Soluble Receptor for Advanced Glycation End Products: a new biomarker in diagnosis of Diabetic Nephropathy

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Abstract: Background: Diabetic nephropathy is a clinical syndrome characterized by persistent albuminuria (>300 mg/d or >200 mcg/min). The interaction of advanced glycation end products with their cellular receptor (RAGE) is implicated in the pathogenesis of diabetic vascular complications. RAGE has a circulating secretory receptor form, soluble RAGE (sRAGE), which, by neutralizing the action of advanced glycation end products, might exert a protective role against the development of cardiovascular disease. Objective: to study the serum levels of sRAGE in type 2 diabetic patients and to clarify the possible association with urinary albumin excretion as an early marker of microvascular damage. Patients and Methods: Eighty subjects divided into two groups; group I (patients group) included 60 type 2 diabetic patients. They were subdivided into 2 subgroups: twenty normo-albuminuric diabetic subgroup and forty micro-albuminuric diabetic subgroup. Group II (control group) included 20 apparently healthy individuals of matched age and sex. All cases were subjected for estimation of sRAGE by sandwich ELISA technique together with routine laboratory investigations including fasting blood glucose, s. creatinine, cholesterol, triglycerides, HDL-C, LDL-C, HbA1C and Microalbumin. Results: sRAGE was significantly lower in microalbuminuric diabetic than normoalbuminuric diabetic and control groups (p<0.05). There was a positive significant correlation between sRAGE and HDL-cholesterol and a negative significant correlation between sRAGE and creatinine, total cholesterol, triglycerides, LDL-cholesterol HbA1C and microalbumin. Conclusion: The present study found that sRAGE blood levels are lower in diabetic patients who have renal complications, supporting the hypothesis that sRAGE, by limiting the interaction of AGE with cell membrane RAGE, can protect vessels against AGE toxicity. Thus, stimulation of sRAGE production should be considered as a potential therapeutic target in diabetes and AGE-related vascular disease.

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Key words: sRAGE, diabetic nephropathy, microalbuminuria.

1. Introduction

Globally as of 2010 it was estimated that there were 285 million people with type 2 diabetes making up about 90% of diabetes cases[(1)]. This is equivalent to about 6% of the worlds adult population[(2)]. In screening for diabetic nephropathy, early testing for glucose intolerance and diabetes are recommended to identify patients who are at risk for developing microalbuminuria, particularly if they have other risks for type 2 diabetes, such as hypertension, lipid abnormalities, or central obesity, approximately one third of type 2 diabetics are believed to be undiagnosed. Once the diagnosis of diabetes has been made, check urinary protein levels to guide therapy and prognosis[(3)].

Diabetic nephropathy is a clinical syndrome characterized by persistent albuminuria (>300 mg/d or >200 mcg/min) that is confirmed on at least 2 occasions 3-6 months apart, a relentless decline in the glomerular filtration rate (GFR), and elevated arterial blood pressure. The main feature of diabetic glomerulosclerosis is excess accumulation of extracellular matrix leading to mesangial matrix expansion as well as glomerular basement membrane thickening, which then becomes targeted by advanced glycation end product (AGE) modification. At a cellular level, release of transforming growth factor-beta (TGF-beta) is the main trigger of this process[(4)].

Early in the course of the disease, mesangial cells may undergo a phase of limited proliferation, but then they typically arrest in the G_1 phase of the cell cycle to produce extracellular matrix[(5)].

Furthermore, mesangial cells exposed to AGE-albumin at concentrations comparable to those found in human pathology showed increased collagen IV and TGF-β expression as well as activation of protein kinase C, the mesangial cells also produced monocyte chemotactic protein-1, which stimulated prostacyclin production by endothelial cells. This pathophysiological sequence may serve as a model for the events leading to chronic glomerular injury in the diabetic kidney *in vivo*[(6)].

The exact cause of diabetic nephropathy is unknown, but various postulated mechanisms are hyperglycemia (causing hyperfiltration and renal injury), advanced glycosylation products, and

activation of cytokines. Hyperglycemia increases the expression of TGF-beta in the glomeruli and of matrix proteins specifically stimulated by this cytokine. TGF-beta may contribute to the cellular hypertrophy and enhanced collagen synthesis observed in persons with diabetic nephropathy[7].

