Low Endogenous Secretory Receptor for Advanced Glycation End-Products Levels Are Associated With Inflammation and Carotid Atherosclerosis in Prediabetes

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Context: Prediabetes is associated with atherosclerotic vascular damage.

Objective: We investigated the correlation of endogenous secretory receptor for advanced glycation end-products (esRAGE), total soluble RAGE (sRAGE) and markers of inflammation, with early cardiovascular disease in subjects with prediabetes. We particularly focused on individuals with prediabetes identified only by glycated hemoglobin A_{1c} (HbA_{1c}) (5.7–6.4%) who had normal fasting glucose and were normotolerant after oral glucose tolerance test.

Design: This was a cross-sectional study.

Setting: The study was conducted in the Department of Clinical and Molecular Medicine, University of Catania, Italy.

Main Outcome Measure: sRAGE, esRAGE, carboxymethyl-lysine, S100A12, HbA_{1c}, fasting glycemia, oral glucose tolerance test, pulse wave velocity, and intima-media thickness were evaluated in subjects with prediabetes.

Patients: Three hundred eighty subjects without previous history of diabetes were stratified into three groups: controls (n = 99), prediabetes (n = 220), and new-onset type 2 diabetes (n = 61).

Results: Subjects with prediabetes exhibited the following: lower esRAGE (0.29 ± 0.18 vs 0.45 ± 0.26 ng/mL; $P < .05$) and higher S100A12 levels than controls. RT-PCR analysis in mononuclear cells revealed that the mRNA expression level of the *esRAGE* splice variant progressively decreased in patients with prediabetes and type 2 diabetes with respect to controls. No difference was observed in sRAGE and carboxymethyl-lysine plasma levels between the groups. After multiple regression analyses, only age, HbA_{1c}, and hs-CRP were independently associated with esRAGE levels. Age, HbA_{1c}, and esRAGE were the major determinants of intima-media thickness, whereas S100A12 and systolic blood pressure were the major determinants of pulse wave velocity. When we analyzed the subjects with HbA_{1c} prediabetes (normal fasting glucose/normotolerant and HbA_{1c} 5.7–6.4%), esRAGE and inflammatory markers plasma levels still remained significantly different in respect to controls.

Conclusions: Subjects with HbA_{1c} prediabetes exhibited significantly reduced esRAGE levels and increased levels of markers of inflammation. These alterations are associated with early markers of cardiovascular disease. (***J Clin Endocrinol Metab*** **101: 1701–1709, 2016)**

Abbreviations: ADA, American Diabetes Association; AGE, advanced glycation end-products; BMI, body mass index; BP, blood pressure; CHD, coronary heart disease; CML, carboxymethyl-lysine; CRP, C-reactive protein; esRAGE, endogenous secretory receptor for advanced glycation end-product; $HbA1_c$, glycated hemoglobin A_{1c}HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment-insulin resistance; hs-CRP, high-sensitivity C-reactive protein; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; IMT, intima-media thickness; LDL, low-density lipoprotein; MNC, mononuclear cell; NFG, normal fasting glucose; NT, normotolerant; OGTT, oral glucose tolerance test; PWV, pulse wave velocity; RAGE, receptor for advanced glycation end-product; sRAGE, soluble RAGE; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; WBC, white blood cell.

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Prediabetes is a high-risk state for diabetes and cardiovascular disease development (1). In 2011, the American Diabetes Association (ADA) revised its criteria for the diagnosis of type 2 diabetes mellitus (T2DM) and the categories at increased risk for diabetes, which already included impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). On the basis of a thorough analysis performed by an international expert committee, the use of glycated hemoglobin A_{1c} (HbA_{1c}) measurement was recommended as another diagnostic test option in addition to glucose values. Specifically for the categories of increased risk for T2DM, the new ADA recommendations state that an HbA_{1c} from 5.7–6.4% identifies individuals at high risk for diabetes to whom the term prediabetes may be applied (2).

