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Glycemic Control and Oxidative Stress Markers and their relationship with the Thioredoxin Interacting Protein (TXNIP) gene in Type 2 Diabetic patients

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Abstract

Objective: **To investigate the relationship between oxidative stress and biochemical parameters and the expression of TXNIP, IL-6, IL-1β and TNF-α in peripheral mononuclear cells (PMCs) from type-2 diabetic patients.**

Methods: **We studied 60 males: 20 normal-weight type-2 diabetic patients (NW), 20 obese diabetic patients (OB) and 20 controls (C). Biochemical and oxidative stress parameters were evaluated. PMCs were isolated and total RNA was extracted in order to determine the expression of TXNIP, IL-6, IL-1β and TNF-α by qRT-PCR.**

Results: **OB had higher weight, BMI and abdominal circumference (One way ANOVA, p<0.0001). NW had higher fasting glycemia (One way ANOVA, p=0.0034) however OB had higher HbA1c (One way ANOVA, p<0.0001). OB also had higher hsCRP (One way ANOVA, p=0.0158). TBARS and AGES were elevated in both NW and OB (One way ANOVA, p<0.0001 and p=0.0008, respectively). Compared to OB and C participants, the expression of TXNIP was significantly higher in NW (Kruskal Wallis, p=0.0074); IL-1β, IL-6 and TNF-α transcripts were higher in NW and OB (Kruskal Wallis, p<0.0001, for all). In NW patients, the expression of TXNIP was positively correlated with fasting glycemia and AGES and negatively correlated with HOMA-β (r=0.72; r=0.59; r=-0.44, respectively, for all p<0.05), in OB there was correlation only with 8-Isoprostanes (r=0.42, p=0.046).**

Conclusions: **Our results suggest that fasting glycemic control, independent of adiposity and nutritional status, represents a risk factor for β-cell dysfunction, increases oxidative stress markers and it is related with an elevation of TXNIP expression.**

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Key words: *TXNIP. β-cell function. Inflammation. AGEs. Glycemic control.*

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CONTROL GLICÉMICO Y MARCADORES DE ESTRÉS OXIDATIVO Y SU RELACIÓN CON EL GEN DE LA PROTEÍNA INTERACTUANTE CON LA TIOREDOXINA (TXNIP) EN SUJETOS DIABÉTICOS TIPO 2

Resumen

Objetivo: **Investigar la relación existente entre parámetros bioquímicos y de estrés oxidativo y la expresión de TXNIP, IL-6, IL-1β y TNF-α en células mononucleares periféricas (CMPs) de sujetos diabéticos.**

Material y métodos: **Se estudió 60 sujetos hombres: 20 con peso normal y diabetes tipo 2 (NW), 20 sujetos obesos con diabetes (OB) y 20 sujetos controles (C). Se evaluaron parámetros bioquímicos y de estrés oxidativo. Además se aislaron CMPs para la extracción de RNA total y se determinó la expresión de los genes TXNIP, IL-6, IL-1β y TNF-α mediante PCR de tiempo real cuantitativo.**

Resultados: **Los OB presentaron mayor IMC y circunferencia abdominal que los NW y los C (ANOVA de una vía, p<0.0001). Los NW tuvieron mayor glicemia en ayuna (ANOVA de una vía, p=0.0034) sin embargo, los OB presentaron mayor HbA1c (ANOVA de una vía, p<0.0001). Los OB además presentaron mayores nivel de PCRus (ANOVA de una vía, p=0.0158). Los TBARS y los AGES se observaron elevadas tanto en OB como en NW (ANOVA de una vía, p<0.0001 y p=0.0008, respectivamente). Comparado con los OB y C la expresión de TXNIP fue significativamente más alta en los NW (Kruskal Wallis, p=0.0074); la expresión de IL-1β, IL-6 y TNF-α se observó más elevada en los NW y OB (Kruskal Wallis, p<0.0001). En los NW la expresión de TXNIP se correlacionó positivamente con la glicemia en ayunas y con los AGES y negativamente con HOMA-β (r=0.72; r=0.59; r=-0.44, respectivamente, p<0.05), en los OB hubo correlación solamente con los 8-isoprostanos (r=0.42, p=0.046).**