High levels of AGE accumulate in diabetic patients, specifically within the vascular intima, the nodular and diffuse lesions of the glomeruli and within hyaline deposits of arterioles, thus again demonstrating the importance of AGE in the pathogenesis of diabetic nephropathy[8]. Receptor for advanced glycation endproducts [RAGE] is a member of the immunoglobulin superfamily, encoded in the Class III region of the major histocompatability complex[9].

The interaction of advanced glycation end products, including *N*ε-(carboxymethyl) lysine-protein adducts (CML) and S100A12 protein, with their cellular receptor (RAGE) is implicated in the pathogenesis of diabetic vascular complications. RAGE has a circulating secretory receptor form, soluble RAGE (sRAGE), which, by neutralizing the action of advanced glycation end products, might exert a protective role against the development of cardiovascular disease[10]. Although several hyperglycemia-elicited metabolic and hemodynamic derangements have been implicated in the pathogenesis of diabetic vascular complication, the process of formation and accumulation of advanced glycation end products (AGEs) and their mode of action are most compatible with the theory 'hyperglycemic memory'. Further, there is a growing body of evidence that AGEs and their receptor (RAGE) axis is involved in the pathogenesis of diabetic vascular complication. Indeed, the engagement of RAGE with AGEs is shown to elicit oxidative stress generation and subsequently evoke inflammatory responses in various types of cells, thus playing an important role in the development and progression of diabetic micro- and macroangiopathy. These observations suggest that down-regulation of RAGE expression or blockade of the RAGE downstream signaling may be a promising target for therapeutic intervention in diabetic vascular complication[11].

The aim of the work is to study the serum levels of sRAGE in type 2 diabetic patients and to clarify the possible association with urinary albumin excretion as an early marker of microvascular damage.

2. Subjects and Methods:

The present study was conducted on 80 subjects divided into two groups; group I (patients group) included 60 type 2 diabetic patients. They were 22 males and 38 females, subdivided into 2 subgroups: Subgroup Ia (normo-albuminuric diabetic group) which included 20 type 2 diabetic patients with normo- albuminuria (8 males and 12 females). Their ages ranged from 45 to 62 years with a mean age of (52.9±6 years) and subgroup Ib (micro-albuminuric diabetic group) which included 40 type 2 diabetic patients with micro-albuminuria (14 males and 26 females). Their ages ranged from 48 to 65 years with a mean age of (51.5±5.5 years). Diabetic patients were selected from those attending Diabetes Outpatient Clinic in Benha University Hospital. Group II (control group) included 20 apparently healthy individuals of matched age and sex. They were 8 males and 12 females. Their ages ranged from 45-62 years (50.8±10.9). The control group consists of healthy volunteers without a history of arterial hypertention, neoplastic, cardiovascular, inflammatory, lung, endocrinal or central nervous system disorder. Exclusion criteria were inflammatory conditions, renal failure patients, cardiac disease and liver disease. All subjects were subjected to:

I. Full history taking (age, sex, duration of diabetes).
II. Through clinical examination.
III. Laboratory investigations:

Blood samples were drawn from all subjects after overnight fasting (10-16 hours) by venipuncture:

1- One ml of blood on 15 µL EDTA for determination of HbA1C.
2- Two milliliters were anticoagulated using sodium fluoride for determination of fasting blood glucose level.
3- Four milliliters were placed in plain tubes and allowed to clot for 30 minutes in water bath at 37°C and then centrifuged for 15 minutes at 1000 xg. Serum was then subdivided into two aliquots:

a- The first aliquot was used for determination of creatinine and lipid profile assays.
b- The second aliquot was used for RAGE assay. This aliquot was kept at – 70°C for subsequent assay.

Second morning urine samples were voided after rising for estimation of microalbumin.

Methodology:

1- Fasting blood glucose, creatinine, cholesterol, triglycerides and HDL-cholesterol were performed by automated enzymatic methods (Cobas Integra 400 analyzer, Roche, Germany). LDL-cholesterol was calculated according to Friedwald formula:

LDL-cholesterol = Total cholesterol – HDL-cholesterol – TG/5.

2- Glycated hemoglobin (HbA1C) level was assessed through HPLC technique using Bio – Rad, Hercules, USA kits.

3- sRAGE level was assessed through enzyme linked immunosorbent assay (ELISA) supplied by RayBiotech Incorporation, Norcross, GA, USA. The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for RAGE (extracellular domain) has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any RAGE present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for RAGE (extracellular domain) is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of RAGE bound in the initial step. The color development is stopped and the intensity of the color is measured using a microplate reader at 540 nm.
4- Microalbumin in urine was assessed by using Micral test strips.

