Association of glomerular and tubular dysfunction with glycaemic control, lipid, lipoprotein, apolipoprotein and antioxidant status in type 2 diabetes mellitus

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ABSTRACT

Introduction: This study was conducted to investigate the relationship of glomerular and tubular dysfunctions with glycaemic control, lipid, lipoprotein, apolipoproteins and antioxidant status in 72 patients with type 2 diabetes mellitus.

Methods: Urine albumin concentration was measured by immunoturbidimetric and urine N-acetyl-beta-D-glucosaminidase (NAG) and alanine aminopeptidase (AAP) activities with colorimetric methods. Glycated haemoglobin was measured using affinity chromatography. Erythrocyte glutathione reductase and glutathione peroxidase activities and serum levels of malondialdehyde, lipids, lipoproteins and apolipoproteins were determined in patients with type 2 diabetes mellitus.

Results: In univariate regression, urinary albumin excretion, and activities of NAG and AAP were associated with glycaemic control. These glycaemic factors included serum glucose concentrations and glycated haemoglobin. Urinary albumin excretion was also inversely correlated with erythrocyte glutathione peroxidase activity, and positively correlated with erythrocyte glutathione reductase activity. No significant associations were found with serum levels of insulin, lipids, lipoproteins, apolipoproteins, malondialdehyde or blood pressure. In multivariate regression, glycated haemoglobin was the most significant predictor of urinary albumin concentration and with erythrocyte glutathione reductase, whereas only glycated haemoglobin was the independent predictor of tubular dysfunctions. Erythrocyte glutathione peroxidase was not an independent predictor of urinary albumin excretion, after adjusting for glycated haemoglobin, glutathione reductase, systolic blood pressure, diastolic blood pressure and apolipoprotein B.

Conclusion: In type 2 diabetes mellitus, both glomerular and tubular dysfunctions are dependent on glycaemic control. Glomerular, but not tubular, dysfunction is also significantly associated with increased glutathione reductase activity.

Keywords: antioxidants, diabetes mellitus, diabetic nephropathy, glomerular dysfunction, tubular dysfunction

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INTRODUCTION

Diabetic nephropathy is a serious complication of diabetes mellitus, and a major cause of mortality and morbidity in these patients.[1,2] High concentrations of glucose and glycolytic intermediates, which are substrates for biochemical pathways, lead to oxidative stress. This stress can lead to the modification of protein and lipid structure and function through glycosidation and peroxidation.[3] Oxidative stress is hypothesised to be a common pathogenesis of late diabetic complications,[3] including nephropathy.[4] Studies in animal models and in humans have demonstrated that diabetes mellitus is associated with oxidative stress which leads to a significant increase in both plasma and tissue lipid peroxidation byproducts[5-8] and reduced levels of antioxidant capacity.[3,9] These modified products could contribute to the morphological and functional abnormalities seen in the kidneys of patients with diabetes mellitus.[6,7,10]

The role of antioxidant enzymes in diabetic renal disease has not been well described. Erythrocyte enzymes have been reported to be changed in type 2 diabetes mellitus,[11] but the effect on nephropathy is

unclear. The relationship between antioxidant vitamins, including vitamins C and E, and renal dysfunction have been conflicting.[11-13] Despite a growing amount of data which show the role of hyperlipidaemia in the aetiology of diabetic nephropathy, there are some contradictory results. A few studies in patients with type 2 diabetes mellitus have suggested an independent role of serum cholesterol concentrations in the subsequent development of incipient or overt diabetic nephropathy.[14,15] The associations between glomerular and tubular dysfunctions, and glycaemic and lipidaemic control, with antioxidant status, have not been adequately investigated. A study of these associations is important because these variables may mediate the effects of diabetes on nephropathic risk factors. The primary aim of this study was therefore to investigate the strength and independence of the associations between urinary albumin excretion, as an indicator of early glomerular dysfunction, and urinary activity of N-acetyl-β-D-glucosaminidase (NAG) and alanine aminopeptidase (AAP), as indicators of tubular dysfunctions, and glycaemic control and lipid, lipoprotein, apolipoprotein and antioxidant status in patients with type 2 diabetes mellitus.

