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Retinol Binding Protien-4 Levels in Early Diabetic Nephropathy in Egyptian Patients with Type 2 Diabetes

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Abstract: Plasma retinol-binding protein 4 (RBP4) is a new adipokine linked to obesity-induced insulin resistance and type 2 diabetes. The impact of diabetic nephropathy on serum RBP4 levels, however, is not well known. This study aimed to investigate the relationships between serum levels of RBP4 and various metabolic parameters in Egyptian patients with T_2DM. Additionally, the present study tested the hypothesis that microalbuminuria is associated with elevated serum concentrations of RBP4 in type 2 diabetic subjects. In a case control study, a total number of 60 type 2 diabetic patients and 20 healthy subjects were enrolled in the present study. The participants were matched by age and sex. Diabetic patients were classified into normoalbuminuria and microalbuminuria subgroups according to the microalbumin level in urine. Serum RBP4 and micoalbumin were measured by an enzyme-linked immunosorbent assay (ELISA).The results demonstrated statistically significant elevation in serum RBP4 concentrations in T2DM subjects compared to control subjects, also serum RBP-4 levels in males were significantly higher in T2DM patients compared with male control group, as well as in females T2DM patients compared with female control group. In T_2DM patients, serum concentration of RBP4 was positively correlated with age, fasting blood sugar, post prandial blood sugar and HbA1c and inversely correlated with female gender. There were no significant correlations between RBP4 and BMI, GFR, cholesterol, triglyceride, HDL, LDL, urea, creatinine, creatinine clearance and uric acid. The serum RBP4 levels in micromacroalbuminuric patients were significantly higher than in normoalbuminuric patients and the control subjects. It could be concluded that serum RBP4 levels were found to be elevated in subjects with T2DM and to be related to various clinical parameters known to be associated with T2DM, so levels of serum RBP4 might play important role in the pathogenesis of T_2DM. Also, the present study suggests that serum RBP4 levels in type 2 diabetic patients are affected by incipient nephropathy.

Key words: Type 2 diabetes mellitus · Retinol bending protein-4 · Microalbuminuria

INTRODUCTION

RBP4 is a lypocalin protein transporting retinol in the circulation. It is produced in the liver and also in mature lipid-laden adipocytes [1, 2]. In the small intestine, vitamin A (retinol) is absorbed, stored in liver and secreted into circulation bound to serum retinol binding protein (RBP4). Circulating retinol may be taken up by extrahepatic tissues or recycled back to liver multiple times before it is finally metabolized or degraded [3]. Following the secretion of retinol-RBP (holo-RBP4), RBP forms complexes with another plasma protein, transthyretin (TTR). RBP4 circulates in a complex with TTR, which maintains the serum RBP4 levels by preventing loss of the smaller protein (21 kDa) from the circulation by filtration in the renal glomeruli [4]. The receptors STRA6 on the surface of the target cells bind to complex with high affinity and mediates vitamin A uptake into cells [5]. After delivery of vitamin A to the target cells, the RBP molecule loses its affinity for TTR. The small RBP molecule (apo-RBP) is filtered through the glomeruli and is subsequently reabsorbed and degraded by the proximal tubular cells [6]. The regulation of RBP-4 is unclear, however RBPR2 was recently identified as a high affinity RBP4 receptor

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expressed primarily in liver and small intestine and induced in adipocytes of obese mice [7]. Many studies showed that RBP4 is decreased with hypocaloric diet [8] as well as weight loss achieved by lifestyle [9] or weight loss achieved by Singapore bariatric surgery [10, 11]. The importance role of RBP4 in the vitamin A metabolism is reflected in the homeostatic regulation of plasma retinol (vitamin A), which guarantees supply a constant and continuous amount of vitamin A to peripheral tissues. Several pathophysiological conditions might be the cause of substantial fluctuations of plasma RBP4. Reduced levels of plasma RBP4 was due to impaired synthesis and/or secretion of RBP4. One of a serious microvascular complication in type 2 diabetic patients was nephropathy [12]. The renal function is decreased at the beginning of diabetic nephropathy which characterized by slight glomerular dysfunction, which is linked closely to elevated urinary albumin excretion in the range of microalbuminuria [13]. The impact of nephropathy on the plasma RBP4 in type 2 diabetic subjects is of specific relevance and needs to be addressed in the discussion on the importance of RBP4 as an adipokine and its role in the pathogenesis of type 2 diabetes mellitus. We therefore investigated if the presence of incipient nephropathy might contribute to elevated levels of RBP4 in type 2 diabetic patients. For this, we studied the combined effect of both type 2 diabetes mellitus and microalbuminuria on serum RBP4, as well as, we investigated relationships between serum levels of RBP4 and various metabolic parameters in Egyptian patients with T_2DM

