวนำด้วยสารสเตรปโตโซโทซิน

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ABSTRACT

In general, diabetes mellitus (DM) causes the decrease of testosterone hormone levels in both men and experimental animals. However, the association between decreasing hormone and expression of steroidogenic acute regulatory (StAR) protein (major precursor of testosterone production) has never been reported. This study aimed to investigate the changing of reproductive parameter and StAR protein in DM mice. Ten mice were divided into 2 groups (control and streptozotocin (STZ) groups). The control group (n=5) was injected with 0.1 M citric buffer while STZ group (n=5) was injected with a single dose of STZ (150 mg/kg BW). After 14 days, all groups were measured glucose levels and testosterone levels then sacrificed to collect reproductive organs and count sperm. The testes were examined for StAR protein expression by immuno Western blotting including histology. The results showed significant decrease of body weight, sperm concentration and testosterone levels in STZ group. In addition, decreased expression of StAR protein depends on the increase of blood glucose levels. Moreover, the testicular tissue in STZ group appears accumulation of vacuole in spermatogonia. In conclusion, expression of StAR protein was correlated with testosterone hormone and blood glucose levels.

บทคัดย่อ

โรคเบาหวานเป็นสาเหตุที่ทำให้เกิดการลดลงของระดับฮอร์โมนเทสโทสเตอโรนทั้งในเพศชายและสัตว์ทดลอง อย่างไรก็ตามความสัมพันธ์ระหว่างการลดลงของฮอร์โมนและการแสดงออกของโปรตีน StAR (ตัวพาสารตั้งต้นของการสร้างฮอร์โมนเทสโทสเตอโรน) ยังไม่มีการรายงาน ในการศึกษาครั้งนี้จึงมีจุดประสงค์เพื่อตรวจสอบการเปลี่ยนแปลงของระบบสืบพันธุ์และโปรตีน StAR ในหนูไมซ์เบาหวาน โดยหนูไมซ์สิบตัวถูกแบ่งออกเป็น 2 กลุ่ม (กลุ่มควบคุมและกลุ่ม STZ) ในกลุ่มควบคุมถูกฉีดด้วย 0.1 M citric buffer ในขณะที่กลุ่ม STZ ถูกฉีดด้วยสารสเตรปโตโซโทซินเพียงครั้งเดียว (150 มิลลิกรัม/กิโลกรัม น้ำหนักตัว) หลังจากนั้น 14 วัน ทุกกลุ่มถูกตรวจวัดระดับน้ำตาลในเลือดและระดับเทสโทสเตอโรน แล้วทำการการุณฆาตเพื่อเก็บอวัยวะสืบพันธุ์และนับอสุจิ ในส่วนอัณฑะถูกตรวจสอบ

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การแสดงออกของโปรตีน StAR โดยวิธีอิมมูโนเวสเทิร์นบล็อททิง รวมถึงลักษณะทางจุลพยาธิวิทยา จากผลการทดลองพบว่ามีการลดลงของน้ำหนักตัว จำนวนอสุจิและระดับของฮอร์โมนเทสโทสเตอโรนในกลุ่ม STZ นอกจากนั้นการแสดงออกของโปรตีน StAR ที่ลดลงขึ้นอยู่กับการเพิ่มขึ้นของระดับน้ำตาลในเลือด ยิ่งไปกว่านั้นเนื้อเยื่ออัณฑะในกลุ่ม STZ เกิดการสะสมของแวคิวโอลในชั้นสเปอร์มาโทโกเนียม สรุปผลการทดลองพบว่าการแสดงออกของโปรตีน StAR มีความสัมพันธ์กับฮอร์โมนเทสโทสเตอโรนและระดับน้ำตาลในเลือด

Key Words: Blood glucose levels, Steroidogenic acute regulatory (StAR) protein, Streptozotocin (STZ)

คำสำคัญ: ระดับน้ำตาลในเลือด โปรตีนที่ควบคุมการสังเคราะห์ฮอร์โมนสเตียรอยด์ สเตรปโตโซโทซิน

Introduction

Diabetes mellitus (DM) is a group of chronic disorders in glucose metabolism caused by insulin impaired secretion from pancreatic beta cells resulting in elevating glucose levels (also called hyperglycemia) (Farmer, 1952). This condition causes the decrease of testosterone hormone levels (Ballester et al., 2004; Jelodar et al., 2009; Navarro-Casado et al., 2010; Fernandes et al., 2011; Khaneshi et al., 2013;) including sperm concentration (Mulholland et al., 2011) in both diabetic patients and diabetic animals induced by chemicals.