METHODS

A cross-sectional study was conducted on 72 patients who have suffered from type 2 diabetes mellitus (35 males and 37 females), aged 30–70 years for at least one year. The patients were recruited from the Institute of Endocrinology and Metabolism. Data on dietary habits, body mass index (BMI), medical history, smoking addiction, medication and dietary supplements were obtained from careful personal interviews. All subjects had to meet the following criteria to be included in the study: non-comsumption of vitamin and/or mineral supplements, thyroid hormones, oestrogens, progesterone, diuretics, lipid- and hypertension-lowering agents, having normal hepatic tests, no history of myocardial infarction, no proteinuria (> 300 mg albumin per g creatinine [Cr]), and in females, not being pregnant. Written informed consent was obtained from all participants. The research protocol was approved by the Ethics Committee of the Tehran University of Medical Sciences.

After 12–14 hours of overnight fasting, from 8 pm to 10 am, and before taking any oral hypoglycaemic agent(s), 20 ml of blood and early morning spot urine samples were collected from each subject. Aliquots of serum and urine were transferred to polystyrene tubes which were then immediately stored at -70°C until analysis. Urinary albumin excretion concentration was measured by immunoturbidimetric assay using commercial kits (Randox, Antrim, UK). Urinary activities of NAG was determined by enzymatic hydrolysis of p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma, MO, USA) at pH 4.4,[16] and AAP by enzymatic hydrolysis of alanine-4-nitroanilide (Sigma, MO, USA) at pH 7.8.[17] Urine Cr was measured using the Jaffe reaction,[18] and all urine results were expressed in relation to gramme Cr excretion. Serum malondialdehyde was determined using a colorimetric method based on thiobarbituric acid reactivity.[19] Erythrocyte glutathione reductase and glutathione peroxidase activities were estimated on haemolysates[20,21] and expressed in relation to gramme haemoglobin.

Fasting serum glucose (FSG) was measured enzymatically. Serum fructosamine concentration was determined by commercial kits (Randox, Antrim, UK). Glycated haemoglobin (Hb_{A1c}) was measured by affinity chromatography method using commercial kits (Sigma, MO, USA). Serum insulin was measured by immunoradiometric assay kits (IMMUOTECH, Prague, Czech Republic). Insulin resistance was estimated using the homeostasis model assessment (HOMA) score.[22] Serum triglyceride and total cholesterol were measured enzymatically. High density lipoprotein cholesterol (HDL-c) was determined after precipitation with phosphotangestate/magnesium, while low density lipoprotein cholesterol (LDL-c) after precipitation with heparin/sodium citrate (Randox, Antrim, UK). Apolipoproteins (apo) A1 and B were measured by the immunoturbidimetric method (Boehringer Mannheim, Mannheim, Germany). Intra- and interassay coefficient variation was less than 5% for all laboratory tests. The systolic and diastolic blood pressure (SBP and DBP) (five minutes seated rest, mean of two readings) were measured using a standard mercury sphygmomanometer. Nutrient intake was estimated using two 24-hour dietary recall questionnaires and analysed using a Food Processor software.

All analysis used the Statistical Package for Social Sciences version 11.5 (SPSS Inc, Chicago, IL, USA). The data were expressed as mean ± standard deviation (SD). Some variables are expressed as geometric means (95% confidence intervals [CI]) due to their non-normal distribution. Skewed data were log-transformed for analysis. Associations were examined by Pearson univariate and by multiple linear regression methods. Variables were compared between patients with low and high levels of microalbuminuria by the Student *t*-test. Statistical significance was defined at the 5% level.

RESULTS

Patient characteristics are summarised in Table I. 22% of patients had microalbuminuria, which is defined as having a urine albumin excretion range of 30–300 mg/g Cr. The lipid, lipoprotein, apolipoprotein and antioxidant levels in patients with and without microalbuminuria are shown in Table II. Compared with patients with normal albuminuria, those with microalbuminuria had

Table I. Characteristics of the 72 patients with type 2 diabetes mellitus.

Characteristics	Mean ± SD
Age (years)	50 ± 9
Gender (male:female)	35:37
BMI (kg/m^2)	28 ± 4.3
Duration of diabetes mellitus (years)	8.5 ± 5.1
SBP (mmHg)	126 ± 16
DBP (mmHg)	81 ± 10
Fasting serum glucose (mg/dL)	179 ± 51
Serum fructosamine (μmol/L)	449 ± 120
Hb_{A1c} (%)	10.0 ± 2.6
Cholesterol (mg/dL)	187 ± 38
Triglycerides (mg/dL)	183 (158–208)
HDL-c (mg/dL)	39 ± 11
LDL-c (mg/dL)	117 ± 31
ApoA1 (mg/dL)	147 ± 24
ApoB (mg/dL)	139 ± 31
Urinary albumin excretion (mg/g Cr)	31.4 (19.7–43.1)
NAG (U/g Cr)	18 (13.6–22.4)
AAP (U/g Cr)	5.6 (4.5–6.8)
SBP > 130 mmHg (%)	45
DBP > 85 mmHg (%)	27

