Original Article

Paraoxonase-1, malondialdehyde and glutathione reductase in Type 2 Diabetic patients with nephropathy

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Abstract. Numerous experimental and epidemiologic studies shown that oxygen-free-radicals are elevated in uncontrolled diabetes mellitus (DM). Paraoxonase-1 (PON1) has been reported to confer antioxidant activities by decreasing the accumulation of lipid peroxidation products. This study was designed to investigate the role of PON1 activity, glutathione reductase (GR), and lipid peroxidation in patients with diabetic nephropathy (DN).

70 subjects were included in the study: 30 as a control, 20 with type 2 DM (T2DM) with nephropathy (DN), 20 T2DM without nephropathy. All studied groups are subjected to the following laboratory investigations after their consents: fasting and postprandial blood glucose, Serum triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL) and low density lipoprotein (LDL), HbA1c, blood urea, serum creatinine, urine microalbumin, serum PON1 activity, serum malondialdehyde (MDA), and serum GR activity.

Fasting and postprandial blood glucose levels, and HbA1c was significantly higher in the DN group than the diabetic or control group (p=0.0001). Serum creatinine show a significant rise in the DN group than diabetic or control group (p=0.0001). Microalbuminuria show a significant rise in the DN group than the control or diabetic group as well as in the diabetic group than control group (p=0.0001). Serum PON1 activity and GR level showed significant decrease in diabetic patients with or without nephropathy compared to the control group with a significant decrease in its activity in the DN group while there was a significant a rise of MDA in diabetic groups than control with a significant rise in DN.

The decreased antioxidant enzyme activities in DN patients, suggesting that oxidative stress may contribute to the development of DN and other microvascular complications beside chronic hyperglycemia and dyslipidemia. Measurement of PNO1, MDA and GR levels may be a helpful marker in the diagnosis and follow up DN.

Key words: Paraoxinase-1, malonaldehyde, glutathione reductase, type 2 diabetes, diabetic nephropathy, oxidative stress

1. Introduction

T2DM accounts for over 90 percent of patients with diabetes (1). Worldwide, the prevalence of T2DM is estimated at 6.4 percent in adults, rates of undetected diabetes may be as high as 50 percent in some areas (2). Because of the associated microvascular and macrovascular disease, diabetes accounts for almost 14% of health care expenditures, at least one-half of which are related to complications such as myocardial infarction, stroke, end-stage renal disease, retinopathy, and foot ulcers (3).

DN is a major microvascular complication of diabetes, representing the leading cause of end-stage renal disease in the western world, and a major cause of morbidity and mortality in diabetic subjects. Clinical hallmarks of DN include a progressive increase in urinary albumin excretion and a decline in glomerular filtration rate (GFR), which occur in association with an increase in blood pressure. DN seems to result from the interaction between genetic susceptibility and environmental insults, primarily metabolic and hemodynamic in origin. It has been determined that both metabolic and hemodynamic stimuli lead to the activation of

key intracellular signaling pathways and transcription factors, thus triggering the production/release of cytokines, chemokines, and growth factors, which mediate and/or amplify renal damage (4).

There is increasing evidence that reactive oxygen species (ROS) play a major role in the development of diabetic complications. Oxidative stress is increased in diabetes. High glucose upregulates transforming growth factor-b1 (TGF-b1) and angiotensin II (Ang II) in renal cells. ROS mediate high glucose-induced activation of protein kinase C and nuclear factor-JB in renal cells. Oxidative stress leads to protein, lipid, and DNA modifications that cause cellular dysfunction and contribute to the pathogenesis of DN (4). A combination of strategies to prevent overproduction of ROS, to increase the removal of preformed ROS, and to block ROS-induced activation of biochemical pathways leading to cellular damage may prove to be effective in preventing the development and progression of chronic kidney diseases (CKD) in diabetes (5).

PON1 is a calcium-dependent esterase closely bound to the apolipoprotein A1- containing HDLfraction in the plasma (6) which has been reported to confer antioxidant activities by decreasing the accumulation of lipid peroxidation products (7). PON1 is capable of hydrolyzing numerous substrates, for example paraoxon, phenyl acetate, lipid peroxides, cholesterol esters, and hydroperoxides. Low activity of PON1 could contribute to vascular dysfunction and diabetic complications (8). MDA is one of the most abundant carbonyl products of lipid peroxidation whose formation is accelerated by oxidative stress (9). GR, is an essential enzyme with the antioxidative system that protects cells against free-radicals (10). GR reduces oxidized glutathione to the reduced sulfhydryl form that is an essential cellular antioxidant (11).

In light of these data, this study was designed to investigate the role of PON1 activity, GR, and lipid peroxidation in patients with DN.