MATERIALS AND METHODS

The study included (60 patients with type 2 diabetes mellitus, 28 males and 32 females) were recruited from the Diabetic Department at Kasr El-Aini Hospital, Cairo University, Egypt. T_2DM was diagnosed if the fasting plasma glucose (FPG) was =126 mg/dl (7.0 mM/L) and/or the patients were receiving treatment for T2DM [14]. T2DM patients were on diet and metformin or sulfonylurea. Patients with known liver disease and those currently treated with Thiazolidinediones were excluded from study. Healthy adults matched for age and BMI were chosen as control group, which included 20 subjects, 7 males and 13 females. Informed consent was obtained from all participants before the study. Detailed history, physical examination and laboratory testing were carried out for all participants. BMI were calculated for each participant by weight in kg divided by the square of the height in meter. Ten ml of venous blood was collected after 14 h overnight fast. Small portion of each blood sample was collected in K2EDTA for or determination of glycosylated hemoglobin. Another aliquot was collected as whole blood in sodium fluoride for plasma separation to be used for the immediate assay of glucose. A third aliquot was allowed to clot. The separated serum was used for the immediate assay of urea, creatinine, uric acid and lipid profile by hitachi 911 (Kits supplied from Roch, USA). The last aliquot was allowed to clot and stored at -20°C for subsequent estimation of RBP4 by ELISA (Abnova, USA) [15]. Twenty four hour urine samples were collected for creatinine clearance and microalbumin measurement. The volume of the collected urine was determined and part volume of urine was centrifugated at 3000g for ten minutes. Part of the urine was used for the immediate assay of creatinine in urine. The rest part volume of urine was divided into aliquots and stored at 20 °c for subsequent estimation of microalbumin in urine by ELISA (NEQAS, UK) [16]. T_2DM patients were classified into two subgroups based on their microalbuminuria according to criteria from American Diabetes Association.

Statistical Analysis: The statistical package for social science (SPSS) program version 16.0 was used for analysis. Results are reported as mean ± SD. One way ANOVA was performed for analysis of more than 2 variables followed by post hoc test for detection of significance. Simple linear correlation (Pearson's correlation) was used for quantitative data. Qualitative data were presented as frequencies (n) and percentages (%). Chi-square (x^2) test was used for comparisons between the three groups. Comparisons of quantitative data were done using the Student's t test for data with normal distribution and nonparametric tests (Mann–Whitney test) for data not assuming normal distribution as in case of microalbuminuria.

RESULTS

Demographic data and clinical parameters of studies groups (mean ± SD) are shown in Table 1. T_2DM patients had significantly higher mean values of systolic blood pressure, diastolic blood pressure, fasting blood sugar, 2h post prandial blood sugar, triglycerides and HbA1c compared with the control subjects. Mean urine creatinine and creatinine clearance levels were significantly lower in T_2DM patients. Mean values of serum RBP-4 levels were significantly higher in T_2DM patients compared with the controls, also mean values of serum RBP-4 levels in males

Table 1: Demographic data and clinical parameters of studies groups.