significantly elevated urine activity of NAG and AAP ($p < 0.05$), erythrocyte glutathione reductase activity and Hb_{A1c} ($p < 0.05$), and significantly lower erythrocyte glutathione peroxidase activity ($p < 0.05$). However, there were no significant group differences in serum lipid, lipoprotein, apolipoprotein levels, and duration of diabetes. SBP and DBP were higher, but were not statistically significant.

The univariate correlations of FSG, fructosamine, Hb_{A1c}, insulin and HOMA score with urinary albumin excretion and urinary activity of NAG and AAP are shown in Table III. Urinary albumin excretion was significantly correlated with FSG and Hb_{A1c}. There were no significant associations with fructosamine, insulin or HOMA score. There were significant associations between glycaemic control and NAG and AAP activities. No correlation was found between urinary activity of NAG or AAP and serum insulin concentration or insulin resistance.

The association between lipids, lipoproteins, apolipoproteins, urinary albumin excretion, and urinary activity of NAG and AAP are shown in Table III. There was no significant correlation between lipids, lipoproteins, apolipoproteins, and glomerular and tubular dysfunctions. In women, there were significant positive associations between apo B, triglycerides and urinary albumin excretion ($r = 0.326$, $p < 0.05$ and $r = 0.339$, $p < 0.05$, respectively). We did not see these correlations in men.

The Pearson correlation coefficients between blood pressure and urinary albumin excretion, NAG and AAP activities are shown in Table III. Urinary albumin excretion and activity of NAG and AAP were not significantly correlated with SBP or DBP. The correlations between urinary albumin excretion, NAG and AAP, and oxidant and antioxidant status are shown in Table III. There was a significant inverse correlation between urinary

Table II. Mean values for nephropathy and glycaemic indices, serum lipid, lipoprotein, apolipoprotein and antioxidant status of normoalbuminuria (urinary albumin excretion < 30 mg/g Cr) and microalbuminuria (urinary albumin excretion 30–300 mg/g Cr) in patients with type 2 diabetes mellitus.

	Normoalbuminuria (n = 56)	Microalbuminuria (n = 16)	p-value
Urinary albumin excretion (mg/g Cr)	9.6 (7.5–11.6)	107.8 (76–140)	< 0.0001
NAG (U/g Cr)	14.8 (12.8–16.8)	29.1 (10.2–48.0)	< 0.05
AAP (U/g Cr)	5.2 (4.0–6.4)	7.3 (3.7–10.9)	< 0.05
Hb_{A1c} (%)	9.7 ± 2.7	11.1 ± 2.0	< 0.05
Cholesterol (mg/dL)	188 ± 38	186 ± 38	NS
Triglycerides (mg/dL)	181 (154–208)	191 (126–255)	NS
HDL-c (mg/dL)	38.4 ± 10.6	40.6 ± 13.6	NS
LDL-c (mg/dL)	116 ± 34	121 ± 37	NS
ApoA1 (mg/dL)	146 ± 24	148 ± 25	NS
ApoB (mg/dL)	138 ± 29	146 ± 39	NS
SBP (mmHg)	125 ± 16	130 ± 15	NS
DBP (mmHg)	80 ± 10	84 ± 10	NS
Glutathione reductase (U/g Hb)	5.7 ± 1.0	6.4 ± 1.3	< 0.05
Glutathione peroxidase (U/g Hb)	55 ± 15	47 ± 10	< 0.05
Malondialdehyde (nmol/ml)	1.40 ± 0.55	1.49 ± 0.53	NS
Duration of diabetes mellitus (years)	8.4 ± 5.3	9.0 ± 4.7	NS

NS: not significant

Table III. Associations (Pearson correlation coefficients) between glycaemic and lipidaemic indices, blood pressure and antioxidant status, with urinary albumin excretion, NAG, and AAP in 72 patients with type 2 diabetes mellitus.

