Original article

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THE RELATIONSHIP BETWEEN SERUM CONCENTRATION OF TNF-α AND INSULIN SENSITIVITY IN RATS WITH DIABETES MELLITUS TYPE 2

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Diabetes and obesity are very common associated metabolic disorders that are linked to chronic inflammation. The development of insulin resistance is driven by multiple factors including an increase in levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α). This study aimed to explore the links between TNF-α -mediated inflammation, insulin sensitivity, and body weight gain in the rat model of type 2 diabetes mellitus (T2DM). The experiment was performed on 10 weeks old Wistar rats randomized into 2 groups. T2DM was induced by intraperitoneal injection of streptozotocin, administered 15 minutes after an intraperitoneal injection of nicotinamide. After 6 weeks, the animals were euthanized. Insulin and TNF-α were determined by using an enzyme-linked immunosorbent assay kit. Insulin sensitivity indices were calculated. The concentration of TNF-α was significantly higher in animals with T2DM when compared to controls ($p < 0.001$). Quantitative Insulin Sensitivity Check Index (QIUCKI) had significantly lower values in animals with T2DM when compared to controls ($p < 0.001$), whereas values calculated for homeostatic model assessment of insulin resistance (HOMA-IR) were significantly higher ($p < 0.001$). TNF-α correlated positively with HOMA-IR ($r = 0.562$, $p < 0.01$) and negatively with QIUCKI ($r = -0.332$, $p < 0.05$). Additionally, TNF-α correlated positively with specific rate of the body weight gain ($r = 0.667$, $p < 0.01$) in the observed period. The results suggest that an increase in circulating TNF-α concentration might be associated with an increase in body weight gain and reduced insulin sensitivity in rats with T2DM.

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Key words: *type 2 diabetes mellitus, TNF-α, insulin sensitivity, body weight, obesity*

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Introduction

Type 2 diabetes mellitus (T2DM) is an expanding global health problem accounting for around 90% of all cases of diabetes around the world. It is characterized by hyperglycemia, impaired insulin secretion, and reduced sensitivity to insulin/insulin resistance (1).

The development of insulin resistance is driven by multiple factors such as gluco-lipotoxicity, discoordinated glucose lowering response, reactive oxygen species (ROS) generation, epigenetics and an increase in levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) (2).

TNF-α is synthesized as a transmembrane protein (tmTNF-α) in several types of cells such as macrophages, monocytes, neutrophils, T-cells, adipocytes, and peripheral tissue cells (2, 3). The soluble form of TNF-α (sTNF) is released through the action of matrix metalloproteinase, TNF-α-converting enzyme (TACE). Both biologically active forms of TNF-α exert their effect through the receptors TNF-R1 and TNF-R2 which activation causes cell death or cell survival respectively (4).

Obesity is a chronic inflammatory condition that has been linked to T2DM and insulin resistance (5). Obese individuals with T2DM have a higher level

of circulating TNF-α when compared to lean individuals (6).

This study aimed to explore the links between TNF-α mediated inflammation, insulin sensitivity, and body weight gain in a rat model of T2DM, by testing the hypotheses that:

1) an increase in TNF-α leads to reduced insulin sensitivity, and

2) that both increases in TNF-α and reduced insulin sensitivity are in connection with an increase in body weight.

Materials and methods

Experimental animals

In this study, 10 weeks old Wistar rats were used. Animals were bred and housed in the vivarium of the Research Center for Biomedicine, Faculty of Medicine, University of Niš, at 22-24 ^{O}C with a 12-hour light cycle. Access to food and water was unrestricted in the course of the experiment. Animals consumed a standard laboratory rodent diet (AIN93M). The experiment was conducted under the Guide for the Care and Use of Laboratory Animals issued by the National Academy of Sciences, Washington, and the Book of Regulations for Work with Experimental Animals adopted by the Faculty of Medicine, University of Niš. The experimental protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Niš (permit number 01-10204-3).

Experimental procedure

The animals were randomly divided into 2 groups (n = 10), control (C), and T2DM. T2DM was induced by an intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ) in citrate buffer (45 mg/kg; Sigma, USA), given 15 minutes after intraperitoneal injections of nicotinamide (NA, 110 mg/kg; Sigma, USA) to the animals in the group T2DM (7). The animals in the group T2DM were injected with citrate buffer and saline respectively, according to the previously described procedure.