Variables	Diabetics (n = 60) Mean ± SD	Control (n = 20) Mean ± SD	*P*-value
Age (years)	51.3 ± 8.8	50.05 ± 4.34	0.560
BMI (kg/m^2)	31 ± 5.3	29 ± 4.5	0.139
Systolic Blood pressure (mmHg)	133.1 ± 16.9	121 ± 14.5	0.005*
Diastolic Blood pressure (mmHg)	85.8 ± 9.1	79.5 ± 8.9	0.008*
G.F.R. (ml/min/1.73cm$^{2)}$	653.6 ± 254.9	737.4 ± 153.5	0.170
Cholesterol (mg/dl)	195.9 ± 39.2	181.2 ± 20.8	0.114
T.G. (mg/dl)	184.6 ± 111.7	133.2 ± 61.2	0.049*
HDL (mg/dl)	42.3 ± 6.8	45.6 ± 10.1	0.098
LDL (mg/dl)	117.6 ± 33	106 ± 32.8	0.185
Urine volume (ml)	1418.3 ± 568	1197.4 ± 388.4	0.084
Urine Creatinine (mg/dl)	75.9 ± 38.5	100.4 ± 44	0.021*
Creatinine clearance (m/min)	82.4 ± 39.1	99.6 ± 26.3	0.021*
F.B.S (mg/dl)	196.4 ± 89.9	90.2 ± 14.5	<0.001*
2h.B.S (mg/dl)	272.2 ± 105.1	109.5 ± 12.5	<0.001*
Urea (mg/dl)	30.6 ± 12	28.3 ± 6.7	0.405
Creatinine (mg/dl)	0.91 ± 0.3	0.83 ± 0.1	0.267
Uric acid (mg/dl)	5.06 ± 1.5	5.14 ± 1.4	0.839
HbA1c %	7.57 ±1.59	3.52 ±0.27	0.0001*
RBP4 (ng/ml)	53.22 ±7.57	40.35 ± 5.02	0.0001*
RBP4 (ng/ml) male	57.18±7.58	43.27±4.51	0.0001*
female	49.76±5.69	38.77±4.77	0.0001*

**: Significant at P= 0.05*, BMI; body mass index, G.F.R.; Glomerular filtration rate, T.G.; triglyceride, HDL; High density lipoprotein, LDL; Low density lipoprotein, F.B.S; fast blood sugar, 2h.B.S; post prandial blood sugar, HbA1c; Glycated hemoglobin, RBP4; retinol binding protein.

Table 2: Comparisons between T_2DM patients and controls according to albuminuria status (Normoalbuminuria and Microalbuminuria).

Variables	Diabetics (n = 60) Normoalbuminuria (n = 49) Mean ± SD	Diabetics (n = 60) Microalbuminuria (n = 11) Mean ± SD	Control (n = 20) Mean ± SD	*P*-value
Age (years)	51.9 ± 8	51.9 ± 12	50.05 ± 4.34	0.807
BMI (kg/m^2)	31.2 ± 5.5	30.2 ± 4.6	29 ± 4.5	0.281
Systolic blood pressure (mmHg)	132.8 ± 14 ^{a}	134.6 ± 27 ^{a}	121 ± 14.5 ^{b}	0.020*
Diastolic blood pressure (mmHg)	86.1 ± 8.4 ^{a}	84.6 ± 12.1 ^{a}	79.5 ± 8.9 ^{b}	0.028*
G.F.R. (ml/min/1.73cm^2)	685 ± 239.5 ^{b}	513.9 ± 285.8 ^{c}	737.4 ± 153.5 ^{a}	0.034*
Cholesterol (mg/dl)	200 ± 37.5 ^{a}	177.4 ± 42.8 ^{b}	181.2 ± 20.8 ^{b}	0.046*
T.G. (mg/dl)	189.8 ± 118.1	161.6 ± 76.8	133.2 ± 61.2	0.125
HDL (mg/dl)	42.2 ± 6.7	42.5 ± 7.2	45.6 ± 10.1	0.256
LDL (mg/dl)	121 ± 31.8	102.4 ± 35.3	106 ± 32.8	0.099
Urine volume (ml)	1454 ± 574.8	1259.1 ± 532.6	1197.4 ± 388.4	0.121
Urine creatinine (mg/dl)	75.7 ± 37.2	77.1 ± 46.2	100.4 ± 44	0.071
Creatinine clearance (m/min)	85.6 ± 39.2 ^{a}	68.1 ± 37.1 ^{b}	99.6 ± 26.3 ^{a}	0.021*
F.B.S (mg/dl)	194.4 ± 92.4 ^{a}	205.2 ± 81.3 ^{a}	90.2 ± 14.5 ^{b}	<0.001*
2h.B.S (mg/dl)	267.1 ± 101.3 ^{a}	295.2 ± 123.4 ^{a}	109.5 ± 12.5 ^{b}	<0.001*
Urea (mg/dl)	29.6 ± 8.7	35.4 ± 21.3	28.3 ± 6.7	0.198
Microalbuminuria (μg/ml)	8.2 ± 5.5 ^{b}	67.1 ± 21.8 ^{a}	9.2 ± 4 ^{b}	<0.001*
Creatinine (mg/dl)	0.87 ± 0.2	1.1 ± 0.6	0.83 ± 0.1	0.076
Uric acid (mg/dl)	5.13 ± 1.5	4.74 ± 1.7	5.14 ± 1.4	0.727
HbA1c %	7.64±1.6^a	7.3±1.38^a	3.52 ±0.27^b	0.0001*
RBP4 (ng/dl)	51.33 ±6.46^b	61.64 ± 6.49^c	40.35 ± 5.02^a	0.0001*

