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Decreased Level of Endogenous Secretory Receptor for Advanced Glycation End-Products in Diabetes With Concomitant Hyperlipidemia

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Summary

Endogenous secretory receptor (esRAGE) for advanced glycation end-product (AGE) acts as decoy for AGEs. The AGE-to-esRAGE ratio was hypothesized to be implicated in diabetic vasculopathy. We investigated an association of esRAGE and methylglyoxal-adducts serum level, as well as AGE-to-esRAGE ratio in subpopulation of diabetic patients with or without concomitant hyperlipidemia and macrovascular disease in history. In diabetes with concomitant hyperlipidemia esRAGE was significantly decreased compared to hyperlipidemia with normal glucose metabolism (0.306±0.2 vs. 0.367±0.1; p=0.019) or diabetes alone (0.306±0.2 vs. 0.404±0.1; p=0.004). High AGE/esRAGE ratio, found in diabetic patients with hyperlipidemia, pointed to increased production of AGEs and low expression of esRAGE. In multivariable analysis adjusted for several confounding factors, increased AGE/esRAGE ratio was recognized as a high risk for vascular disease outcomes.

Key words

Advanced glycation pathway • Type 2 diabetes • Hyperlipidemia • Endogenous secretory receptor for AGE • AGE-to-esRAGE ratio

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Introduction

Macrovascular complications associated with diabetes include cardiovascular, cerebrovascular and lower-extremity arterial disease. The increased risk of vascular disease in diabetic patients is promoted by some specific pathobiochemical processes, amidst which advanced glycation holds a prominent place (Brownlee 2005). The pathway leading to advanced glycation end-product (AGE) formation is associated with hyperglycemia at a fundamental level (Singh *et al.* 2001, Turk 2010). The formation and subsequent accumulation of AGEs in target tissues at an accelerated rate are an outcome of high blood glucose. The knowledge of AGE-pathways has expanded considerably over the years and a large body of evidence has documented their implication in diabetic vasculopathy.

AGEs cause a wide range of deleterious effects binding to cell membrane receptor, termed RAGE. This may lead to activation of intracellular pathways and release of cytokines, which may induce endothelial dysfunction and other detrimental vascular effects (Yan *et al.* 2010). In addition to cell surface RAGE, soluble forms of RAGE (sRAGE) circulate in the plasma. Total sRAGE consists of two isoforms: endogenous secretory receptor (esRAGE) secreted from cells, and cleaved RAGE (cRAGE), formed by proteolytic cleavage of the cell surface receptor (Kalea *et al.* 2011). These two secretory isoforms of soluble RAGE may have different (patho-) physiological functions (Koyama *et al.* 2005, Katakami *et al.* 2005, 2008, Lu *et al.* 2010, Nin *et al.* 2010, Raposeiras-Roubin *et al.* 2010, Colhoun *et al.* 2011, Wang *et al.* 2011). There are studies showing that esRAGE alone, but not total sRAGE, has a protective role in cardiovascular disease (Koyama *et al.* 2005, Katakami *et al.* 2005, 2008, Wang *et al.* 2011).

Table 1. Baseline characteristics of the study population.