2. Subjects and methods

The current study was performed on 40 T2DM patients selected from the Internal Medicine Department, Tanta University Hospital classified into two groups: Group I: twenty patients with T2DM with nephropathy (11 female, 9 male). The diagnostic criteria of diabetic nephropathy were depending on clinical examination and detection of microalbumin or macroalbuminuria in urine samples of the patients. Microalbuminuria is defined as an albumin creatinine ratio (ACR)

between 30-300 mg/g. Macroalbuminuria is defined as an ACR > 300 mg/g. 2 of 3 samples should fall within the microalbuminuric or the macroalbuminuric range to confirm classification (12). Group II: Twenty patients with T2DM without nephropathy (10 female, 10 male). Thirty healthy persons (16 female, 14 male) of matched age and sex were included in the study as a control group. All patients and controls are aged from 40 - 63 years.

2.1. Exclusion criteria

Patients have causes that lead to transient elevations in urinary albumin excretion for example: urinary tract infections, heart failure, acute febrile illness and thyroid disease.

After their consent, all subjects were subjected to:

1. Full history taking with particular emphasis on age, duration of DM and treatment, history of any systemic diseases, e.g., Hypertension, dyslipidemia or history of any associated diseases and drug intake.

2. Thorough clinical examination with special stress on systolic and diastolic blood pressure (SBP & DBP).

3. Blood sampling and analysis: after overnight fasting, blood samples were collected from a vein in the antecubital fossa. All collections were made between 8:00 and 9:00 am. The samples were immediately centrifuged (3000 xg, 10 min) for serum separation and stored at -20°C for assay of:

   a. Fasting and two hours postprandial blood glucose, according to glucose - oxidase method using kits from Diamond Diagnostic.

   b. Glycated hemoglobin A1c was measured using column chromatography method by commercial Kit from Biosystem diagnostic.

   c. Serum TG, TC was estimated according to (GPO-POD) method using kits from Spinreact company.

   d. Serum HDL-Cholesterol Level was estimated by "enzymatic colorimetric method using kits from Spinreact. Serum LDL-Cholesterol Level (LDL) was calculated using Friedewald's formula if the triglycerides were less than 4.5 mmol/l, as following: LDL-cholesterol = total cholesterol–HDL-cholesterol–triglycerides/5.

   e. Serum urea level and serum creatinine level were estimated by "urea enzymatic kit" and "creatinine kinetic kit"

respectively, using kits from diamond diagnostics.

f. Microalbumin in urine was estimated using a human microalbumin ELIZA kit from Elabscience product ID E-EL-H0115, detection range 3.125~200 ug/mL and sensitivity of 1.876 ug/mL.

PON1 activity toward paraoxon "(O, O-diethyl-O-p-nitrophenyl phosphate, Sigma) was determined by measuring the initial rate of substrate hydrolysis to p-nitrophenol, whose observance was monitored in the assay mixture (950μl containing 1mM Paraoxon, 1 mm CaCl2, 100 mm Tris/HCl buffer, pH 8.0, incubated at 37°C for 3 minutes). 50μl serum was added to this mixture and the change in absorbance at 405nm was immediately recorded spectrophotometrically. The enzyme activity was calculated from the molar extinction coefficient (ε405) of p-nitrophenol (18053 M-1·cm-1), and expressed in U/mL; where 1 U of enzyme hydrolyzes 1 nmol of paraoxon/ minute" (13).

Malondialdehyde is formed as an end product of lipid peroxidation. It was measured following the method of Kalghatgi et al. (14) using Abcam's Lipid Peroxidation (MDA) Assay Kit (ab118970). The MDA in the sample reacts with Thiobarbituric Acid (TBA) to generate the MDA-TBA adduct. The MDA-TBA adduct can be easily quantified colorimetrically (λ = 532 nm). This assay detects MDA levels as low as 1 nmol/well.

Glutathione reductase measured using the commercial Kit from RANDOX laboratories. GR catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by NADPH. The specterophotometric assay for glutathione reductase is based on observing the decrease in absorbance at 340nm caused by the conversion of NADPH to NADP+. The enzyme activity was expressed in U/mL; where one unit/mL/min is equivalent to the oxidation of 1 (mole of NADPH in 1 min at 37 °C.

2.2. Statistics

Values were expressed as mean ±SD. The statistical analysis of the results was performed based on the conventional standard statistical procedures using computed statistical analysis by SPSS, version 22.0 for Microsoft windows 7. All variables were tested for normality of distribution. Paired-samples t test was applied to compare between parametric values; Pearson's correlation with correlation coefficient was applied for parametric results. The significant difference was considered at p<0.05.

