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Research Article

Plasma proteins production and excretion in diabetic nephropathy in type II diabetic patients

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ABSTRACT

Diabetic nephropathy is the leading cause of chronic renal disease and a major cause of cardiovascular mortality in both developed and developing countries. In type II diabetes patients with normoalbuminuria, fibrinogen production is increased, whereas that of albumin is normal. It is not known whether hepatic albumin production in albuminuric type II diabetes patients is also increased, and whether fibrinogen production is further increased in these patients. Knowledge of these potential relationships is important to understand both the mechanistic associations between albuminuria and hyperfibrinogenaemia. Therefore, this study was designed to measure fibrinogen and albumin concentrations in patients with type II diabetes who had normal or increased urinary albumin excretion. Subjects, materials, and methods: Plasma albumin, and fibrinogen concentrations and urinary albumin are measured in macroalbuminuric diabetic patients (n=16), microalbuminuric diabetic patients (n=16), and healthy controls (n=8).Results: A direct relationship was found between albuminuria and albumin concentration ($r=0.59$, $p<0.05$). Direct relationship also found between albuminuria and fibrinogen concentration ($r=0.65$, $p<0.002$), and fibrinogen pool ($r=0.66$, $p<0.002$). Conclusions: this study demonstrates that albumin and fibrinogen levels are increased in macroalbuminuric type II diabetes subjects compared with type II diabetes patients with microalbuminuria and healthy subjects , showing an upregulation of hepatic secretory proteins in this clinical condition. Such an upregulation seems to be responsible for the relative hyperfibrinogenaemia observed in the albuminuric diabetic patients.

INTRODUCTION

Diabetic nephropathy is the leading cause of chronic renal disease and a major cause of cardiovascular mortality in both developed and developing countries (Bruno and Gross, 2000), (Valmadrid *et al*.,2000). Hyperfibrinogenaemia and albuminuria are established cardiovascular risk factors in diabetic and in non-diabetic populations (Ganda and Arkin, 1992), (Fuller *et al*., 1979). Hyperfibrinogenaemia is common in type II diabetes, and is often associated with albuminuria (Jain *et al*., 2001), (Festa *et al*., 2002). Production of albumin and fibrinogen is increased in non-diabetic nephrotic syndromes (, suggesting coordinate changes in hepatic protein production in response to albuminuria (De Sain *et al*., 1998), (Zanetti *et al*., 2001). In type II diabetes patients with normoalbuminuria, fibrinogen production is increased, whereas that of albumin is normal (Barazzoni *et al*., 2000), (Tessari *et al*., 2006). It is not known whether hepatic albumin production in albuminuric type II diabetes patients is also increased, and whether fibrinogen production is further increased in these patients. Knowledge of these potential relationships is

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important to understand both the mechanistic associations between albuminuria and hyperfibrinogenaemia. Therefore, this study was designed to measure fibrinogen and albumin synthesis in patients with type II diabetes who had normal or increased urinary albumin excretion.

SUBJECTS, AND METHODS

Subjects

Thirty two male subjects with type II diabetes were included in this study (disease duration >3 years) from patients attending the Diabetes Centre at Suez General Hospital. Sixteen patients had a low urinary Albumin/cretinine ratio ACR (<300 mg/g; Alb−), whereas the remaining sixteen had an increased ACR (>300 mg/g; Alb+), based on two morning fresh void urine collections. The albuminuric patients were slightly older and had a slightly greater BMI, than the non-albuminuric patients, although the differences were not significant Table 1. Diabetes duration was, however, longer in the patients with albuminuria. All subjects had been adapted for 1 month to a standard weight-maintaining diet containing ≈50% of calories as carbohydrates, ≈20% as proteins and ≈30% as lipids in order to reduce the effect of diet on plasma proteins production. Daily protein intake was unrestricted and was at least > 1g/kg body weight in both patients' groups. The hypoglycaemic therapy comprised diet only in two Alb− subject, diet plus oral hypoglycaemic agents (glyburide or gliclazide) in ten Alb− and in eight Alb+ subjects, oral hypoglycaemic agents plus insulin in 4 Alb− and 2 Alb+ subjects, and split insulin doses in the remaining subjects. The patients' metabolic control was poor, as shown by the elevated HbA1c concentration (Table 1), and it was not different in the two groups. Plasma creatinine concentration was normal in the Alb− patients, whereas it was moderately increased in the Alb+ patients (Table 1). Seven Alb− and all the Alb+ patients were on anti-hypertensive therapy, with combinations of angiotensin-converting-enzyme inhibitors, diuretics, anti-adrenergic agents and/or calcium antagonists. All drugs were suspended the night before the study day. No patient had clinical signs of either oedema or pleural or abdominal liquid effusion. Background retinopathy and peripheral vascular insufficiency were found in three and four, respectively, of the Alb−, and in six and five, respectively, of the Alb+ patients. No Alb− subject had any clinical or biochemical evidence of ongoing inflammatory disease, as shown by normal leucocyte counts, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP). In the Alb+ patients, the ESR was elevated in eleven subjects and CRP was mildly elevated in two subjects. No subject was a current smoker or had being smoking for at least 6 months prior to the study. Control studies were done in eight healthy male subjects Control subjects also recorded their food intake for 3 days and collected urine and fasting blood samples. Urine was analyzed for urea, creatinine, protein, albumin, and fibrinogen. All patients and volunteers agreed to participate after signing an informed consent form, in accordance with the Helsinki Declaration of Human Rights. The procedures have been approved by the local ethics committee.