	Diabetes with hyperlipaemia (DM+HL, n=98)	Diabetes without hyperlipaemia (DM, n=32)	Hyperlipaemia (HL, n=38)	p-value
**Age (years)*	62 (56-69)	55.5 (40-63.5)	60 (48-68)	NS
esRAGE (ng/ml)	0.306±0.2	0.404±0.1	0.367±0.1	0.001
HbA1c (%)	6.8±1	7.0±1.3	4.9±0.4	<0.001
Fasting glucose (mmol/l)	8.1±2.3	8.1±3.2	5.2±1.1	<0.001
**AGE (μgEq/l)*	116 (84-131)	127.9 (107-133)	101.4 (65-127)	0.011
**MG-adduct (μgEq/l)*	91.7 (62-121)	119.2 (93-136)	76.1 (50-97)	0.001
**Urine MG-adduct (μgEq/l)*	150 (42-337)	224 (81-254)	133 (70-339)	0.042
Total cholesterol (mmol/l)	5.0±1.3	4.4±0.7	6.0±1.7	<0.001
HDL-cholesterol (mmol/l)	1.36±0.3	1.57±0.4	1.51±0.4	0.006
LDL-cholesterol (mmol/l)	2.8±1.1	2.4±0.5	3.6±1.4	<0.001
Triglyceride (mmol/l)	2.46±0.39	1.05±0.4	2.05±1.92	NS
Lp(a) (mg/dl)	35.8±56	20.3±16	62.9±86	NS
CRP (mg/l)	3.47±5.1	1.8±1.5	2.41±3.7	NS
Homocysteine (μmol/l)	15.1±5.6	12.7±4.0	15.6±6.9	0.011

Values are mean ± SD or * median (quartiles). Continuous variables were log-transformed and compared by ANOVA. NS – not significant.

To our knowledge, no previous studies have evaluated the association between esRAGE and methylglyoxal-adduct, a major member of AGE family. Methylglyoxal is an extremely reactive intermediary metabolite generated mainly in the glycolytic pathway, and additionally produced in the course of lipid metabolism (Kalapos 2008). Recently published study has shown a tight correlation of LDL and triglycerides with MG-adduct biogenesis in diabetic subjects (Turk *et al.* 2011). In keeping with these finding, we hypothesized that intensified biogenesis of methylglyoxal-derived AGE may have influenced the expression of esRAGE. The purpose of this study was (*a*) to evaluate the circulating esRAGE in type 2 diabetes with or without concomitant hyperlipidemia, (*b*) to establish whether glycemia control and which of metabolic parameters are determinants of esRAGE humoral level and (*c*) to assess a possible association between AGE/esRAGE ratio and macrovascular disease in diabetes type 2.

Patients and Methods

Study population

Methylglyoxal-adducts, total AGEs and esRAGEs were measured in 168 subjects with either type 2 diabetes without hyperlipidemia (DM, n=32), type 2 diabetes with hyperlipidemia (DM+HL, n=98) or hyperlipidemia with normal glucose metabolism (HL, n=38). Table 1 shows baseline characteristics in the groups of investigated patients.

Exclusion criteria were acute diabetes complications (ketoacidosis or lactic acidosis), impaired liver or renal function, active inflammatory disease, autoimmune disease and cancer.

Any patient taking lipid-lowering drugs or having at least one of the following plasma levels: total cholesterol >5.0 mmol/l, LDL-cholesterol >3.0 mmol/l or triglyceride >1.7 mmol/l was defined as being hyperlipemic. A history of macrovascular events was recorded in 72 subjects. Coronary heart disease was established in 37 patients, of whom six had cerebral vasculopathy and two additionally suffered from peripheral vascular disease. Cerebral vasculopathy was noted in 30 patients, six of whom had peripheral arterial disease. Peripheral arterial disease alone was observed in five of the investigated patients. Diagnostic modalities for the detection and evaluation of myocardial ischemia were: clinical history of myocardial infarction, angina pectoris or coronary artery surgery, positive ECG stress testing and coronary arteriography. Significant stenosis was defined as a 70-percent occlusion of major coronary arteries. Diagnosis of cerebral vasculopathy was made

based on the history of symptoms of transient ischemic attack and/or stroke or a significant stenosis (50 %) as assessed by Doppler ultrasound. Diagnosis of peripheral vascular disease included the ankle-brachial index test.

The control group consisted of healthy volunteers (n=30) recruited from the hospital staff, median age 38 years (25-42), BMI <25, without a history of metabolic disease (diabetes, dyslipidemia, hypertension, obesity), cancer, cardiovascular or renal disease. None of the control subjects were taking any medications.

The study was designed and carried out in accordance with the Declaration of Helsinki of the World Medical Association. The Hospital Ethics Committee approved the study protocol, and an informed consent was obtained from each subject before entering the study.