The diagnosis of T2DM was established by measuring glucose concentration in venous blood. Hyperglycemia was confirmed using an automated blood glucose meter Accu-Chek Performa (Roche). Blood was collected from the tail vein, after 2 and 8 hours fast, on the 3rd and 7th day after the STZ and NA injections. The cut-off values for the blood glucose were > 8.3 mmol/L and > 6.1 mmol/L, after 2 and 8 hours fast, respectively.

After six weeks, the animals were euthanized by exsanguination after bilateral thoracotomy under deep anesthesia (Ketamidor, Richter Pharma AG; 100 mg/kg, i.p.). The blood was taken by cardiac puncture (terminal). Before the animals were euthanized, the concentration of blood glucose was measured under the same conditions as at the beginning of the experiment.

Animal measurements

The body weight was measured weekly after night fasting.

The specific rate of body weight gain (SBWG) was calculated using formula (g/kg) = ΔMM/M, where ΔMM represents an increase in body weight in the observed period (dt = t2-t1) and M body weight at the time point t1.

Sample Preparation

Serum and plasma were separated by centrifugation (15 minutes at 3000 g) and stored at 20 ^{O}C. Na-EDTA was used as an anticoagulant.

Determination of serum glucose

Serum glucose levels were determined by using an enzymatic method on an automated clinical chemistry analyzer (Dimension RxL Max, Siemens, USA) using original reagents from Siemens Healthcare Diagnostics.

Determination of serum insulin and TNF-α

Insulin levels were determined by using a commercially available rat enzyme-linked immunosorbent assay (ELISA) kit (Mercodia Rat Insulin ELISA, Uppsala, Sweden). TNF-α levels were determined by using a commercially available rat ELISA kit (Rat TNF-α ELISA Kit, Abcam Cambridge, UK).

Calculation of insulin sensitivity indices

Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated assuming an average HOMA-IR of 1 in control young adult rats, analogous to the assumptions in humans (8). The equation used for HOMA-IR calculation was as follows HOMA-IR = (fasting glucose (mg/dl) * fasting insulin (μU/ml))/2430 (8). Quantitative insulin sensitivity check index (QUICKI) was calculated according to the original formula

QUICKI = 1/[log (fasting glucose (mg/dl) + log(fasting insulin (μU/ml)] (8).

Statistical analysis

The data were expressed as mean ± standard deviation. Statistical analysis was performed using the IBM® SPSS® Statistics 21.0. The Kolmogorov-Smirnov test was used to test the normality of distribution. Comparison of continuous variables was performed using a Student's t-test. The linear relationship between quantitative variables was determined using the Pearson test for parametric data. The level of significance for all statistical tests was set at 5%.

Results

Specific rate of body weight gain, non-fasting, fasting glucose, insulin levels and QUICKI and HOMA-IR values in the control and experimental rats are shown in Table 1. Specific rate of body weight gain (SBWG) was significantly higher in the T2DM group ($p < 0.01$). There were significant differences in both non-fasting (T2DM vs. C, $p < 0.001$) and fasting glucose levels (DM vs. C, $p < 0.001$) between the T2DM and the control group. Insulin levels did not differ between groups (T2DM vs. C, NS). Both QUICKI and HOMA-IR values were significantly different in group T2DM when compared to C (QUICKI, T2DM vs. C, $p < 0.05$; HOMA-IR, T2DM vs. C, $p < 0.001$).

Table 1. Specific rate of body weight gain, biochemical parameters of glucose metabolism and insulin sensitivity indices in healthy (C) and rats with type 2 diabetes mellitus (T2DM).

	C	T2DM
Specific rate of body weight gain (g/kg bw)	0.12 ± 0.02	0.17 ± 0.03*
Non-fasting glucose (mmol/l)	6.53 ± 0.75	10.81 ± 0.61*
Fasting glucose (mmol/l)	4.82 ± 0.19	6.51 ± 0.34*
Insulin (μU/ml)	30.49 ± 2.70	33.58 ± 3.54
QUICKI	0.46 ± 0.01	0.44 ± 0.01*
HOMA-IR	1.14 ± 0.12	1.64 ± 0.19*

Values are mean±standard deviation of the mean
Means with superscript differ significantly (Student's t-test), $p < 0.05$