*: Significant at P = 0.05, Means with different letters are statistically significantly different

Table 3: Results of correlation between RBP4 and different variables in diabetics and control groups

	Diabetic patients (n = 60)	
Variables	Correlation coefficient (r)	*P*-value
Age (years)	0.281	0.030*
Gender [a]	-0.435	0.0001*
BMI (kg/m^2)	0.106	0.350
Systolic blood pressure (mmHg)	0.191	0.09
Diastolic blood pressure (mmHg)	0.180	0.110
G.F.R. (ml/min/1.73cm^2)	-0.152	0.179
Cholesterol (mg/dl)	0.043	0.708
T.G. (mg/dl)	0.220	0.050
HDL (mg/dl)	-0.149	0.189
LDL (mg/dl)	-0.005	0.961
Fasting blood glucose (mg/dl)	0.346	0.002*
2h. blood glucose (mg/dl)	0.389	0.0001*
Creatinine clearance (mg/dl)	-0.044	0.7
Urea (mg/dl)	0.103	0.363
Creatinine (mg/dl)	0.167	0.138
Uric acid (mg/dl)	-0.073	0.518
HbA1c %	0.464	0.0001*

**: Significant at P = 0.05,* [a] scored as 1 for male and 2 for female in the analysis.

were significantly higher in T_2DM patients compared with male control group, as well as, in females T_2DM patients compared with female control group. Moreover, mean values of serum RBP-4 in males were significantly higher than females in control and T_2DM patients. No significant differences were found between T_2DM and controls in age, BMI, G.F.R., cholesterol, urea, creatinine and uric acid. Diabetic patients were subdivided into 2 subgroups according to their albuminuria levels as normoalbuminuric or macroalbuminuric. The clinical variables of the normoalbuminuric, macroalbuminuric and control subjects have been displayed in Table 2. No significant differences between three groups in age, BMI, cholesterol, urea, creatinine and uric acid.

The serum RBP4 levels in micromacroalbuminuric patients were significantly higher than in normoalbuminuric patients and the control group, while systolic blood pressure, diastolic blood pressure, fasting blood sugar, 2h post prandial blood sugar and HbA1c in normoalbuminuric and micromacroalbuminuric patients were significantly higher than in the control group. Mean G.F.R in control group showed the statistically significant highest. This was followed by normoalbuminuric group, while microalbuminuric group showed the statistically significant lowest mean G.F.R. There was no statistically significant difference between mean creatinine clearance in normoalbuminuria and control groups which showed the statistically significantly highest values. Microalbuminuria group showed the statistically significant lowest mean creatinine clearance. Correlation between serum RBP4 and other clinical characteristics have been displayed in Table 3. In T_2DM patients, serum concentration of RBP4 was positively correlated with age, fasting blood sugar, 2h post prandial blood sugar and HbA1c and inversely correlated with female gender. There were no significant correlations between RBP4 and BMI, GFR, cholesterol, triglyceride, HDL, LDL, urea, creatinine, creatinine clearance and uric acid.