Methylglyoxal-adduct measurement

A competitive DELFIA® with MG-derived AGE antibody was used to assess serum and urine MG-adducts (Turk *et al.* 2009). Briefly, the immunoplate was coated with the MG-derived HSA and subsequently blocked. After washing, 100 µl of MG-HSA standards (0; 7.8; 15.6; 31.3; 62.5; 125 and 250 mg/l) or samples of human serum (diluted 1:10) or urine (diluted 1:2) were added to the wells, followed by 50 µl of anti-MG-AGE antiserum. The plate was incubated at room temperature for 2 h and washed. A volume of 100 µl/well of europium-labeled anti-rabbit IgG antibody (Perkin Elmer, Boston, USA, cat. no. AD0105) was then added to a final concentration of 0.1 mg/l in dilution buffer (0.05 mol/l TRIS-HCl, pH 7.8 containing 0.5 ml/l Tween-20 and 2 % of BSA). The plate was incubated while shaking for 1 h at room temperature and washed again. Afterwards, 200 µl of DELFIA Enhancement solution (Wallac, Turku, Finland) were added to each well and incubated for another 5 min. Europium-ion chelate-specific fluorescence was finally measured in a 1234 DELFIA Fluorometer at 615 nm. A calibration curve was plotted from serial double dilutions of MG-derived AGE-albumin, corresponding to a protein concentration of 250 to 15 mgEq/l.

Measurement of advanced glycation end-products and their endogenous secretory receptors

(*i*) Competitive-type ELISA with polyclonal anti-AGE antibodies was used for the detection of total AGE content, as described in detail in our previous report (Turk *et al.* 2001). The assay utilizes AGE-human serum albumin (AGE-HSA) as a standard. Competitive immunoreactivity of the samples was read from the calibration curve and expressed relative to AGE-albumin standard in µgEq/ml.

(*ii*) esRAGEs were measured using a commercially available sandwich ELISA kit (manufactured by Daiichi Fine Chemicals, Takaoka, Japan and distributed by B-Bridge International, CA, USA) according to the manufacturer´s protocol. In short, samples and esRAGE antibody conjugated with horseradish peroxidase were incubated in an anti-RAGE antibody microtiter plate. After incubation and washing, a substrate was added and the plates were additionally incubated. The enzymatic reaction was stopped and the resulting absorbance measured using an ELISA plate reader. The absorbance was proportional to the concentration of esRAGE. The interassay coefficient of variation (CV) for repeated esRAGE measurements (concentrations of 0.270 and 0.600 ng/ml) ranged from 2.7 % to 5.2 %. Thirty healthy controls recruited from the hospital staff had mean esRAGE level 0.458±0.074 ng/ml.

Statistical analysis

Variables with a skewed distribution (i.e. MG-adduct, AGEs, esRAGE, Lp(a), CRP, homocysteine, BMI) were logarithmically transformed prior to further analyses. Differences between continuous variables were evaluated by ANOVA or Mann-Whitney U test, as appropriate. Categorical variables were expressed as percentages and compared using chi-square test (i.e. diabetes with or without hyperlipidemia; macrovascular disease). Linear regression was used to examine the extent to which esRAGE level was associated with parameters of advanced glycation (MG-adduct and total AGEs), as well as with parameters of lipid profiles and glycemic control. All variables that had shown independent and significant correlation in bivariate analyses of correlation were additionally tested by multiple regression analysis conducted by a stepwise model.

Results

The analysis of circulating esRAGE with respect to the study groups revealed significantly lower levels in diabetic patients with concomitant hyperlipidemia in comparison with hyperlipemic subjects (0.306±0.2 vs. 0.367±0.1 ng/ml; p=0.019) or those with diabetes alone (0.306±0.2 vs. 0.404±0.1; p=0.004). In addition, as

shown in Figure 1, circulatory esRAGE level in all three patient groups was significantly lower than in the control subjects.