Statistical Analysis:

The data were coded, entered and processed on an IBM-PC compatible computer using SPSS (version 11). The level $p < 0.05$ was considered the cut-off value for significance. Descriptive statistics of the different studied groups were done using the mean and standard deviation.

Student "t" test was used for the comparison between each 2 groups according to all measured parameters. Correlation analysis was used for assessing the strength of association between two variables. The correlation coefficient denoted symbolically *r*, defines the strength and direction of the linear relationship between serum levels of sRAGE and other variables among cases.

3. Results:

Parameters including fasting blood glucose, S. creatinine, total cholesterol, triglycerides, LDL-cholesterol and HbA_1C were significantly higher in diabetic than control group while HDL-cholesterol and sRAGE were significantly lower in diabetic than control group.

Table (1): Comparison of different laboratory parameters between the control group (GI) and the diabetic 2 patients group (GII)

Parameter	Control group (N=20) Mean ± SD	Type 2 diabetic patients (N= 60) Mean ± SD	t	*p*
Age (years)	50.8 ± 10.9	51.9 ± 5.7	0.58	>0.05
Fasting Blood Glucose (mg/dl)	86.3 ± 8.8	168.3 ± 29.2	19.3	<0.001
S. Creatinine (mg/dl)	0.6 ± 0.1	0.95 ± 0.2	7.5	<0.001
Total cholesterol (mg/dl)	98.3 ± 8.6	198.2 ± 47.7	15.49	<0.001
Triglycerides (mg/dl)	76.7 ± 7.8	131.6 ± 60.6	6.86	<0.001
HDL-cholesterol (mg/dl)	74.1 ± 17.5	33.6 ± 4.3	10.49	<0.001
LDL-cholesterol (mg/dl)	49.1 ± 4.4	98.5 ± 24.8	14.75	<0.001
HbA1C(%)	4.9 ± 0.5	10.7 ± 0.8	38.6	<0.001
sRAGE (pg/ml)	1452.1 ± 854.7	940 ± 680	2.42	<0.05

p value >0.05 is considered non significant. *p* value <0.05 is considered significant.
p value <0.01 is considered highly significant.

Table (2): Comparison of different laboratory parameters between control group and normoalbuminuric diabetic group

Parameter	Control group (N=20) Mean ± SD	Normoalbuminuric diabetic group (N= 20) Mean ± SD	t	*p*
Age (years)	50.8 ± 10.9	52.9 ± 6.0	0.96	>0.05
Fasting Blood Glucose (mg/dl)	86.3 ± 8.8	165.6 ± 26.1	10.8	<0.001
S. Creatinine (mg/dl)	0.6 ± 0.1	0.6 ± 0.1	-----	-----
Total cholesterol (mg/dl)	98.3 ± 8.6	163.9 ± 27.4	10.4	<0.001
Triglycerides (mg/dl)	76.7 ± 7.8	90.2 ± 30.2	1.95	<0.05
HDL-cholesterol (mg/dl)	74.1 ± 17.5	38.1 ±10.1	8.0	<0.001
LDL-cholesterol (mg/dl)	49.1 ± 4.4	82.1 ± 13.5	4.95	<0.001
HbA1C(%)	4.9 ± 0.5	8.0 ± 0.7	20.6	<0.001
sRAGE (pg/ml)	1452.1 ± 854.7	1050 ± 410	1.9	<0.05

Parameters including fasting blood glucose, total cholesterol, triglycerides, LDL-cholesterol and HbA_1C were significantly higher in normoalbuminuric diabetic than control group while HDL-cholesterol and sRAGE were significantly lower in normoalbuminuric diabetic than control group.

Table (3): Comparison of different laboratory parameters between control group and microalbuminuric diabetic group.

Parameter	Control group (N=20) Mean ± SD	Microalbuminuric diabetic group (N= 40) Mean ± SD	t	*p*
Age (years)	50.8 ± 10.9	51.5 ± 5.5	0.33	>0.05
Fasting Blood Glucose (mg/dl)	86.3 ± 8.8	173.8 ± 35.4	13.2	<0.001
S. Creatinine (mg/dl)	0.6 ± 0.1	0.97 ± 0.2	7.8	<0.001
Total cholesterol (mg/dl)	98.3 ± 8.6	215.4 ± 46.6	11.1	<0.001
Triglycerides (mg/dl)	76.7 ± 7.8	152.3 ± 61.5	5.5	<0.001
HDL-cholesterol (mg/dl)	74.1 ± 17.5	27.1 ±9.2	11.2	<0.001
LDL-cholesterol (mg/dl)	49.1 ± 4.4	106.8 ± 25.1	10.1	<0.001
HbA1C(%)	4.9 ± 0.5	11.2 ± 0.8	42	<0.001
sRAGE (pg/ml)	1452.1 ± 854.7	858.3 ± 488.7	5.1	<0.05

Parameters including fasting blood glucose, S. creatinine, total cholesterol, triglycerides, LDL-cholesterol and HbA_1C were significantly higher in microalbuminuric diabetic than control group while HDL-cholesterol and sRAGE were significantly lower in microalbuminuric diabetic than control group.