Previous studies have confirmed that events portending accelerated atherosclerosis are under way prior to the formal diagnosis of diabetes. According to these reports, prediabetes is associated with more advanced vascular damage compared with normoglycemia (3, 4). In a recent study, we analyzed the cardiovascular risk profile in subjects with prediabetes identified according to HbA_{1c} (5.7–6.4%) and reported an alteration of early markers of cardiovascular damage in subjects with higher HbA_{1c} (5).

To explain the association between hyperglycemia and vascular complications in diabetes, several studies have emphasized the role of advanced glycation endproducts (AGE) and their receptors (RAGE). The interaction between RAGE and its ligands (AGEs and other molecules, such as S100A12) effectively modulates several steps of atherogenesis, triggering an inflammatory-proliferative process and critically contributing to the propagation of vascular perturbation, mainly in diabetes (6). RAGE has a secretory isoform, which is termed soluble RAGE (sRAGE). sRAGE is primarily formed by the proteolytic cleavage of membrane-bound RAGE and, secondarily, by a secreted, nonmembrane-bound form of the receptor resulting from alternative splicing of the *RAGE* gene, which is known as endogenously secreted RAGE (esRAGE). esRAGE may contribute to the removal/neutralization of circulating ligands, thus functioning as a decoy by competing with cell-surface RAGE for ligand binding (7).

sRAGE has been recently associated with a greater risk of cardiovascular complications. Several studies have demonstrated an inverse cross-sectional association between sRAGE plasma levels and coronary heart disease (CHD) or atherosclerosis in nondiabetic men (8). Prospective studies have shown that low levels of sRAGE predict cardiovascular mortality in diabetic and nondiabetic subjects (9). Moreover, common oral agents such as thiazolidinediones and statins are known to modulate the AGE-RAGE system, even if long-term prospective studies are needed to evaluate whether the modulation of sRAGE can be helpful in preventing macrovascular disease (10–12).

Although numerous data have been reported in subjects with both type 1 diabetes mellitus (T1DM) and T2DM, to date, very little information is available regarding circulating sRAGE levels in subjects with prediabetes and their possible link with vascular damage in this population.

In this study, we measured sRAGE and esRAGE levels and examined their associations with other proinflammatory factors and early markers of atherosclerosis in subjects with prediabetes.

Although different criteria for the diagnosis of prediabetes have good predictive values for the development of T2DM (13), there are scarce and conflicting data as to which best predicts cardiovascular disease (8, 14, 15). Therefore, we studied the AGE/RAGE axis and proinflammatory profile, focusing on individuals with prediabetes identified only by HbA_{1c} (5.7–6.4%) who had normal fasting glucose (NFG) and were normotolerant (NT) after oral glucose tolerance test (OGTT).

Materials and Methods

Study subjects

Three-hundred eighty subjects with no previous diagnosis of diabetes who attended our university hospital for diabetes and cardiovascular risk evaluation were consecutively recruited for the study. The inclusion criteria were ages ranging from 18 to 65 years. All patients were Caucasian and underwent a physical examination and review of their clinical history, smoking status and alcohol consumption. The exclusion criteria were: a previous history of diabetes, a previous history of overt cardiovascular events (stroke, ischemic heart disease, chronic obstructive peripheral arteriopathy, or heart failure), anemia, or hemoglobinopathies, the use of medications known to affect glucose metabolism, clinical evidence of liver or renal disease, chronic diseases, and/or recent history of acute illness, malignant disease, and drug or alcohol abuse.

After an overnight fasting venous blood samples were obtained for the measurement of biochemical parameters. Low-density lipoprotein (LDL) cholesterol concentrations were estimated using the Friedewald formula. All subjects underwent a 75-g OGTT with sampling for glucose and insulin, as previously described (16). Glucose tolerance status was defined on the basis of OGTT according to ADA recommendations (2).

Biochemical analyses

Plasma glucose, total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, and high-sensitivity C-reactive protein (CRP) (hs-CRP) were measured using available enzymatic methods, as previously described (17).