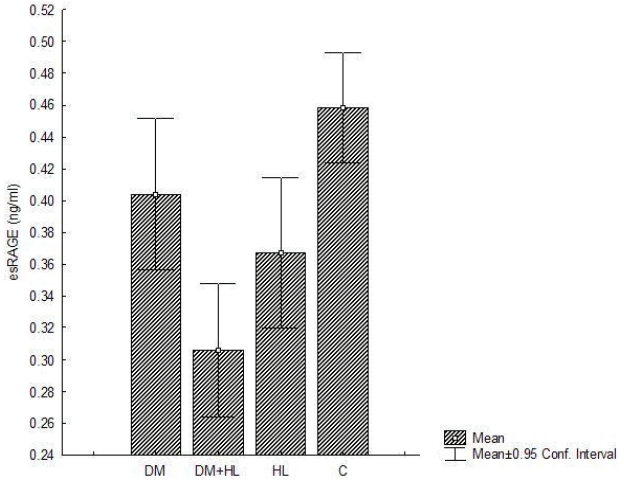

Fig. 1. Endogenous secretory receptor for advanced glycation end-products (esRAGE) is significantly decreased in diabetic patients with concomitant hyperlipidemia (DM+HL, n=98) compared to hyperlipemic persons (HL, n=38) with normal glucose metabolism (0.306±0.2 vs. 0.367±0.1 ng/ml; p=0.019) or diabetic patients (DM, n=32) without hyperlipidemia (0.306±0.2 vs. 0.404±0.1; p=0.004). As shown in the Figure, circulatory esRAGE level in all three patient groups was significantly lower than in the control (C, n=30) subjects: DM vs. C, p=0.031; DM+HL vs. C, p<0.001; HL vs. C, p=0.008.

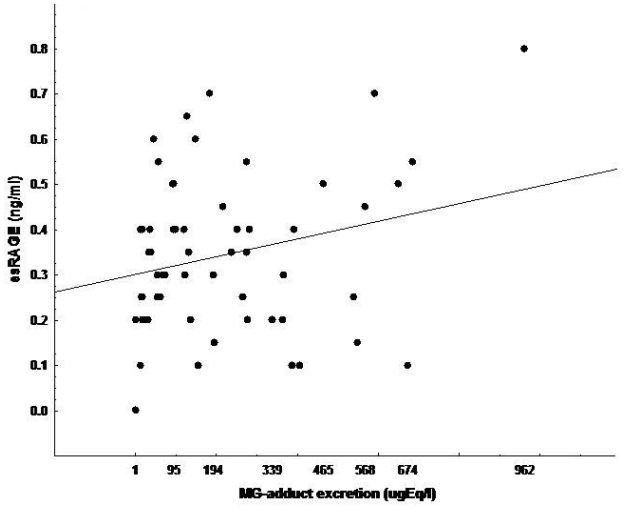

Fig. 2. Urinary excretion of methylglyoxal-adducts (MG-adducts) tightly correlated (r=0.45, p=0.001) with circulatory level of endogenous secretory receptor for advanced glycation end-products (esRAGE).

Table 2 shows results of univariate and multivariate regression analyses of esRAGE level as a dependent variable and metabolic parameters as independent variables. Multiple stepwise regression analysis was performed to determine which of these parameters best predicted esRAGE level. Finally, urinary MG-adduct excretion (p<0.001) (Fig. 2), HbA1c (p<0.001), HDL-cholesterol (p<0.001), CRP (p=0.025, inversely) and Lp(a) (p=0.003) remained significant and independently correlated with esRAGE.