3. Results

Regarding the clinical characteristics, there was no significant difference between all the studied groups regarding age and sex. There was a significant increase in blood pressure in DN group compared to diabetic patients without nephropathy (group II). There is a positive correlation between duration of diabetes and DN (group II) (Table 1). There was a significant increase in TC, TG, and LDL levels in diabetic groups compared to the control group with more increase in the DN group than the non nephropathy one (p=0.0001). There was a significant decrease in HDL level in diabetic groups compared to control group (p=0.0001, p=0.0001) respectively, with more decrease in its level in DN group than the non nephropathy one (p=0.0001). Fasting and postprandial blood sugar and HbA1c were significantly higher in the diabetic groups compared to the control (p=0.0001). Both serum creatinine and microalbuminuria showed significant increase in DN group than the diabetic group or the control and in the diabetic group than control group (p=0.0001 for all) (Table 2).

There was a significant decrease in PON1 activity in diabetic groups compared to control (p=0.001, p=0.0001) respectively, with a significant decrease in DN patients than patients without DN (p=0.0001) (Table 3). Our study revealed a negative correlation between PON1 activity and MDA, TC, TG, LDL, creatinine and microalbumin levels in diabetic groups with a positive correlation between PON1 activity and both GR and HDLlevels (Table 4). There was a significant increase in MDA level in diabetic groups compared to control (p=0.0001, p=0.0001)

Table 1. Comparison between the different studied groups for their clinical characteristics

	C (N=25)	DM (N=25)	DN (N=30)	p
Age (years)	50.43±9.00	53.80±6.81	54.75±12.92	>0.05
Gender Male/female	16 / 14	12 / 13	11 / 14	>0.05
Blood pressure (Normal / hypertensive)	------	15 / 5	7 / 13	<0.05
Duration of diabetes (years)	------	7.55±4.84	12.00±6.88	<0.05

Table 2. Comparison between the different studied groups as regard chemical parameters

	C		DM		DN		P
	M	SD	M	SD	M	SD	
TC (mg/dl)	148.10	10.41	197.05	9.99	261.77	18.68	0.0001
TG (mg/dl)	86.90	5.86	178.10	10.61	241.89	16.91	0.0001
HDL-C (mg/dl)	51.10	3.27	43.95	2.50	25.30	2.95	0.0001
LDL-C (mg/dl)	79.89	10.86	117.48	10.67	188.54	18.18	0.0001
FBS (mg/dl)	91.30	9.79	154.65	14.05	287.20	16.88	0.0001
PPBS (mg/dl)	113.75	9.81	201.65	16.74	345.65	25.86	0.0001
HbA1c %	5.33	0.91	7.33	0.50	11.77	1.21	0.0001
Creatinine (mg/dl)	0.59	0.09	0.67	0.16	2.79	0.52	0.0001
microalbuminurea (mg/dl)	5.99	1.32	11.87	1.98	126.56	21.46	0.0001

Table 3. Shows a comparison between the different studied groups as regard levels of PON1, MDA and GR in different studied groups

	C		DM		DN		P
	M	SD	M	SD	M	SD	
PON1 (U/mL)	49.13	4.26	42.15	1.98	24.12	2.77	0.0001
MDA (nmol/mL)	2.20	0.38	6.63	0.75	11.88	2.09	0.0001
GR (U/mL)	9.89	0.91	5.21	0.64	3.32	0.43	0.0001

Table 4. Correlation of PON1 activities with the different parameters in the studied groups

Parameter	P		
	PON1	MDA	GR
TC	-821**	0.851**	-0.767**
TG	-0.813**	0.843**	-0.765**
HDL	0.990 **	-0.967**	0.914**
LDL	-0.863**	0.889**	-0.821**
Creatinine	-0.962**	0.979**	-0.884**
Microalbuminurea	- 0.910**	0.962**	-0.865**
GR	0.965**	-0.903**	-------
MDA	-0.962**	-------	-0.903**

**indicates high significance
*indicates significance

respectively, with significant increase in its level in a DN group than the non nephropathy group (p=0.0001) (Table 3). There was a positive correlation between MDA levels and TC, TG, LDL, creatinine, and microalbumin in diabetic groups with a negative correlation between MDA levels and HDL. While there was a negative correlation between MDA and GR in patients with DN with no correlation in patients without nephropathy (Table 4). There was a significant decrease in GR levels in diabetic groups compared to control (p= 0.0001, p=0.0001) respectively, with significant increase in its level in a non nephropathy group than the DN group (p=0.0001) (Table 3). There was a negative

correlation between GR levels and TC, TG, LDL-c creatinine, microalbumin and MDA level in diabetic groups with a positive correlation was found between GR levels and HDL-c (Table 4).