Methods

Biochemical measurements

After ten hour fasting, Urine and intravenous blood samples are collected. Plasma insulin, glucagon, C-peptide and fibrinogen concentration were measured according to (Jacques Wallach: Interpretation of Diagnostic Tests 2000). Plasma glucose, albumin, triglyceride, total and HDL cholesterol, and creatinine concentrations were determined by automatic methods using a COBAS Mira Auto Analyzer (Roche Italia, Milan, Italy) and specific reagents (Dade-Behring, Marburg, Germany). Plasma protein profiles were determined with standard electrophoresis. Circulating blood cells and the erythrocyte sedimentation rate were determined by photometrical capillary stopped-flow kinetic analysis (Test1TH; Alifax, Padova, Italy). C-reactive protein was measured by nephelometry on a BN2 analyzer (Dade-Behring) (a non–high-sensitive assay). Glycated hemoglobin (HbA1C) was measured and a value of less than 7% was taken to indicate good glycemic control.

Albuminuria assessment

The "gold standard" to assess albuminuria is 24-hour urinary albumin excretion (UAE). Because 24-hour urine collection is cumbersome, American society of nephrology guidelines suggest measuring albuminuria in a first morning void, either as urinary albumin concentration (UAC) or adjusted for creatinine concentration, the albumin:creatinine ratio (ACR) (Hiddo *et al*., 2008),(Holly *et al*., 2003). The albumin creatinine ratio is done to compare the amount of albumin that is passing into the urine from the kidneys compared to the amount of creatinine present. Although one can always check for albumin levels in the urine by doing a simple test, the advantage of calculating the albumin creatinine ratio is that this ratio remains unaffected by any kind of variation in the concentration in urine. The normal ratio of albumin to creatinine is seen to be around less than 30 mg/g of creatinine. In men, the level is seen to be less than or equal to 17

mg/g of creatinine. When there is a high albumin creatinine ratio, that is, around 30 - 300 mg/g of creatinine, then it is known as microalbuminuria. This means that there is a small amount of albumin in the urine, which indicates that the kidneys may be slightly diseased. If the ratio of albumin to creatinine is more than 300 mg/g of creatinine, then the condition is known as macroalbuminuria, which means, there is a large amount of albumin in the urine.

Table 1
Clinical and biochemical characteristics of the type II diabetic patients with a lower (Alb−) or an increased (Alb+) urinary albumin excretion