AGE-to-esRAGE ratio was calculated, its high value pointing to an increased AGE production and a low esRAGE expression. A significantly higher AGE/esRAGE ratio was found in the population of diabetic patients with hyperlipidemia (p<0.001) (Fig. 3). A history of macrovascular events was recorded in 72 patients. This subgroup showed a good correlation of esRAGE with the urinary excretion of methylglyoxal-derived AGE (r=0.42 p=0.001). Multiple regression model was used to evaluate the relationship between macrovascular disease as a dependent variable, and metabolic and advanced glycation parameters as independent variables. The results pointed to LDL (β=0.40 p<0.001), HbA1c (β=0.37 p<0.001), AGE/esRAGE (β=0.31 p=0.007) and homocysteine (β=0.25 p=0.013) as significant independent contributors to diabetic macrovascular disease. It is worth noting that AGE/esRAGE values were strongly associated with serum homocysteine only in patients with established vascular disease. Given that hyperhomocysteinemia promotes atherosclerosis, this association is indicative.

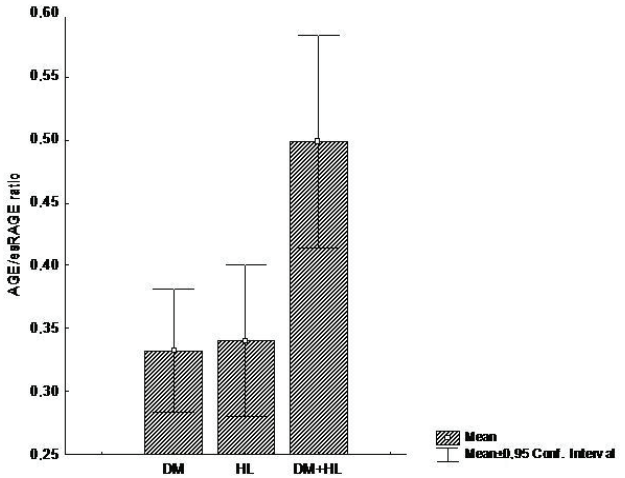

Fig. 3. The calculated ratio of advanced glycation endproducts-to-endogenous secretory receptor (AGE/esRAGE) pointed to an increased production of AGEs and low esRAGE expression. The AGE/esRAGE values were significantly higher in hyperlipemic diabetic patients (DM+HL) as compared to hyperlipemic subjects with normal glucose metabolism (HL) (0.5±0.3 vs. 0.34±0.1, p=0.026) or diabetes alone (DM) (0.5±0.3 vs. 0.33±0.1, p=0.026).

Table 2. Determinants of endogenous secretory RAGE obtained by forward stepwise multiple regression analysis.

Parameters	Bivariate analysis β	p value	Multivariate regression $\beta^{\#}$	p value
HbA1c (%)	0.188	0.039	0.514	0.0008
Fasting glucose (mmol/l)	0.063	NS		
**AGE (μgEq/l)*	0.134	0.049		
**MG-adduct (μgEq/l)*	-0.04	NS		
**Urine MG-adduct (μgEq/l)*	0.458	0.001	0.523	0.000023
Total cholesterol (mmol/l)	0.189	0.014		
HDL-cholesterol (mmol/l)	0.283	0.001	0.421	0.00069
**LDL-cholesterol (mmol/l)*	-0.290	0.008		
Triglyceride (mmol/l)	0.341	0.045		
**CRP (mg/l)*	-0.170	0.029	-0.265	0.025
**Homocysteine (μmol/l)*	-0.400	0.001		
**Lp(a) (mg/dl)*	0.300	0.003	0.372	0.0034
**BMI (kg/m^2)*	0.037	NS		

β – bivariate coefficient, $\beta^{\#}$ – multivariate regression coefficient, * – log-transformed values were used, NS – not significant.

Discussion

In the current study we were focused on the association between esRAGE and several metabolic endpoints in diabetes and hyperlipidemia, which may help to better predict macrovascular disease progression. The data showed down-regulation of esRAGE in diabetic patients with concomitant hyperlipidemia as compared to hyperlipidemic patients with normal glucose metabolism or patients with diabetes alone. It is worth noting that in our diabetic population with and without hyperlipidemia, was no between-group difference in glycemia control. A significantly higher AGE-to-esRAGE ratio was documented in the hyperlipidemic diabetic patients, indicating an increased production of AGE and a low expression of esRAGE, and that was statistically recognized as an independent contributor of macrovascular disease.