4. Discussion

DN is the most common cause of progressive renal damage and end stage renal failure in patients with DM. While the exact cause of DN remains unknown, oxidative stress coupled with chronic hyperglycemia may have an important role in the pathogenesis of glomerular and tubular functional and structural abnormalities. Both serum creatinine and microalbuminuria show significant increase in DN group than the diabetic or the control groups and in the diabetic group than the control group. Fasting and postprandial blood sugar and HbA1c were significantly higher in the DN group and the diabetic group compared to the control group (15). There was a significant increase in TC, TG, and LDL levels in diabetic patients with or without DN compared to control group with more increase in the DN group. A significant decrease in HDL level was found in diabetic patients compared to control group, with more decrease in its level in the DN group than the non nephropathy one.

Rosario RF and Prabhakar S. (2006), (16) said that, although several factors may mediate the development and progression of DN, hyperlipidemia is now considered an independent and major determinant of progression of renal disease in diabetes. One of the major risk factors

for development and progression of DN is dyslipidemia. Lipids may induce both glomerular and tubulo-interstitial injury through mediators such as cytokines, ROS and through hemodynamic changes (17). A number of studies reported that increase in TG and LDL has also been linked to increased oxidative stress (18). In the present study, we observed high levels of TG, LDL and low levels of HDL in DN compared to controls. Oxidative degradation of lipids is referred as a lipidperoxidation and one of the most abundant carbonyl products of lipidperoxidation is MDA, whose formation is accelerated by oxidative stress (9). Lipid peroxidation also induces endothelial damage and inflammatory response, impairs vasodilatation and activates macrophages (19, 20).

The current study showed increases in MDA level in diabetic patients than control, and there was an increase in its level in a DN group than the non nephropathy group. Maha et al, (2012) (21) concludes that DM is associated with excessive production of ROS, which can damage cellular macromolecules. The percent of DNA damage of peripheral blood mononuclear cells was higher in diabetic patients compared to healthy controls with a significant positive correlation of DNA damage with fasting blood glucose and glycated hemoglobin, but not with serum TC, TG, HDL and LDL and poor glycemic control may aggravate this damage. Dyslipidemia is not a contributing factor in DNA damage in diabetes.

PON1 may directly act on lipid peroxides, or even more likely, lipid peroxides are first transferred to HDL after which destroyed by PON1 (22). The present study show decrease in PON1 activity in diabetic patients compared to control group with more decrease in patient with DN. This result in agreeing with other investigators (23). Some clinical data indicate that low PON1 activity contributes to the development of microvascular complications, (24) other do not (25). These conflicting results may be explained by the effect of several factors as sex, age, and PON1 polymorphisms, on PON1 activity. The present study shows positive correlation between PON1 and HDL in patients with and without DN. Mastorikou et al. (2008), (26) founded that isolated HDL from patients with T2DM showed dramatically lower PON1 activity after *in vitro* nonenzymatic glycation versus HDL that was not treated in this process.

Other researchers reported that low activity of PON1 in the diabetic group suggests that HDL could show functional deficiency in T2DM patients, despite high HDL concentration (27).

There is a negative correlation between PON1 activity and TC in diabetic patients DN. This has coincided with Aksoy et al, 2009 (28) and Younis et al, 2013; (29) who found a positive correlation between PON1 activity and TC as well as LDL. There was a significant decrease in GR levels in diabetic patients compared to control with an increase in its level in a non nephropathy group than nephropathy group. Other studies revealed that there had been significant variations in serum GR concentration and highly significant variations in GR activity between hypercholesteremic patients and T2DM patients with hypercholestermia (30). Sailaja et al in 2003 (31) reported an increase in lipid peroxidation and decrease in levels of reduced glutathione, GR, and glutathione peroxidase activities in diabetic humans. Krishan and Chakkarwar (2011), (15) said that, timely and judicious use of recent therapies to maintain good glycemic control, adequate lipid levels and blood pressure, along with lifestyle measures such as regular exercise, optimization of diet and smoking cessation, may help to reduce oxidative stress and endothelial cell dysfunction and slow the progression of diabetic nephropathy.

5. Conclusion

The decreased antioxidant enzyme activities in DN subjects, suggesting that oxidative stress may contribute to the development of DN beside chronic hyperglycemia and dyslipidemia. Measurement of PNO1, MDA and GR levels may be a helpful marker in the diagnosis and follow up of DN. Supplementing the antioxidant therapy seems to be promising in preventing the induction and progression of DN.

Wide scale retrospective study on different stages of DN is recommended. Further researches are strongly recommended to examine genetic polymorphism distribution in large population to accomplish a concise overview that may explain variations in PON1 activity and its particular relationship with the additional factors that associate DM as well as its complications.

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