	Alb^- type II diabetes (n=16)	Alb^+ type II diabetes (n=16)
Age (years)	51±3	56±3
BMI (kg/m^2)	29.0±1.5	32.5±1.4
Duration of disease (years)	9±2	16±3[b]
HbA_{1c} (%)	9.4±0.5	10.5±1.5
Fasting glucose (mg/dl)	130±10	165±9[a]
Insulin (nmol/l)	100±7	110±8
Glucagon (pg/ml)	139±12	158±24
C-peptide (ng/ml)	1.8±0.14	3.23±0.62[a]
Erythrocyte sedimentation rate (mm)	9.9±2.8	56.4±13.4[b]
Urinary albumin excretion rate (mg/24 h)	14.11±2.7	33.40±1.25[b]
C-reactive protein (ng/ml)	3.32±0.02	4.56±0.55
Creatinine (µmol/l)	76±3	145±25[a]
Albumin (g/l)	41±1	44±2[a]
Albumin pool (g)	142±4	130±8
Fibrinogen (g/l)	3.65±0.27	4.86±0.41[a]
Fibrinogen pool (g)	11.2±0.8	17.1±1.2[b]
Total cholesterol (mmol/l)	5.37±0.28	5.38±0.28
HDL cholesterol (mmol/l)	1.13±0.08	0.95±0.08
Triglycerides (mmol/l)	1.55±0.25	1.32±0.17

[a] $p<0.05$; [b] $p<0.02$ or less, Alb^+ vs Alb^- type II diabetes

RESULTS

The patients with albuminuria had greater concentrations of plasma glucose, albumin, fibrinogen, C-peptide, creatinine and fibrinogen pool than the patients without albuminuria (Table 1).

The patients with albuminuria had greater concentrations of total plasma proteins, albumin, and fibrinogen compared to both patients with lower albuminuria and healthy control subjects (Table 2).

The patients with albuminuria had greater loss of albumin in urine, greater albumin concentration in urine and higher albumin/creatinine ratio compared to both lower albuminuric and healthy control subjects (Table 3).

A direct relationship was found between albuminuria and albumin concentration ($r=0.59$, $p<0.05$). Direct relationship also found between albuminuria and fibrinogen concentration ($r=0.65$, $p<0.002$), and fibrinogen pool ($r=0.66$, $p<0.002$).

Table 2
Biochemical parameters in plasma from diabetic patients and control subjects

	Alb− type II diabetes (n=16)	**Alb+ type II diabetes (n=16)**	**Control subjects (n=8)**
Total protein g/L	71.11±1.4	74.31±0.5[a,b]	67.4±1.5
Albumin g/L	41±1	44±2[a,b]	36.2±0.5
Fibrinogen g/L	3.65±0.27	4.86±0.41[a,b]	3.0±0.2

[a]Statistically significant difference vs the Alb^- type 2 diabetic patients ($p<0.05$ or less), whereas [b]denotes a statistically significant difference vs the healthy control subjects ($p<0.05$ or less), by one-way ANOVA.

Table 3
Biochemical parameters in urine from patients and control subjects

	Alb− type II diabetes (n=16)	**Alb+ type II diabetes (n=16)**	**Control subjects (n=8)**
24-hour urine Albumin (µg/kg/24 h)\	211.21±2.68	471.40±3.25[a,b]	9.25±2.4
Albumin concentration (mg/l)	10.16±1.5	23.75±2.4[a,b]	5.21±1.25
Albumin/ creatinine ratio (mg/g)	49±2.3	356.97±0.8[a,b]	8.2±1.25

[a]Statistically significant difference vs the Alb^- type 2 diabetic patients ($p<0.05$ or less), whereas [b]denotes a statistically significant difference vs the healthy control subjects ($p<0.05$ or less), by one-way ANOVA.

DISCUSSION

Albuminuria is a marker of renal damage and a hallmark of progression to renal insufficiency (Parving *et al*., 1992). It increases cardiovascular risk in type 2 diabetes mellitus, probably because it reflects widespread increased vascular permeability causing organ damage (Parving *et al*., 1996), (Nannipieri *et al*., 1995). Albuminuria and hyperfibrinogenaemia, another cardiovascular risk factor, are frequently associated in diabetes (Jain *et al*., 2001), (Festa *et al*., 2002). Such an association is important in that fibrinogen, an acute-phase protein, is a powerful and independent cardiovascular risk factor (Ganda and Arkin, 1992),

(Fuller *et al*., 1979) . However, the mechanism(s) of the association between hyperfibrinogenaemia and albuminuria, as well as the response of hepatic albumin synthesis to albuminuria in type 2 diabetes, are not known.

In non-diabetic nephrotic syndromes albuminuria is associated with an upregulation of albumin synthesis, probably mediated by the decreased oncotic pressure at the hepatic level, which counteracts the increased urinary albumin loss. In these conditions, fibrinogen synthesis is also increased (De Sain *et al*., 1998), (Zanetti *et al*., 2001) suggesting an upregulation of hepatic secretory proteins. Thus, a link between albuminuria and the altered fibrinogen metabolism can be suspected also in type II diabetes, possibly at the site of liver production. However, whether these mechanisms are operating in type II diabetes as well is not known.