Conclusiones: **Nuestros resultados sugieren que la alteración de la glicemia en ayunas, independiente de la adiposidad y del estado nutricional, representa un factor de riesgo para la disfunción de la célula beta, aumenta los marcadores de estrés oxidativo y se relaciona con el aumento de la expresión de TXNIP.**

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Palabras Claves: *TXNIP. Célula beta. Inflamación. AGEs. Control glicémico.*

Abreviations

TXNIP: Thioredoxin interacting protein.
IL-6: Interleukin-6.
IL-1β: Interleukin-1beta.
TNF-α: Tumor Necrosis Factor-alpha.
hs CRP: High sensitivity-C-reactive protein.
HOMA-β: Homeostasis model assessment-beta.

Introduction

Type-2 diabetes (T2D) is a chronic disease that is characterized by defects in insulin secretion, failure to suppress hepatic glucose output, and impaired glucose uptake in target tissues, such skeletal muscle and fat[1]. Hyperglycemia results in oxidative stress and inflammation and it is considered a risk factor for cardiovascular disease.

Thioredoxin interacting protein (TXNIP) is a pro-apoptotic and pro-oxidant protein, which is elevated in insulin resistance and diabetes. It has been demonstrated that glucose stimulates TXNIP transcription through a carbohydrate response element in the TXNIP promoter, resulting in elevated TXNIP mRNA expression[2]. TXNIP through a reactive oxygen species sensitive manner links NLRP3 which activates the inflammasome that allows the increased expression of IL-1β[3]. In turn this cytokine triggers the raising of IL-6 and TNF-α, these cytokines are recognized risk factors for insulin resistance and diabetes development, especially when accompanied by obesity[4]. Therefore, glucose may trigger a cycle between oxidative stress and inflammation induced by TXNIP and for the increasing of circulating cytokines.

Since it was discovered this top glucose-induced gene in a human pancreatic islet, is that TXNIP has emerged as a key player in beta cell biology[5]. The overexpression of TXNIP induces beta cell apoptosis, and is essential for glucotoxicity-induced beta cell death[2]. Even more, TXNIP has been implicated in important cellular process including redox state[6], apoptosis[7], inflammation 3 and endoplasmic reticulum stress[8].

TXNIP is found increased in type 2 diabetes, and its down-regulation is related to the protection in the development of type 2 and 1 diabetes[2, 9], but also in type 2 diabetes accompanied for obesity is found high levels of circulating glucose and fatty acids, nutrients that could exacerbate the expression of TXNIP and the inflammation markers.

Type 2 diabetes is more frequent in obese subjects; the obesity especially the abdominal obesity is characterized for a mild chronic inflammation with increased levels of markers such as: IL6, TNFα, IL-1β between others. The obesity, the inflammation and the lack of glycemic control could be in part, related to TXNIP.

Since there is no evidence that shows that TXNIP expression may be modified for nutritional status, in this study, we explore the relationship between the expression of TXNIP in PMCs from normal weight and obese type-2 diabetic patients, compared to healthy controls, with respect to β-cell functioning, oxidative, biochemical and inflammatory markers.

Methods

Study Subjects

We studied 20 diabetic subjects with normal nutritional status (NW); 20 obese with T2D (OB) and 20 controls (C). All participants were male and > 35 years. The inclusion criteria for T2D participants included: <10 years since diagnosis, not currently being treated with insulin, and BMI <27 kg/m^2 for NW and C and > 30 kg/m^2 for OB. Exclusion criteria included having any chronic disease or inflammatory condition other than T2D. The protocol was approved by the ethical committee of the Institute of Nutrition and Food Technology (INTA), and an informed consent was obtained from all participants.