To quantify the plasma concentration of sRAGE (Human sRAGE ELISA; Biovendor), esRAGE (B-Bridge esRAGE ELISA Kit), carboxymethyl-lysine (CML), [CircuLex, ELISA Kit for CML-Nε_(Carboxymethyl)lysine], and S100A12 (Cloud-Clone Corp., ELISA Kit for S1000A12), fasting blood samples were centrifuged and stored at −80°C. The interassay and intra-assay coefficients of variation were respectively 5.5–8.8% and 2.6–5.3% for sRAGE, 5.9–7.5% and 0.7–1.5% for esRAGE, 4.7–15.2% and 5.2–7.4% for CML, and <10 and 12% for S100A12.

HbA_{1c} was measured via HPLC using a National Glycohemoglobin Standardization Program and was standardized to the Diabetes Control and Complications Trial (10) assay reference. Chromatography was performed using a certified automated analyzer (HLC-723G7 hemoglobin HPLC analyzer; Tosoh Corp.) (normal range, 4.25–5.9%); intra- and interassay coefficients of variation were 1.7 and 2.6%, respectively.

Carotid ultrasound examination

Ultrasound scans were performed using a high-resolution B-mode ultrasound system (MyLab 50 Xvision; Esaote Biomedica SpA) equipped with a 7.5-MHz linear array transducer. Scans were performed by a single physician, as previously described (18). The obtained values were averaged and are presented as the mean of the intima-media thickness (IMT) of the common carotid artery.

Arterial stiffness evaluation

The SphygmoCor CvMS (AtCor Medical) system was used for the determination of the pulse wave velocity (PWV), as already described (5). The PWV was calculated on the mean basis of 10 consecutive pressure waveforms to cover a complete respiratory cycle.

Mononuclear cell isolation

Mononuclear cells (MNCs) were isolated from blood samples (10–12 mL) using lympholyte medium (LymphoprepTM, Stemcells Technologies) according to the manufacturer's instructions.

Total RNA isolation and RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified by spectrophotometry. First-strand cDNA was produced from 1 μg of total RNA using Superscript II and OligodT primers (Invitrogen, Life Technologies) according to the manufacturer's instructions.

The transcripts of human advanced glycosylation end product-specific receptor (*RAGE* gene, NM_001136) and its splice variant (*esRAGE*, NM_001206940) were obtained by PCR using the primers 5′-CAGCATCATCGAACCAGGC-3′ and 5′-TGGATGGGATCTGTCTGTGG-3′. The forward 5′-CTCTTCCAGCCTTCCTTCCT-3′ and reverse 5′-AGCACTGTGTTGGCGTACAG-3′ primers specific to the human *actin beta* sequence (NM_001101.3) were used as housekeeping gene. With these primer pairs, the expected bands were 282 bp for the *RAGE* gene and 214 bp for the *esRAGE* gene; the expected band for the actin beta gene was 116 base pairs.

Statistical analyses

We based the power calculation on previous studies examining sRAGE differences among patients with altered glycemic homeostasis and control subjects to compare the means of three groups using ANOVA; the level of significance (α) was set to 5% and power (1-β) to 80%. The ρ value selected was 0.25.

Statistical comparisons of parameters were performed using Stat View 6.0 for Windows. The data are presented as the mean ± SD or median and interquartile range. Each variable's distributional characteristics were assessed by the Kolmogorov-Smirnov test. Statistical evaluation among groups consisted of ANOVA followed by Bonferroni post-hoc test. The χ^2 test was used for categorical variables. $P < .05$ was considered significant. When necessary, numerical variables were logarithmically transformed to reduce skewedness.

Simple regression analysis was performed to relate esRAGE, IMT, and PWV to clinical and biochemical variables. To identify variables independently associated with variations of esRAGE, IMT, and PWV, we performed two multivariate regression models. The first model included several cardiovascular risk factors (age, sex, body mass index [BMI], smoking status, systolic and diastolic blood pressure [BP], HDL cholesterol, triglycerides, LDL cholesterol, non-HDL cholesterol, statin therapy, HbA_{1c}, fasting glycemia, and fasting insulin). Subsequently, variables reaching significance were inserted in a second multiple regression model that included glycation and inflammatory markers (sRAGE, esRAGE, CML, hs-CRP, S100A12). The variance inflation factor was used to check for the problem of multicollinearity in multiple regression analysis (19).