Hyperlipidemia is often considered as a pathogenic force in diabetic vascular disease. Lipoprotein peroxidation reactions can also form a subclass of AGE-compounds termed ALE (advanced lipooxidation endproduct). To be more precisely, reactive dicarbonyl intermediates generate ALEs in the course of lipid peroxidative process (Miyata *et al.* 1998). Among AGE precursors, the α-dicarbonyl methylglyoxal is considered as one of the key intermediates (Kalapos 2008, Turk 2010). In diabetes, the majority of methylglyoxal production arises from hyperglycemia in cells accumulating a high level of glucose. However, methylglyoxal is also produced in the course of lipid and amino acid metabolism, but their percentile share has not yet been examined. Previously published study by Turk *et al.* suggested that methylglyoxal might be a common factor linking the two dominant metabolic changes in diabetes, hyperglycemia and intensive lipolysis (Turk *et al.* 2011). AGE binding to its receptor RAGE elicit oxidative stress and provokes inflammatory and thrombogenic responses. Soluble forms of RAGE may neutralise formation of cell-bound AGE-RAGE complex (Katakami *et al.* 2008). The key finding of current study is that esRAGE level is significantly decreased in diabetic patients with concomitant hyperlipidemia. We have speculated therefore, that esRAGE might act as a scavenger receptor. But, under condition of excessive AGE-ligand production, as in poorly controlled diabetes or diabetes with hyperlipidemia, the receptor might lose its scavenger function. The efficiency of AGE-esRAGE clearance would be then abolished, leaving free AGEs to cause a wide range of deleterious effects. Several studies suggest that decreased level of soluble RAGE may be useful marker of hyperactivity of AGE-pathway and inadequate endogenous protective response (Santilli *et al.* 2009, Tam *et al.* 2011). Our clinical data are in good agreement with these observations. Thus, results obtained in our study suggest that humoral esRAGE could be

a protective factor rather than a proatherogenic one, although regulatory mechanisms remain to be elucidated.

Our study provides evidence that high AGE/esRAGE ratio found in diabetes with hyperlipidemia is a significant independent contributor to diabetic macrovascular disease. Literature data additionally show that esRAGE level inversely correlate with carotid intima-media thickness (Katakami *et al.* 2005, 2009), severity of diabetic retinopathy (Katakami *et al.* 2005), and overt albuminuria (Marcovecchio *et al.* 2009). Circulating esRAGE has also been reported to be inversely associated with components of the metabolic syndrome (Koyama *et al.* 2005, Chen *et al.* 2011). In the current study esRAGE was found to correlate positively with MG-adduct excretion and HbA1c values, and inversely with LDL, CRP and homocysteine, parameters known to be involved in the atherosclerosis. The results of clinical trials may therefore be said to be in accordance with the previously published report on experimental animals. Thus, administration of a recombinant soluble form of RAGE consisting of the extracellular ligand-binding domain has been shown to block the AGE-RAGE signaling pathway (Bucciarelli *et al.* 2002). Endogenously administered sRAGE may capture and eliminate circulating AGEs, thus protecting against the AGE-elicited tissue damage by acting as a decoy receptor. Moreover, endogenously administered sRAGE was shown not only to suppress the development of atherosclerosis but also to stabilise existing atherosclerosis in diabetic mice. However, the published literature on serum levels of esRAGE as predictive biomarker of cardiovascular disease in patients with diabetes is conflicting and more study in different diabetic population are warranted.

In conclusion, high AGE-to-esRAGE ratio and esRAGE down-regulation observed in diabetes with concomitant hyperlipidemia was statistically recognized to be an independent contributor of macrovascular disease. Prospective data with a longer follow-up are needed to corroborate these findings.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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