Table (4): Comparison of different laboratory parameters between normoaluminuric and microalbuminuric diabetic subgroups.

Parameter	Normoalbuminuric diabetic group (N= 20) Mean ± SD	Microalbuminuric diabetic group (N= 40) Mean ± SD	t	*p*
Age (years)	52.9 ± 6.0	51.5 ± 5.5	0.88	>0.05
Fasting Blood Glucose (mg/dl)	165.6 ± 26.1	173.8 ± 35.4	0.92	>0.05
S. Creatinine (mg/dl)	0.6 ± 0.1	0.97 ± 0.2	9.55	<0.001
Total cholesterol (mg/dl)	163.9 ± 27.4	215.4 ± 46.6	5.37	<0.001
Triglycerides (mg/dl)	90.2 ± 30.2	152.3 ± 61.5	5.24	<0.001
HDL-cholesterol (mg/dl)	38.1 ± 10.1	27.1 ±9.2	4.07	<0.001
LDL-cholesterol (mg/dl)	82.1 ± 13.5	106.8 ± 25.1	4.95	<0.001
HbA1C(%)	8.0 ± 0.7	11.2 ± 0.8	21.3	<0.001
sRAGE (pg/ml)	1050 ± 410	858.3 ± 488.7	1.61	<0.05

Parameters including S. creatinine, total cholesterol, triglycerides, LDL-cholesterol and HbA_1C were significantly higher in microalbuminuric diabetic than normoalbuminuric diabetic groups while HDL-cholesterol and sRAGE were significantly lower in microalbuminuric diabetic than normoalbuminuric diabetic groups.

There was a positive significant correlation between sRAGE and HDL-cholesterol.

There was a negative significant correlation between sRAGE and creatinine, total cholesterol, triglycerides, LDL-cholesterol HbA1C and microalbumin.

Table (5): Correlation coefficients (r) & probability value (p) between sRAGE and other parameters among type 2 diabetic patients group:

Parameters	sRAGE	
	r	*p*
Age (years)	0.0496	>0.05
FBS (mg/dl)	0.084	>0.05
S. creatinine (mg/dl)	-0.349	<0.05
Total cholesterol (mg/dl)	-0.804	<0.001
Triglycerides (mg/dl)	-0.656	<0.01
HDL-C (mg/dl)	0.719	<0.001
LDL-C (mg/dl)	-0.817	<0.001
HbA1C(%)	-0.554	<0.01
Microalbumin (mg/l)	-0.545	<0.01

4. Discussion:

Advanced glycation is one of the pathways by which cellular injury is induced in diabetes and lead to formation of advanced glycation end products (AGEs). There is substantial evidence to support the involvement of advanced glycation end-products (AGE) binding to its receptor (RAGE) in the development of diabetic microvascular complications as atherosclerosis and nephropathy [12].

The comparative study of serum creatinine among the studied groups showed a significant increase in the serum level of creatinine in type 2 diabetic group as compared to the control group (p<0.001), and significant increase in the serum level of creatinine in microalbuminuric diabetic group as compared to normoalbuminuric diabetic group (p<0.001) and control group (p<0.001). These results showed increased serum level of creatinine with the progression of diabetic nephropathy but it was within normal range in normoalbuminuric and control group.

The results of creatinine among the studied groups were in agreement with many investigators[13,14]. They demonstrated that, increased serum level of creatinine in microalbuminuric diabetic patients as a marker of diabetic nephropathy than normoalbuminuric diabetic patients.

In this study, there is a significant difference in serum levels of lipid profile in microalbuminuric diabetic group compared to normoalbuminuric diabetic group (p<0.001) and control group (p<0.001).