	Urinary albumin excretion		NAG		AAP	
	r	p-value	r	p-value	r	p-value
FSG	0.335	< 0.005	0.414	< 0.001	0.347	< 0.005
Fructosamine	0.204	0.089	0.462	< 0.001	0.345	< 0.005
Hb_{A1c}	0.286	< 0.05	0.424	< 0.001	0.375	< 0.001
Insulin	-0.104	0.385	-0.021	0.860	-0.180	0.131
HOMA score	0.054	0.650	0.201	0.091	0.024	0.844
Total cholesterol	-0.001	0.994	0.095	0.426	-0.036	0.765
Triglycerides	0.177	0.094	0.077	0.522	-0.059	0.622
HDL-c	0.038	0.754	0.179	0.140	0.076	0.525
LDL-c	0.017	0.889	0.011	0.924	-0.008	0.946
Apo A-I	0.085	0.480	0.004	0.971	0.028	0.813
Apo B	0.039	0.746	0.053	0.660	-0.079	0.307
SBP	0.060	0.615	-0.023	0.846	0.136	0.253
DBP	0.151	0.207	-0.045	0.711	0.105	0.378
Glutathione reductase	0.279	< 0.05	-0.038	0.751	0.047	0.615
Glutathione peroxidase	-0.259	< 0.05	-0.067	0.578	-0.016	0.897
Malondialdehyde	-0.040	0.736	0.016	0.893	-0.036	0.763

albumin excretion and erythrocyte glutathione peroxidase activity, as well as a significant positive correlation with erythrocyte glutathione reductase activity. Urine activity of NAG or AAP were not significantly correlated with any variables, in particular, erythrocyte glutathione peroxidase and glutathione reductase activity. In multiple regression analysis, Hb_{A1c} and erythrocyte glutathione reductase activity were significantly correlated with urinary albumin excretion, after adjusting for erythrocyte glutathione peroxidase activity, triglyceride, BMI, age and duration of diabetes mellitus (Table IV). Hb_{A1c} was the best predictor of urinary activity of NAG and AAP.

DISCUSSION

The present study examined the association of glomerular and tubular dysfunction with glycaemic control, lipid, lipoprotein and antioxidant status in patients with type 2 diabetes mellitus. A novel finding in the present study was that in these patients, glycaemic control was the best predicted by urine albumin concentration and urinary activity of NAG and AAP. We also found that high urine levels of albumin were closely associated with erythrocyte glutathione reductase activity (positive) and with erythrocyte glutathione peroxidase activity (negative). In addition, in the group with microalbuminuria, glutathione reductase activity was strongly elevated and glutathione peroxidase activity was suppressed. Tubular dysfunction was not related to erythrocyte activity of glutathione reductase or glutathione peroxidase.

Recent studies have shown that several risk factors are associated with the presence of microalbuminuria in patients with type 2 diabetes mellitus;[23-26] however, all are based on chronic hyperglycaemia.[25-28] Intensive blood-glucose control (Hb_{A1c} 7.0% compared with 7.9%) decreases the risk of microvascular complications in patients with type 2 diabetes mellitus.[27] In type 2 diabetes mellitus, there is a significant association between index of metabolic control and albuminuria[28] as well as urinary NAG activity.[29] Taking these reports and our results into consideration, glycaemic control may be one of the most important risk factors for the progression of nephropathy in patients with type 2 diabetes mellitus. It is hypothesised that glucose-dependent processes are involved in diabetic nephropathy. The effects of glucose may occur through the generation of advanced glycated proteins. These substances accumulate in the kidneys and cause a decline in renal functions.[30] Another glucose-dependent pathway is polyol producing sorbitol via aldose reductase. The presence of sorbitol in any given organ or cell is a problem. The kidneys have several different types of cells containing aldose reductase. Sorbitol causes a fluid imbalance within these cells and this disruption of cellular osmoregulation might alter cell function and cause organ impairment and failure.[31]

Oxidative stress is hypothesised to play an important role in the development of late diabetic complications. Chronic hyperglycaemia increases oxidative stress, and modifies the structure and function of proteins and lipids considerably through glycosidation and peroxidation.[3] It has also been reported that antioxidant capacity

Table IV. Multiple linear regression models showing association between predictor variables (glycated haemoglobin, glutathione reductase, glutathione peroxidase, triglyceride, BMI, age and duration of diabetes) and (a) urinary albumin excretion, (b) NAG and (c) AAP.