In process of testosterone production, steroidogenic acute regulatory (StAR) protein transports the cholesterol into matrix of mitochondria. Then, cytochrome P450 side chain cleavage (CYP11A1) enzyme cleaves the cholesterol side chain to be pregnenolone molecules. Pregnenolone will be transported into smooth endoplasmic reticular (sER). In the sER, pregnenolone is metabolized by 3 β hydroxyl steroid dehydrogenase, 17α-hydroxylase, C17-20-lyase and 17-ketosteroid reductases to be testosterone before secreting into the bloodstream (Strauss and Barbieri, 2009).

In general, streptozotocin (STZ), a chemical, is produced by *Streptomyces achromogenes* (Rakieten et al., 1963). Previously, STZ was used as an antibiotic (Vavra et al., 1959). In addition, STZ is commonly used as a type I-DM inducer in animal model research (Khaneshi et al., 2013). It is known that STZ can damage DNA (Yamamoto et al., 1981a; Yamamoto et al., 1981b; LeDoux et al., 1986; Takasu et al., 1991; Morgan et al., 1994; Bedoya et al., 1996; Chan et al., 2008; Koo et al., 2011; Zhang et al., 2011) and selectively toxic to the beta cells of Islets of Langerhans (Weiss, 1982; Szkudelski, 2001; Lenzen, 2008; Szkudelski, 2012). Consequently, the pancreatic beta cells induced with STZ have significantly reducing production of the insulin into the bloodstream causing hyperglycemia or type I-DM condition (Rorsman & Braun, 2013).

However, the association between decreasing testosterone hormone and expression of StAR proteins has never been reported.

Objective of the study

The aim of this study was to investigate the changing of reproductive parameters and StAR protein in DM mice.

Methodology

STZ-induced diabetes

Ten male mice (6-weeks old), initially weighing 35-37 g, were divided into 2 groups. The control mice (n=5) were injected with 0.1 M citric buffer, pH 4.5 while STZ group (n=5) were injected a single intraperitoneal injection of STZ (150 mg/kg BW in 0.1 M citric buffer, pH 4.5) (Muralidhara, 2007). All mice groups were starved for 16 to 18 hours. After 72 hours post-injection and before euthanized, all mice were measured the glucose levels from tail prick blood samples using blood glucose oxidase reaction monitoring system (trademark: Johnson and Johnson Ltd.). The diabetic mice were considered mild hyperglycemia (250-450 mg/dL).

Serum testosterone and cholesterol assays

At day 14 of the experiment, all mice were euthanized by cervical dislocation. They were cleaned the anterior abdominal external wall by 70% alcohol. The abdominal and thoracic walls and opened perform the cardiac puncture in blood collection at right ventricles. The blood samples were centrifuged by microcentrifuge (Microfuge 22R Centrifuge), 800 rpm at 4° C for 5 minutes to collect serum. The blood serum was delivered to the diagnostic clinical chemistry laboratory of Srinagarind hospital, Faculty of Medicine, Khon Kaen University for measurement the levels of testosterone hormones and cholesterol.

Testicular testosterone and cholesterol assays

The left side testis was rapidly kept at 4 °C. Then, the testicular total protein lysate prepared by homogenization of testis with cocktail protease inhibitors added-RIPA buffer (Cell Signaling Technology Inc., USA). The testicular homogenate was centrifuged at 14000 rpm for 10 minutes and the supernatant was collected to measure levels of testosterone hormones and cholesterol.

Investigation of testicular histology

The right side testis was fixed with 10% formalin and embedded with paraffin, and sectioned by microtome about 5 μm thicknesses. The testicular sections were deparaffinized with xylene, rehydrated with serial alcohols and water. The rehydrate sections were stained by hematoxylin and eosin (H&E), mounted with DPX and observed under light microscope.