Anthropometrics were measured in all patients (weight, height, and waist circumference). A blood sample (35 ml) was obtained after overnight fasting, of which 20 ml was used for biochemical determinations. Fasting glycemia was evaluated by glucose oxidase reaction (Dialab, Austria), HbA1c by turbidimetry, high-sensitivity C-reactive protein (hsCRP) by liquid-phase immunoprecipitation (Orion Diagnostica, Espoo, Finland), lipid profile by colorimetric method, insulin by radioinmmunoassay (Coat-A-Count Insulin Kit, Siemens, USA), advanced glycation end products (AGES) by ELISA (Oxiselect™, Cell Biolabs, USA), TBARS (OxiSelect™ TBARS Assay Kit, Cell Biolabs, San Diego, CA) and 8-Isoprostanes (ELISA kit, Cayman Chemical Com, Michigan, USA). The HOMA-β index was calculated according to Wallace et al.[10]

Peripheral Mononuclear Cells (PMCs) Isolation

Fifteen mls of the blood sample was collected with EDTA anticoagulant and processed within 1-2 hrs. PMCs were separated by Ficoll-Histopaque gradient sedimentation (density 1.119, Sigma, St. Louis, MO). The mononuclear layer was removed and washed twice with PBS, an aliquot was used for determination of heme oxygenase (HO) activity according to Arredondo et al.[11] and total RNA was extracted and stored at -80º C.

RNA from PMCs was isolated using Trizol Reagent according to manufacturer protocol (Invitrogen). RNA (1.5 μg) was reverse transcribed using the AffinityScript cDNA synthesis kit (Stratagene). qRT-PCR was performed using Fast SYBR®Green Master Mix (Applied Biosystems, USA) on StepOne equipment

(Applied Biosystem, USA). As housekeeping gene was used Beta-2 microglobulin (B2M). The studied genes were TXNIP, IL-6, IL-1β and TNF-α. The primer list is available upon request.

Statistical Analysis

Anthropometric, biochemical and oxidative stress parameters are presented as means ± SD. The differences were evaluated using one-way ANOVA and the the Dunnett post-hoc test. Gene expression is presented as geometric mean and range (±1SD), and differences were evaluated using Kruskal Wallis and Dunn's post-hoc tests. Relationships between TXNIP and anthropometry, biochemical and oxidative stress parameters were evaluated using Spearman correlation. Statistical significance was assigned as $p<0.05$. GraphPad Prism 6 (USA) was used for statistical analysis.

Results

As we expected OB had higher weight, BMI and abdominal circumference, compared to NW and C participants (Table I). However, NW subjects had higher fasting glycemia than OB and C, but OB had increased HbA1c than NW and also hsCRP (Table I). β-cell functioning, measured by HOMA-β, was significantly lower in NW, compared to OB and C, subjects (Table I).

Respect to the oxidative stress markers, TBARS and AGES were increased in both OB and NW, but HO activity was higher just in NW (Table I).

In the expression of genes, TXNIP was significantly higher in NW, compared to the other two groups (Table II). IL-1β, IL-6 and TNF-α expression were increased in both NW and OB, compared to C subjects, but IL6 and IL-1β were especially increased in OB (Table II).