(a) log urinary albumin excretion

Predictor variables	Regression coefficient	SEM	p-value
Hb_{A1c}	**0.068**	**0.028**	**0.014**
Glutathione reductase	**0.013**	**0.007**	**0.045**
Glutathione peroxidase	-0.007	0.005	0.154
Log triglyceride	0.121	0.327	0.712
BMI	0.016	0.117	0.352
Age	0.004	0.008	0.625
Duration of diabetes mellitus	-0.017	0.014	0.247

(b) log NAG

Predictor variables	Regression coefficient	SEM	p-value
Hb_{A1c}	**0.047**	**0.013**	**0.0001**
Glutathione reductase	-0.001	0.003	0.792
Glutathione peroxidase	0.002	0.002	0.305
Log triglyceride	0.018	0.147	0.904
BMI	-0.004	0.008	0.599
Age	-0.002	0.004	0.613
Duration of diabetes mellitus	-0.002	0.007	0.772

(c) log AAP

Predictor variables	Regression coefficient	SEM	p-value
Hb_{A1c}	**0.050**	**0.016**	**0.003**
Glutathione reductase	0.003	0.004	0.411
Glutathione peroxidase	0.001	0.003	0.643
Log triglyceride	-0.280	0.190	0.144
BMI	-0.003	0.010	0.764
Age	0.000	0.005	0.926
Duration of diabetes mellitus	0.012	0.008	0.163

is decreased in patients with diabetes mellitus.[9] In particular, patients with complicated diabetes mellitus have been shown to have elevated levels of peroxidation products in plasma and erythrocytes.[32] Erythrocyte glutathione peroxidase activity was significantly reduced and glutathione reductase activity enhanced in the group of patients with microalbuminuria, suggesting a putative role of these enzymes in the pathogenesis of early renal damage in type 2 diabetes mellitus. Glutathione peroxidase detoxifies hydrogen peroxide and converts lipid hydroperoxides to nontoxic alcohols.[33] Decreased activity of this antioxidant enzyme may reflect its known sensitivity to radical-induced inactivation.[34,35] As there is no significant association between glutathione peroxidase activity and microalbuminuria after adjusting for Hb_{A1c}, one cannot exclude the possibility that a decrease in activity of the antioxidant system in diabetic patients may be linked to progressive glycation of enzymatic proteins. The increment of glutathione reductase activity reported in other studies,[36,37] may be due to an increased recycling of oxidised glutathione. Further studies are required to examine the role of glutathione reductase in glomerular dysfunction. However, oxidative stress, which plays an important role in the pathogenesis of gomerular damage in this study, has not played an important part in the tubular functions.

Although animal studies have demonstrated a significant deleterious role for hyperlipidaemia in the initiation and progression of renal injury,[38,39] data remain more conflicting in humans. In patients with type 2 diabetes mellitus, the relationship between lipid and lipoprotein concentrations and nephropathy have been reported.[14,15,40,41] Fagot-Campagna et al found that HDL-c was a predictor for abnormal excretion of albumin in women, but not in men.[42] Clinical trials have suggested a beneficial effect of hyperlipidaemic

treatment for microalbuminuria in these patients.[43] In agreement with our results, a number of other studies have failed to demonstrate any independent effect of any serum lipid parameters on either the evaluation of microalbuminuria or the decline in renal function.[15,28] In our present study, lipids, lipoproteins and apolipoproteins were either not significantly associated with glomerular and tubular damage or, in the case of triglyceride in women, significant associations were not independent of glycaemic control.

Despite a growing amount of data which show the role of hypertension in the aetiology of nephropathy, there are some contradictory results. Elevated blood pressure is not necessarily associated with the prevalence of microalbuminuria and when followed longitudinally, it did not predict later progression to overt proteinuria.[28] However, since high levels of blood pressure was part of the exclusion criteria, we could not address the relationship between glomerular and tubular dysfunction and hypertension. Our study is limited by its cross sectional designs. Definitive evidence of the role of glutathione reductase and glutathione peroxidase in the pathogenesis of early renal damage in type 2 diabetes mellitus will require further investigation. While we reported significant associations in the present study, a large proportion of the variation in urinary albumin excretion and NAG and AAP activity remained unexplained. We attempted to account for the variation in nutrient intake and exercise, but this was based on historical details. Our estimate of urine excretion of albumin and activities of NAG and AAP were based on gramme Cr excretion. Hourly collections of urine over a duration of 24 hours would be required for exact excretion determination.

Several risk factors which have recently been suggested to have an effect on renal functions include hyperglycaemia, hyperlipidaemia, hypertension and oxidative stress. Our study suggests that among these variables, glycaemic control significantly and independently regulates glomerular and tubular functions. Our results also indicate the importance of erythrocyte glutathione reductase and glutathione peroxidase activity as a determinant of microalbuminuria.

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