The study was approved by the local ethic committee. Informed consent was obtained from each participant.

Results

The study population (380 subjects) was divided into three groups based on fasting glucose, OGTT, and HbA_{1c} levels: 99 control subjects (NFG and NT and $HbA_{1c} < 5.7\%$), 220 patients with prediabetes (IFG and/or impaired glucose tolerance [IGT] and/or HbA_{1c}, 5.7–6.4%) and 61 patients with new-onset T2DM (fasting glucose ≥ 126 mg/dL and/or 2-hour glucose post-OGTT ≥ 200 mg/dL and/or $HbA_{1c} \geq 6.5\%$).

The clinical and biochemical characteristics of the study subjects are presented in Table 1. The prediabetic patients were older than the controls but were similar with regard to BMI, plasma levels of total cholesterol, HDL cholesterol, and systolic and diastolic BP. There were no differences between subjects with prediabetes and T2DM with respect to anthropometric and metabolic characteristics except for the lower systolic BP, lower fasting glycemia, higher HDL cholesterol, and lower homeostasis model assessment–insulin resistance. (HOMA-IR).

The circulating plasma levels of S100A12, hs-CRP, and white blood cells (WBCs) were higher in the subjects with prediabetes and T2DM compared with the

Table 1. Clinical and Metabolic Characteristics of the Study Population According to Glucose Tolerance

	Controls (n = 99)	Prediabetes (n = 220)	T2DM (n = 61)	P[c]
Age, y	46 ± 11.2	49.1 ± 9.8[a]	50.9 ± 8[a]	<.0001
BMI, kg/m^2	28.8 ± 4.5	29.4 ± 3.9	30 ± 4.9	.12
Fasting glucose, mg/dL	86.4 ± 8.6	93.4 ± 11[a]	120.2 ± 19[a,b]	<.0001
Fasting insulin, microu/mL	7 (4.9–9.3)	7.6 (5.6–11.7)	8.2 (6.6–11.3)	.53
2-h glucose post-OGTT, mg/dL	117 ± 28.7	150.5 ± 42.6[a]	222.2 ± 39.7[a,b]	<.0001
HbA_{1c}, %	5.3 ± 0.2	6 ± 0.3[a]	7.1 ± 0.5[a,b]	<.0001
Total cholesterol, mg/dL	188.9 ± 36.6	195.5 ± 39.3	189.3 ± 41.5	.1
HDL cholesterol, mg/dL	47.8 ± 12.4	46 ± 11.5	39.1 ± 8.3[a,b]	<.01
Triglycerides, mg/dL	86 (66–122)	109.5 (78–151)[a]	117 (83–146)[a]	.004
LDL cholesterol, mg/dL	120.2 ± 32.8	129.2 ± 35.5[a]	126.2 ± 36	.01
Non-HDL cholesterol, mg/dL	140.4 ± 35.4	154 ± 38.6	150.1 ± 40.7	.09
Systolic BP, mm Hg	119 ± 15.4	122.6 ± 14.2	126.7 ± 15.4[a,b]	.02
Diastolic BP, mm Hg	71.8 ± 10.2	73 ± 10.6	76.2 ± 12.8[a]	.02
HOMA-IR	1.5 (1–2)	1.5 (1.6–3.3)	2.6 (1.9–0.72)[a,b]	<.0001
Hypertension, %	18	24	26	.24[d]
Active smokers, %	30	33	36	.63[d]
Statin therapy, %	20	24	23	.23[d]
Sex, M/F	37/62	97/93	27/34	.34[d]

The data are presented as the mean ± SD or median (interquartile range).

Smoking was quantified, and smoking status was classified as active smokers or nonsmokers.

Hypertension was defined as systolic BP ≥ 135 mm Hg or diastolic BP ≥ 85 mm Hg or as the prescription of any hypertension medications.