Table I
Sample characteristics, Biochemical and Oxidative Stress Parameters

Parameters	*C n=20*	*NW n=20*	*OB n=20*	*p*
Age (years)	43.9±6.4	64.9±7.6	56.9±8.9	**<0.05**
Weight (Kg)	66.9±11.3	73.9±7.8	92.9±16.3***	**<0.0001**
BMI (kg/mt^2)	23.6±2.9	25.7±2.1	32.4±5.9***	**<0.0001**
Waist Circumference(cm)	84.4±6.7	95.6±5.9*	109.8±13.1***	**<0.0001**
SBP (mmHg)	128.4±16.7	132.8±20.4	137.8±12.7	NS
DBP (mmHg)	81.3±8.9	78.5±12.4	83.1±11.8	NS
Biochemichal Parameters				
Fasting Glycemia (mg/dl)	105.4±9.3	189.0±72.9***	156.0±63.2*	**0.0034**
Fasting Insulin (μUI/mL)[1]	11.2 (7.2-12.4)	21.6 (5.7-34.9)	14.4 (9.0-15.4)	NS
Homa-β[1]	101.3 (62.3-109.1)	66.1 (18.4-92.9)*	80.3 (37.6-81.2)	**0.0153**
Hb1Ac (%)[1]	0.9 (0.5-1.6)	2.4 (1.3-4.3)	5.3 (3.9-7.2)***	**<0.0001**
hsCRP[1]	2.1 (1.1-4.2)	2.3 (1.3-4.1)	5.6 (3.9-8.2)*	**0.0158**
Total Cholesterol (mg/dl)	200.6±37.9	197.6±53.9	195±59.9	NS
LDL (mg/dl)	136.3±3.1	122.4±37.7	115.0±42.0	NS
HDL (mg/dl)	35.0±10.3	35.3±7.6	34.0±7.7	NS
Tryglycerides (mg/dl)[1]	134.1 (109.2-164.8)	163.2 (115.3-231.1)	165.6 (116.0-236.5)	NS
Oxidative Stress Parameters				
HO[1] (nmole bilirubin/mg protein/h)	2.7 (2.1-3.1)	8.6 (4.7-15.7)**	5.3 (3.0-9.3)	**0.0024**
TBARS[1] (nmoles/mL)	1.0 (0.8-1.2)	1.6 (1.2-2.0)**	1.3 (1.2-1.4)**	**<0.0001**
AGES (μg/mL)	12.2±0.9	13.7±1.5**	13.3±1.3**	**0.0008**
8-isoprostanes (pg/mL)	6.6±1.4	6.8±1.6	6.9±2.5	NS

Results are expressed as mean ± SD; [1]Geometric mean (± 1SD).
One way ANOVA, post-hoc Dunnett's: *p<0.05, **p<0.01, ***p<0.001.
BMI: Body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: glycosylated hemoglobin; hsCRP: high-sensitive C-reactive protein; HO: Heme oxygenase; TBARS: Thiobarbituric acid reactive species; AGES: Advanced glycation end products.

Table II
Expression of TXNIP, IL-1β, IL-6 and TNF-α in PMCs from diabetic patients

Gen	*C* *n= 20*	*NW* *n=20*	*OB* *n= 20*	*p*
TXNIP	0.6 (0.4-1.1)	6.2 (0.6-6.6)***	3.3 (1.2-3.6)**	0.0074
IL-1β	0.7 (0.4-1.4)	7.2 (0.4-14.8)***	21.5 (2.3-45.4)***	<0.0001
IL-6	0.4 (0.4-1.4)	7.4 (6.2-21.3)*	34.9 (10.8-79.8)***	<0.0001
TNF-α	0.8 (0.3-2.6)	3.6 (1.1-8.4)*	6.2 (0.6-12.0)***	<0.0001

Results are expressed as geometric means (± 1SD).
Kruskal Wallis, post-hoc Dunn's: *p<0.05, **p<0.01, ***p<0.001.

TXNIP expression was positively correlated with fasting glycemia and AGES in NW patients (r=0.72; r= 0.59, respectively, both p<0.05), and negatively correlated with HOMA-β (r=-0.44, p= 0.0386). In OB subjects we found a positive correlation with 8-isprostanes (r= 0.42, p= 0.046) (Table III).