In this study we show that both albumin and fibrinogen productions are greater in type II diabetic patients with albuminuria than in patients with a lower urinary excretion rate. Positive correlations between the degree of albuminuria and both albumin and fibrinogen plasma levels have been found. These observations suggest that upregulation of hepatic protein synthesis, probably in response to the increased urinary albumin loss, operates in type II diabetes with nephropathy. Albuminuria was also directly correlated with fibrinogen concentrations and the circulating pool of fibrinogen. These data indicate that albuminuria in type II diabetes may represent a trigger for increased albumin production, as well as for a further increase of fibrinogen concentrations and production. This may be mediated by decreased oncotic pressure caused by albuminuria.

That fibrinogen synthesis is increased in type II diabetes, even in the absence of micro- or macroalbuminuria, has been previously demonstrated (Barazzoni *et al*., 2001), (Barazzoni *et al*., 2003). The present study showed that a further increase in fibrinogen concentration occurs when type II diabetes patients are also albuminuric (Table 1). On the whole, the additional cardiovascular risk associated to albuminuria may be, at least partly, the result of the increased fibrinogen concentration of these patients.

The mechanism(s) possibly associated with the increased fibrinogen production in type II diabetes have been previously discussed (Barazzoni *et al*., 2001), (Tessari *et al*., 2006) and may include insulin resistance, hyperglucagonaemia, increased fibrinogen degradation products acting as stimulators of fibrinogen production in the liver, and possibly, also a subclinical inflammatory state otherwise not detectable by standard assays. In our albuminuric type II diabetes patients, the increased ESR, which is a common finding in albuminuria (Liverman *et al*., 1988), may indicate the occurrence of a mild inflammatory state, despite the normality of other biochemical (leucocytic count, urine analysis) and clinical indices of inflammation, with the exception of a mild increase of CRP. Therefore, in addition to the previously mentioned factors, a subclinical inflammatory condition, with the associated expected changes in inflammatory cytokines (that were not measured in this study), may represent a stimulus towards increased fibrinogen production. On the other hand, that inflammation cannot be the only cause of the observed metabolic increase in hepatic protein synthesis is supported by the fact that albumin is a negative acute-phase protein (Gordon, 1976), therefore its synthesis should be depressed, and not increased, by inflammation, which is in contrast with our present findings. Since diabetes duration was greater in the albuminuric patients than in the non-albuminuric patients, disease duration could represent an additional variable in the observed findings.

Albumin synthesis is physiologically stimulated by insulin and amino acids (Doweiko and Nompleggi, 1991), (De Feo *et al*., 1991). Although insulin concentration was similar in both groups, the increased C-peptide concentration, an index of increased endogenous insulin secretion, observed in the albuminuric subjects could constitute a factor contributing to their increased albumin synthesis.

Age difference between the two groups may have a confounding role on albuminuria-associated increased albumin and fibrinogen production in type II diabetes.

The albuminuric diabetic patients had a moderate increase of creatinine concentration (Table 1). Impaired kidney function in these patients may theoretically affect plasma protein production in the albuminuric type II diabetes group.

The fact that all the albuminuric patients were receiving hypotensive drugs, as opposed to a lower number of subjects being treated in the normoalbuminuric group, might have affected the results because fibrinogen concentration can be reduced by angiotensin-converting-enzyme inhibitor (Fogari *et al.*, 1998). However, because the fibrinogen concentration was greater in the albuminuric patients despite their drug therapy, their fibrinogen levels might have been even greater than those observed here.

In conclusion, this study demonstrates that albumin and fibrinogen levels are increased in macroalbuminuric type II diabetes subjects compared with type II diabetes patients with microalbuminuria and healthy subjects , showing an upregulation of hepatic secretory proteins in this clinical condition. Such an upregulation seems to be responsible for the

relative hyperfibrinogenaemia observed in the albuminuric diabetic patients. Albuminuria, through as yet unknown mechanisms, could thus represent a key factor. This study casts new light on the pathophysiological mechanisms of the association between albuminuria and hyperfibrinogenaemia in type II diabetes.

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