The results of lipid profile among the studied groups were in agreement with many investigators [15-18]. They demonstrated that diabetic dyslipidemia is characterized by an elevation of TG and a reduction in HDL-C in type 2 diabetes mellitus. Also they reported that serum total cholesterol and LDL-C may be elevated in type 2 diabetic patients and are considered as cardiovascular risk factors in type 2 diabetes mellitus.

In the present work, comparative study of HbA1C as a marker of glycemic control among studied groups showed poor glycemic control state in microalbuminuric diabetic patients compared with normoalbuminuric diabetic patients.

The present study showed significant decrease in serum level of sRAGE in diabetic group compared to control group (p<0.001). This is in agreement with ***Basta et al.***[19] who reported that plasma sRAGE levels were diminished in type 1 and type 2 diabetes and correlated inversely with intima-media thickness, suggesting a protective role of high sRAGE levels in the development of late vascular complications.

These findings go on line with another study performed by ***Grossin et al.***[20], they found that patients with renal and retinal complications had significantly lower blood levels of sRAGE compared with patients without complications.

Glomerular hyperperfusion and renal hypertrophy occur in the first years after the onset of DM and are reflected by an increased glomerular filtration rate (GFR). Before the onset of overt proteinuria, there are various renal functional changes including renal hyperfiltration, hyperperfusion, and increasing capillary permeability to macromolecules then diabetic nephropathy develops[21]. In this study, the patients with type 2 diabetes were subdivided into 2 subgroups (normoalbuminuric and microalbuminuric diabetic patients) according to urinary microalbumin excretion, and the study showed a significant negative correlation of sRAGE with microalbuminuria in patient with type 2 diabetic nephropathy at the early stage.

The comparative study of sRAGE among the studied groups showed significant decrease in sRAGE in microalbuminuric diabetic patient (p<0.001) than normoalbuminuric diabetic patient, and decrease in sRAGE level in microoalbuminuric diabetic patients than control group.

These findings go online with many studies as ***Tan*** [22] who found that serum sRAGE levels and circulating AGEs are associated with the severity of nephropathy in type 2 diabetic patients, and ***Bruno et al.***[23] also reported that low sRAGE with high CML-protein levels in diabetic patients developed severe diabetic complications and patients with higher sRAGE levels did not exhibit vascular complications.

Another study made by Humpert ***et al.***[24] showed that plasma sRAGE was associated with albumin excretion in type 2 diabetic patient. Hence, they reported that plasma sRAGE levels might represent an early marker of microvascular dysfunction and diabetic nephropathy in type 2 diabetes. Also previous reports stated an inverse correlation of intima-media thickness with sRAGE levels in type 1 and type 2 diabetes [25].

This study showed that sRAGE was negatively correlated with HbA1C and its level was significantly higher (p<0,05) in good glycemic control diabetic patients compared with lower level of sRAGE in poor control glycemic patients. These results revealed that sRAGE is inversely associated with HbA1c as a marker of glycemic control in diabetic subject. This is in agreement with ***Nakamura et al.***[26] who found that sRAGE was inversely associated with HbA1c in their diabetic subjects.

On the other hand ***Yamagishi et al.***[27] suggested that endogenous sRAGE may capture and eliminate circulating AGEs and decrease its serum levels. However, AGEs up-regulate tissue RAGE

expression and endogenous sRAGE could be generated from the cleavage of cell surface RAGE, so sRAGE may be positively associated with circulating AGEs and HbA1c (as one of the early glycation products) by reflecting tissue RAGE expression.

Basta et al.[10] also found that circulating soluble receptor for advanced glycation end products is inversely associated with glycemic control and S100A12 protein as plasma level of sRAGE is down-regulated in chronic hyperglycemia; among its ligands, S100A12 protein.

The present study also showed that, low serum sRAGE levels were associated with hyperlipidemia as there was a significant negative correlation with s.cholesterol, s.triglyceride and LDL-C, and a significant high positive correlation with HDL-C among diabetic group. ***Lehmann et al.***[28], demonstrated that type 2 diabetes patients with a state of chronic hyperglycemia, and glucose-dependent processes are likely to be involved in the pathogenesis of diabetic complications, including nephropathy. Glucose-induced tissue injury may be mediated by generation of advanced glycated proteins which have been implicated in nephropathy.

In conclusion, the present study found that sRAGE blood levels are lower in diabetic patients who have renal complications, supporting the hypothesis that sRAGE, by limiting the interaction of AGE with cell membrane RAGE, can protect vessels against AGE toxicity. Thus, stimulation of sRAGE production should be considered as a potential therapeutic target in diabetes and AGE-related vascular disease.

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