[a] $P < .05$ vs controls.

[b] $P < .05$ vs prediabetes.

[c] By ANOVA.

[d] By χ^2.

controls. CML plasma levels were similar among the three groups (Table 2).

Circulating plasma levels and MNC RNA expression of esRAGE in subjects with prediabetes

The plasma levels of esRAGE were significantly lower in the subjects with prediabetes than in the controls (0.32 ± 0.18 vs 0.52 ± 0.26 ng/mL; $P < .05$) but were similar to those with T2DM. As shown in Table 2, no differences were observed in the sRAGE levels among the three groups.

In the simple regression analysis, esRAGE was inversely associated with age ($r = -0.19$; $P < .05$), BMI ($r = -0.15$; $P < .04$), HbA_{1c} ($r = -0.18$; $P < .01$), hs-CRP ($r = -0.17$; $P < .05$) and S100A12 ($r = -0.28$; $P < .01$). To estimate the independent contributions of cardiovascular risk factors, glycation and inflammation markers to esRAGE levels, we performed multiple regression analysis using two models (see Statistical Analysis). The first model exhibited a significant correlation between esRAGE and age ($P < .001$), BMI ($P = .04$) and HbA_{1c} ($P = .02$). In the second model, the variables that

Table 2. Circulating Levels of sRAGE, esRAGE, Inflammatory Makers, CML, Intima-media Thickness (IMT) and Pulse Wave Velocity (PWV) According to Glucose Tolerance

	Control (n = 99)	Prediabetes (n = 220)	T2DM (n = 61)	P[c]
sRAGE, ng/mL	1.464 ± 596.1	1.328 ± 580.1	1.437 ± 532.3	.56
esRAGE, ng/mL	0.52 ± 0.26	0.32 ± 0.18[a]	0.3 ± 0.19[a]	<.0001
S100A12, ng/mL	5.35 ± 3.38	7.13 ± 5.4[a]	8.41 ± 4.44[a,b]	.04
hs-CRP, mg/dL	0.14 (0.08–0.27)	0.34 (0.11–0.47)[a]	0.45 (0.18–0.72) [a,b]	.001
WBC, $10^3/\mu L$	6.4 ± 1.6	7.1 ± 1.8[a]	7.2 ± 1.8[a]	.03
CML, $\mu g/mL$	2.64 ± 1.18	2.45 ± 1.26	2.46 ± 1.44	.51
IMT, mm	0.67 (0.6–0.73)	0.75 (0.65–0.78)[a]	0.78 (0.7–0.92)[a]	<.0001
PWV, m/sec	7.1 ± 1.7	7.6 ± 1.6[a]	8.6 ± 1.7[a,b]	<.0001

The data are presented as the mean ± SD or median (interquartile range).

[a] $P < .05$ vs controls.

[b] $P < .05$ vs prediabetes.

[c] By ANOVA.

Table 3. Multiple Regression Analysis Evaluating esRAGE, IMT and PWV as Dependent Variables

	Coefficient β	*P*
esRAGE		
Multiple regression—model 1[a]		
Age, y	−0.07	<.001
HbA_{1c}, %	−0.05	.02
BMI, kg/m^2	−0.007	.04
Multiple regression—model 2[b]		
Age, y	−0.005	<.001
HbA_{1c}, %	−0.09	.007
hs-CRP, mg/dL	−0.21	.01
IMT		
Multiple regression—model 1[a]		
Age, y	0.002	<.001
HbA_{1c}, %	0.03	.01
Multiple regression—model 2[b]		
Age, y	0.003	<.001
HbA_{1c}, %	0.04	.03
esRAGE, ng/mL	−0.06	.001
PWV		
Multiple regression—model 1[a]		
Age, y	0.025	<.001
Systolic BP, mm Hg	0.015	.01
Multiple regression—model 2[b]		
Systolic BP, mm Hg	0.02	.001
S100A12, ng/mL	1.1	.02

[a] Model 1 was adjusted for age, sex, smoking status, BMI, systolic BP, diastolic BP, HDL cholesterol, LDL cholesterol, non-HDL cholesterol, triglycerides, statin therapy, HbA_{1c}, fasting glycemia, and insulin.