DISCUSSION

The number and diversity of identified adipokines are growing rapidly [17]; and understanding of the diverse effects of distinct adipokines as well as the interplay between these bioactive mediators is still incomplete [18] and, if fully elucidated, would provide much better understanding for the molecular basis of T_2DM. Type 2 DM is a metabolic disorder resulting from the combination of resistance to insulin action, deficiency of insulin secretion and excessive or inappropriate glucagon secretion [19]. T_2DM is associated with some disease such as hypertension and cardiovascular disease and is characterized by target-tissue resistance to insulin [20]. The present study showed that serum RBP4 levels were significantly elevated in diabetic patients in comparison with control subjects. Our results agreed with different studies [21-28] that demonstrated elevated serum RBP4 levels in type 2 diabetic subjects. Several mechanisms linking RBP4 to insulin resistance and type 2 diabetes have been investigated. To our knowledge, the RBP-4 gene is located on chromosome 10 (10q23-q24) in humans in a region that contains at least 1 interesting gene, hexokinase 1, the gene encoding a key enzyme in the initial step of glucose metabolism [29]. Furthermore, increased serum RBP-4 levels are known to stimulate hepatic gluconeogenesis through stimulation of phosphoenolpyruvate carboxykinase and the attenuated insulin signaling in skeletal muscle [30]. Insulin signaling in primary human adipocytes was affected by RBP4 through blocking the insulin-stimulated phosphorylation of insulin receptor substrate-1 at serine in position 307 [31]. The link between RBP4 and type T_2DM could also be mediated through impaired insulin secretion, insulin levels were not measured in the present study. However, Nehal *et al.* [22] found circulating RBP4 concentration was negatively associated with insulin secretion in Egyptian T_2DM. In fact, it is well known that retinolis

pathophysiologically linked to â-cell function. RBP-4 circulates in serum, forming a complex with transthyretin. Borch *et al.* [32] found that transthyretin constitutes a functional component in pancreatic â-cell stimulus-secretion coupling. Transthyretin blocked RBP receptors which prevent binding of RPB to its receptor. Thus, increased serum RBP4 may prevents transthyretin from exerting its â-cell stimulus-secretion effects [33].

The present study showed that male patients with type 2 diabetes mellitus had significantly higher serum RBP4 concentration than females, as well as, in control group. These findings are in consistent with the findings of Cho *et al.* [26], Chiba *et al.* [34], Jia *et al.* [35] and Kim *et al.* [36] this difference can be partly explained by variations between-gender in body fat and sex hormone levels. Recently, Kos *et al.* [37] found higher RBP4 mRNA expression in adipose tissue in females when compared with males; however, RBP4 mRNA expression was not reflected in the circulating levels. Many studies have declared the relationship between the increased circulating RBP4 and increased fasting plasma glucose levels [38,39], in light of our result, we found positive significant correlation between RBP4 and fasting blood glucose as well as with 2h post prandial blood glucose concentrations which might suggest that the over-secretion of RBP4 may negatively affect B-cell function and this is in agreement with Broch *et al.* [32], who concluded that RBP4 could be one signal from insulin-resistant tissues that β-cell secretion and this impacts on mechanism could be behind the association between increased circulating RBP4 and type 2 diabetes. However, Cho *et al.* [6] reported no association between circulating RBP4, glucose concentrations and B-cell function [28]. Data regarding the relationships between RBP4 levels and BMI are inconclusive [4, 22] and in line with our results, no correlations were found between the serum RBP4 level and BMI. This might due to the study population which comprised patients with type 2 diabetes mellitus, with narrow BMI range and poor islet function and received glucose lowering treatments. In our patient group, no correlations were found between the RBP4 and lipid parameters, which may be due to that most of our patients treated with antilipidemic agents which masked the real relation between RBP4 and lipid parameters. This comes in line with the finding of Akbay *et al.* [4].