Investigation expression of StAR protein by immuno Western blotting

The left side testis was rapidly kept at 4 °C. Then, the testicular total protein lysate prepared by homogenization of testis with cocktail protease inhibitors added-RIPA buffer (Cell Signaling Technology Inc., USA). The testicular homogenate was centrifuged at 14000 rpm for 10 minutes and the supernatant was collected to measure the total protein concentration by NANO drop (NanoDrop ND-1000 Spectrophotometer V3.5 User's Manual, NanoDrop Technologies Inc., USA). Fifty micrograms of total testicular proteins pooled from triplicate samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane to detect the intensity of StAR protein by using the Anti-StAR and beta actin primary antibody (1:1000 (v/v), Santa Cruz Biotechnology INC., USA) overnight, followed by another incubation of HRP goat anti-rabbit (1:10000 dilution) and anti-mouse secondary antibody (Santa Cruz Biotechnology INC.,USA). In immuno Western blotting, beta-actin and StAR lysate (293 Lysate; Santa CruzBiotecnology INC., USA) were used as

the internal control and positive control. To detect the phosphorylated proteins, the enhanced chemiluminescence (ECL) substrate was used before visualization under Gel Doct 4 (ImageQuant 400, GH Healthcare, USA).

Assessments of sperm concentration

Epididymis plus vas deferens of both sides of mice were cut and separated from the testes using surgical scissors, then they were squeezed gently by using small non-tooth forceps. The squeezed sperm fluid looks like milky fluid. The sperm fluid was dipped into 1 ml of PBS, pH 7.4 at 37° C. It was resuspended gently (at 500 rpm for 5 minutes) for washing and separation out of cell debris. After centrifugation, the sperm suspension was separated into 2 layers (upper supernatant and lower sperm pellet containing mature sperm). Supernatant was pipetted out form the sperm pellet. The pellet in the eppendorf tube was gently resuspended again with 2 ml 0.9% normal saline. The mixture was called "the sperm suspension". Sperm suspension prepared above was diluted with 0.9% normal saline (1:20 dilution factors) to analyze its sperm concentration ($\times 10^6$/ml). The diluted sperm suspension was loaded onto the neubauer double counting chamber of haemacytometer and covered by glass slip. Then the haemacytometer was placed under light microscope using 40X magnifications of objective lens to view and count sperm. After counting sperm, sum numbers of sperm were calculated of the sperm concentration according the formula of Wolf et al., 1988:

$$n = a \times 5 \times b \times 10000$$

Data Analysis

The data were analyzed by one-way ANOVA and represented as means ± SD. The differences were considered to be significant when the calculated $P<0.05$.

Results

The results in figure 1 showed significant decrease of body weight in STZ group compared with control group ($P<0.05$).

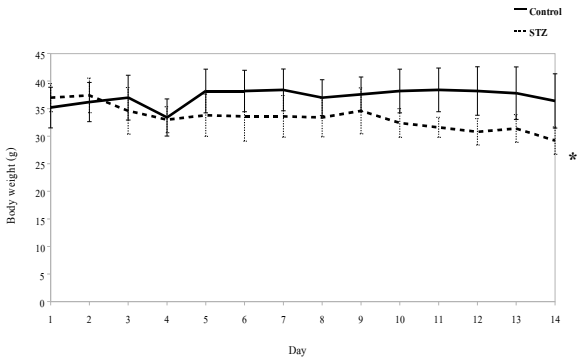

Figure 1 Body weights of control and STZ groups for 14 days after STZ induction

Figure 2 showed that mice in STZ group were not different in cholesterol levels in serum but tend to be higher in testis as compared to the control.

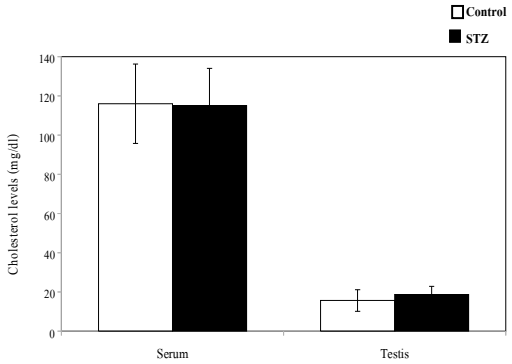

Figure 2 Cholesterol levels in blood serum and testis of control and STZ mice

Figure 3 showed that serum testosterone levels were significantly decreased in STZ group as compared with the control ($P<0.05$).

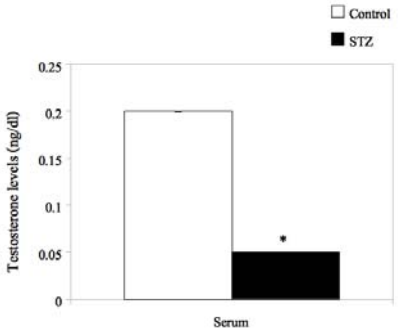

Figure 3 Serum testosterone levels in of control and STZ mice

Table 1 showed no differences of male reproductive organs between control and STZ groups.