Table III
Correlations between TXNIP expression, anthropometric, biochemical and oxidative stress parameters

Parameters	*NW* *n=20*	*OB* *n=20*
Weight	r= 0.27 p= 0.147	r= -0.13 p= 0.275
BMI	r= 0.35 p= 0.086	r= -0.046 p= 0.4202
Abdominal Cir	r= 0.04 p= 0.4326	r= -0.12 p= 0.2939
Fasting glycemia	**r= 0.72 p= 0.006**	r= 0.13 p= 0.2713
Fasting insulin	r= -0.31 p= 0.1127	r= -0.062 p= 0.3913
HbA1c	r= -0.3 p= 0.121	r= 0.2742 p= 0.1145
hsPCR	r= -0.2889 p= 0.1304	r= 0.2621 p= 0.1193
HOMA-β	**r= -0.44 p= 0.0386**	r= -0.06 p= 0.3943
HO	r= 0.004 p= 0.4944	r= 0.3854 p= 0.0516
TBARS	r= 0.2218 p= 0.1961	r= 0.2120 p= 0.1718
AGES	**r= 0.59 p= 0.0067**	r= 0.2928 p= 0.0067
8-Isoprostanes	r= 0.2132 p= 0.2056	**r= 0.4240 p= 0.246**

Discussion

Hyperglycemia in diabetes is considered a major risk factor for the development of diabetes complications and for cardiovascular disease in absence of diabetes[12]. It has been recently discovered that TXNIP, under hyperglycemic environment, migrates from the nucleus to mitochondria where it activates apoptosis signaling in β-cells[13]. Thus, TXNIP overexpression in pancreatic β-cells in hyperglycemic conditions has profound effects in diabetes and its complications[13].

In this study, we evaluated the expression of TXNIP, inflammatory cytokines and their relationship with oxidative stress, biochemical, anthropometric, and inflammatory parameters. Our findings were unexpected. We observed that diabetic patients with normal nutritional status had less metabolic control of their fasting glycemia, had oxidative markers comparable to obese diabetic patients, and TXNIP expression was higher in these participants. HsCRP levels were comparable between NW and C, and the expression of IL-1β, TNF-α and IL-6 were lower compared to OB participants. In NW participants only, we found a significant correlation between TXNIP expression and AGES, fasting glycemia and HOMA-β. These results suggest that fasting glycemia is a better predictor of β-cell functionality than a chronic marker (i.e. HbA1c).

Also, we intended to elucidate if nutritional status had an impact over TXNIP expression. Our NW patients that had lesser adiposity (measured by abdominal circumference) than OB *but* they did not have less visceral adiposity than C subjects, and NW also had the highest expression of TXNIP, therefore our data also suggest that the expression of this gene is independent of the adiposity and nutritional status.

AGES are produced under hyperglycemic conditions, but are also produced by diet. AGES bind to specific cell surface receptors (RAGE) and lead to post-receptor signaling and further generation of

ROS[14]. AGES also may activate intracellular transcription factors such as NF-אB, which in turn could activate the inflammatory pathway through the increasing of pro-inflammatory cytokines[4]. Besides the formation of AGES, hyperglycemia also activates TXNIP, which is the endogenous inhibitor of thioredoxin protein—one of the major intracellular thiol-reducing mechanisms. Thioredoxin reduces ROS through reversible oxidation of thioredoxin at two cysteine residues[15]. The relationship found in this study with fasting hyperglycemia and the expression of TXNIP could be explained because intermittent high glucose is able to induce overproduction of ROS and both high glucose and ROS trigger the expression of TXNIP initiating an inflammatory pathway that can persist even after glycemia is normalized[12]. We could observe that NW patients had higher HO activity, which could suggest that this group had higher oxidative stress, thus explaining the elevated expression of TXNIP and alteration in fasting glycemia.

In this study, we showed that alteration in fasting glycemia is a risk factor for β-cell dysfunction and is related to the TXNIP expression, independent of adiposity and nutritional status. Findings could be explained, in part, by the oxidative stress in this group, but further study is necessary to replicate these findings in a bigger sample and to explain the differences found between obese and normal-weight patients.

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Statement of Authorship

Andrews M designed the study, performed the statistical analysis and interpreted the data.

Arredondo M and Olivares M participated in data interpretation and provided helpful comments about the manuscript.

Conflict of Interest

The authors declare do not have any conflict of interest.

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