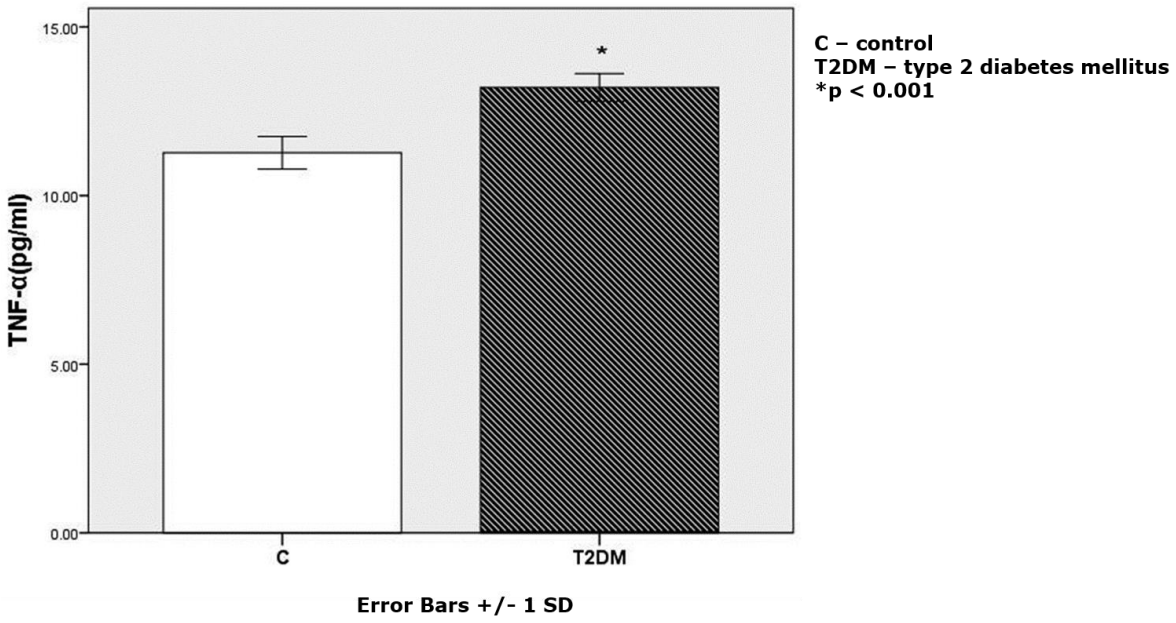

Figure 1. Circulating TNF-α concentration in animals with type 2 diabetes mellitus

Table 2. Pearson correlation coefficient (r) between TNF-α, insulin sensitivity indices and specific rate of body weight gain (SBWG)

	TNF-α		SBWG	
	r	P	r	P
TNF-α	1	N/A	0.667	< 0.001
HOMA-IR	0.562	< 0.001	0.334	0.043
QUICKI	-0.332	0.03	-0.335	0.024

Compared to controls, the concentration of TNF-α (Figure 1) was also significantly higher in animals with T2DM (T2DM vs. C, $p < 0.001$).

There was negative correlation between serum TNF-α and QUICKI (Table 2) and positive correlation between TNF-α and HOMA-IR (Table 2). Specific body weight gain was correlated to TNF-α, QUICKI, and HOMA-IR (Table 2).

Discussion and conclusion

Both T2DM and obesity are recognized as serious public health concerns with a significant impact on the quality of life. Moreover, concerning trends of rising prevalence have been recorded in high and low-income countries (9, 10).

Our first hypothesis was that animals with T2DM would have higher circulating TNF-α and reduced insulin sensitivity. The hypothesis was accepted since the circulating TNF-α levels found in animals with T2DM were higher when compared to healthy animals. There was a correlation between TNF-α levels and values of surrogate measures of insulin sensitivity, QUICKI, and HOMA-IR. Both serum TNF-α levels and values of QUICKI and HOMA-IR were in correlation with the increase in body weight gain, although the correlation with surrogate measures of insulin sensitivity was found to be weak.

The animal model of T2DM used in this experiment is characterized by impairment in insulin secretion and reduced glucose tolerance caused by both metabolic and non-metabolic disturbances in pancreatic β cells (7, 11). The severity of diabetes induced by STZ/NA application depends on the doses of STZ and NA given (11). Serum insulin levels in this study were not significantly different than levels measured in healthy controls, whereas diabetic animals had mild to moderate hyperglycemia.