Table 1 Weight of male reproductive organs in control and STZ groups.

Group	Testis (g)		Seminal vesicle (g)		Epididymis + vas defferen (g)	
	left	right	left	right	left	right
Control-1	0.1351	0.1514	0.1989	0.0569	0.0507	0.0569
Control-2	0.1718	0.1725	0.3146	0.0739	0.0706	0.0739
Control-3	0.1132	0.1091	0.2655	0.0596	0.0644	0.0596
Control-4	0.1650	0.1713	0.3085	0.0710	0.0739	0.0710
Control-5	0.1588	0.1714	0.3347	0.0831	0.0757	0.0831
mean	0.1488	0.1551	0.2844	0.0689	0.0671	0.0689
SD	0.0242	0.0272	0.0541	0.0107	0.0101	0.0107
STZ-1	0.1486	0.1570	0.1681	0.0975	0.0736	0.0975
STZ-2	0.1312	0.1395	0.3467	0.0732	0.0575	0.0732
STZ-3	0.1466	0.1544	0.2431	0.0569	0.0608	0.0569
STZ-4	0.0917	0.0924	0.2318	0.0481	0.0464	0.0481
STZ-5	0.1607	0.1781	0.1871	0.0638	0.0672	0.0638
mean	0.1358	0.1443	0.2354	0.0679	0.0611	0.0679
SD	0.0268	0.0321	0.0695	0.0189	0.0103	0.0189

Figure 4 revealed the significant decrease of sperm concentration in STZ group compared with that of the control ($P<0.05$).

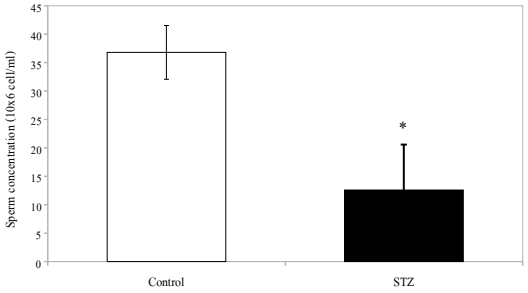

Figure 4 Showing sperm concentration of control and STZ groups

Figure 5 demonstrated that the expression of StAR proteins was decreased when the blood glucose levels (BGL) was elevated.

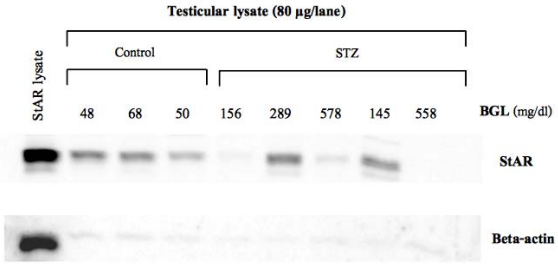

Figure 5 Expression of StAR protein in testicular lysate (80 µg/ lane) showing with BGL in control and STZ groups.

In histology of the testis, the results showed that seminiferous of STZ group is present the accumulation of vacuole in spermatogonia as compared with control group.

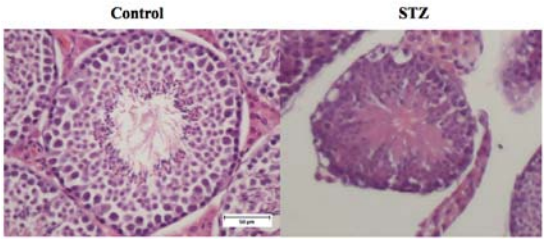

Figure 6 Histology of seminiferous tubules of control and STZ groups.

Discussion and Conclusions

The results of this study are generally consistent with previous studies, which showed that body weights were reduced in animals induced with STZ for 14 days. However, STZ did not affect the weights of all male reproductive organs implying that it does not direct affect those organs. This study showed for the first time that STZ could reduce sperm concentration. It is possible that STZ could significantly decrease the testosterone hormone levels (Fig. 3) which is similar to that of Muralidhara (2007). It can also be explained that STAR protein expression responsible for transport cholesterol precursors for testosterone production were significantly decreased when increasing of BGL. Moreover, we found that STZ could partially damage the seminiferous tubules by accumulating the vacuoles in spermatogonia. We concluded that STZ affects sperm concentration and testicular histology including StAR expression associated with elevated blood glucose levels.

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