The present study showed that both systolic and diastolic blood pressures were significantly higher in T_2DM patients and correlated positively with RBP4, but this correlation was not significant. Recently, Wang *et al.* [40] found that there were positive correlations between the serum RBP4 concentration and both systolic and diastolic blood pressures. These authors used multiple linear regression analysis which showed that serum RBP4 concentration was independently associated with systolic blood pressure. This was in accordance with the findings of others, who speculated that RBP4-mediated cellular uptake of retinol activates inflammation and induces arteriosclerosis, resulting in elevated blood pressure [34]. In this study, whether the presence of microalbuminuria as an early marker of diabetic nephropathy is associated with changes in the concentrations of serum RBP4. Our results showed that the RBP4 levels of patients who had microalbuminuria were significantly higher than the normoalbuminuric diabetic subjects and controls consistent with the findings of Raila *et al.* [41] and Li *et al.* [42]. These results imply that excretion or degradation of RBP4, together with other adipocytokines, may have been impaired from the early stages of diabetic nephropathy Moreover there were significant positive correlation between microalbuminuria and levels of RBP4, so these increased in the levels of RBP4 might be a consequence of incipient diabetic nephropathy as measured by microalbuminuria. In this study we found that GFR was different between the groups indicating that the presence of microalbuminuria was related to changes in the GFR. Might be to the renal clearance of low-molecular weight proteins is close to the GFR [43], it was found an inverse correlation between RBP4 levels and GFR but was not significant; it is therefore, unlikely that changes in the GFR are responsible for the elevated RBP4 levels. Raila *et al.* [41] showed that plasma of type 2 diabetic subjects had the lower molar ratios of retinol to RBP4 and were the lowest in patients with microalbuminuria, in additionally they found the higher percentage of circulating apo-RBP4. This might be due to damaging effect of filtered protein on the proximal tubules which decreased renal uptake and catabolism of apo-RBP4 [44], because apo-RBP4 has been considered as a physiological positive-feedback signal from peripheral tissues for the hepatic release of the RBP4 complex [45]. Therefore, RBP4 elevates in serum of type 2 diabetic subjects with miocroalbuminuria as a result of increases of apo-RBP4 which seems to be a plausible mechanism. However, Masaki *et al.* [46] found that RBP4 levels were not changed in patients with microalbuminuria compared with normoalbuminuria. In addition, Cabre´ *et al.* [15] reported that RBP4 was related to creatinine and GFR but the presence of microalbuminuria was not associated with

RBP4 levels in type 2 diabetic patients. These data suggests that RBP4 does not change in the early stage of diabetic nephropathy but deterioration of kidney function contributes to the elevated RBP4 concentrations in diabetic nephropathy.

Several explanations could be postulated to explain the controversy of different studies: different ethnic populations in the studies, RBP4 genetic variation, different methods of measurement of RBP4, sex-specific dimorphism of RBP4, the characteristics of the patients, or the treatment used. Different assays have been used to measure RBP4 levels and this could account for the varied results reported by different laboratories. A recent study reported a strong correlation between RBP4 measured by western blot and by ELISA, but neither method was able to detect a difference in plasma RBP4 concentrations between insulin sensitive and insulin-resistant individuals [38]. It was suggested that sex-specific dimorphisms in circulating levels of adipokines can be related to direct effects of sex hormones on adipocyte expression and secretion. In case of RBP4, this possible mechanism is not supported by the fact that serum RBP4 levels are similar in pubertal and prepubertal subjects that significantly differ in levels of circulating sex hormones [47]. A possible direct role of gonadotropins on the expression of RBP4 is also proposed, as circulating RBP4 levels are higher in post menopausal compared with premenopausal women and in women older than 50 years when compared with those younger than 50 years [48].

In conclusion, our results showed that concentrations of serum RBP4 were increased in type 2 diabetic patients and were related to the presence of incipient nephropathy measured by microalbuminuria. Diabetic nephropathy is an additional factor that might be responsible for elevated serum RBP4 in type 2 diabetes mellitus and should be considered when discussed RBP-4 in T_2DM.

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