In humans with T2DM increased levels of pro-inflammatory cytokines, such as TNF-α and IL-6, are found in the systemic circulation (12). Increased concentrations of TNF-α were reported previously in an animal model similar to the one used in this experiment (13). Hyperglycemia could trigger the release of TNF-α by peripheral monocytes and adipocytes (14). Additionally, the expression of TNF-α appears to be increased in adipose and muscle tissue of both obese rodents and humans (15). An increase in circulating TNF-α might be linked to hyperinsulinemia and insulin resistance in peripheral tissues in obese and diabetic humans (2, 16-18). This study reported a correlation between levels of circulating TNF-α and insulin sensitivity indexes. The indexes used exhibited good sensitivity and specificity and provided an easy and accurate measure of insulin sensitivity in rats (8). Besides, we observed an increase in circulating TNF-α which appears to be in correlation with the specific body weight gain in the observed 6-week period. A positive correlation between serum TNF-α and HOMA-IR in obese humans with T2DM has been reported recently (19). Increased production of TNF-α in adipose tissue could be related to the obesity-associated insulin resistance that leads to the development of T2DM (20).

TNF-α-induced insulin resistance might be driven by the molecular mechanisms that involve the activation of Jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway (2). By activating transcriptional factor NF-kB, TNF-α induces the apoptosis of pancreatic β-cells that might lead to insulin secretion suppression (2, 21). JNK phosphorylates IRS-1 at Ser307, which inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 that might result in inhibition of insulin signaling (2, 21, 22). Additionally, TNF-α reduces the expression of insulin-dependent glucose transporter type 4 (GLUT4) located in adipocytes and skeletal muscles (23).

In conclusion, an increase in circulating TNF-α might be associated with reduced insulin sensitivity and an increased body weight gain in rats with T2DM.

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POVEZANOST KONCENTRACIJE TNF-α U SERUMU I INSULINSKE SENZITIVNOSTI KOD PACOVA SA DIJABETESOM MELLITUSOM TIP 2

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Dijabetes melitus i gojaznost su metabolički poremećaji povezani sa hroničnom inflamacijom, koji se često javljaju u opštoj populaciji. Razvoj insulinske rezistencije povezan je sa mnogobrojnim faktorima, uključujući povećanje koncentracije proinflamatornih citokina, poput faktora nekroze tumora alfa (TNF-α). Cilj ove studije bio je da ispita povezanost koncentracije TNF-α, insulinske senzitivnosti i povećanja telesne mase na animalnom modelu dijabetesa melitusa tipa 2 (T2DM). Eksperiment je izveden na 10 nedelja starim Vistar pacovima nasumično podeljenim u 2 grupe. T2DM indukovan je intraperitonealnom injekcijom streptozotocina, primenjenom 15 minuta nakon intraperitonealne injekcije nikotinamida. Posle 6 nedelja, životinje su žrtvovane. Koncentracije insulina i TNF-α određene su korišćenjem komercijalnog enzimskog imunoeseja. Indeksi insulinske senzitivnosti određeni su pre-računavanjem uz upotrebu odgovarajućih formula. Koncentracija TNF-α bila je značajno veća kod životinja sa T2DM u poređenju sa životinjama iz kontrolne grupe ($p < 0,001$). Kvantitativni indeks provere insulinske senzitivnosti (QIUCKI) imao je značajno niže vrednosti kod životinja sa T2DM u poređenju sa životinjama iz kontrolne grupe ($p < 0,001$), dok su vrednosti izračunate za homeostatski model procene insulinske rezistencije (HOMA-IR) bile značajno veće ($p < 0,001$). TNF-α bio je u pozitivnoj korelaciji sa vrednostima HOMA-IR ($r = 0,562$, $p < 0,01$) i negativnoj korelaciji sa vrednostima QIUCKI ($r = - 0,332$, $p < 0,05$). Pored toga, TNF-α je bio u pozitivnoj korelaciji sa specifičnim indeksom porasta telesne mase ($r = 0,667$, $p < 0,01$) u posmatranom periodu. Rezultati sugerišu na to da bi povećanje koncentracije TNF-α u cirkulaciji moglo biti povezano sa povećanjem telesne mase i smanjenom insulinskom senzitivnošću kod pacova sa T2DM.

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Ključne reči: *dijabetes melitus tipa 2,* TNF-α*, insulinska senzitivnost, telesna masa, gojaznost*