[b] Model 2 was adjusted for sRAGE, esRAGE, WBC, S100A12, hs-CRP, and CML.

Table 4. Multiple Regression Analysis Evaluating esRAGE, IMT, and PWV as Dependent Variables for Prediabetic Group

	Coefficient β	*P*
esRAGE		
Multiple regression—model 1[a]		
Age, y	−0.07	<.001
BMI, kg/m^2	−0.04	.03
Multiple regression—model 2[b]		
Age, y	−0.006	.03
hs-CRP, mg/dL	−0.16	.01
IMT		
Multiple regression—model 1[a]		
Age, y	0.003	<.001
Systolic BP, mm Hg	0.01	.01
Multiple regression—model 2[b]		
Age, y	0.003	<.001
esRAGE, ng/mL	−0.04	.04
hs-CRP, mg/dL	0.07	.004
PWV		
Multiple regression—model 1[a]		
Age, y	0.025	<.0001
Systolic BP, mm Hg	0.031	.001
Multiple regression—model 2[b]		
S100A12, ng/mL	0.9	.02

[a] Model 1 was adjusted for age, sex, smoking status, BMI, systolic BP, diastolic BP, HDL cholesterol, LDL cholesterol, non-HDL cholesterol, triglycerides, statin therapy, HbA_{1c}, fasting glycemia, and insulin.

[b] Model 2 was adjusted for sRAGE, esRAGE, WBC, S100A12, hs-CRP, and CML.

remained significantly associated with esRAGE were HbA_{1c} ($P = .007$), hs-CRP ($P = .01$), and age ($P < .001$) (Table 3).

When the patients with prediabetes were analyzed separately from controls and T2DM, age ($P = .03$), hs-CRP ($P = .01$) remained significantly associated with esRAGE plasma levels (Table 4).

esRAGE is produced from the alternative splicing of the *RAGE* gene and is formed from the inclusion of part of intron 9 and the removal of exon 10, which changes the reading frame sequence of the protein and leads to the loss of both the transmembrane and cytosolic domains (20). To determine whether *esRAGE* gene expression was changed in our population, we performed an RT-PCR analysis of MNC from prediabetic and T2DM patients compared with control subjects (n = 10 for each group). The mRNA expression level of the *esRAGE* splice variant progressively decreased in patients with prediabetes and T2DM with respect to the controls (Figure 1).

IMT and PWV in subjects with prediabetes

IMT was higher in the patients with prediabetes than in the control group [0.75 (0.65–0.78) vs 0.67 (0.6–0.73) mm, $P < .05$]. Furthermore, subjects with new-onset T2DM exhibited no significant difference in IMT with respect to the prediabetic patients (Table 2).

IMT was associated with age (r = 0.55; $P < .0001$), HbA_{1c} (r = 0.4; $P < .001$), fasting glycemia (r = 0.34; $P < .001$), and esRAGE (r = −0.2; $P < .02$) in the simple regression analysis.

Next, we performed multiple regression analysis using two models (see Statistical Analysis). The first model exhibited a significant correlation among IMT, age ($P < .001$) and HbA_{1c} ($P = .01$). In the second model, the variables that remained significantly associated with IMT were HbA_{1c} ($P = .03$), age ($P < .001$), and esRAGE ($P = .001$) (Table 3).

In the subgroup analysis including only subjects with prediabetes the variables that were significantly associated with IMT were age ($P = .003$), esRAGE ($P = .04$), and hs-CRP ($P = .004$) (Table 4).

The PWV was significantly higher (7.6 ± 1.6 vs 7.1 ± 1.7 m/sec, $P < .05$) in the patients with prediabetes than in the control subjects (Table 2). Furthermore, these subjects exhibited lower PWV with respect to new-onset T2DM patients.

PWV was associated with age (r = 0.29; $P < .0001$), HbA_{1c} (r = 0.25; $P < .001$), fasting glycemia (r = 0.18;

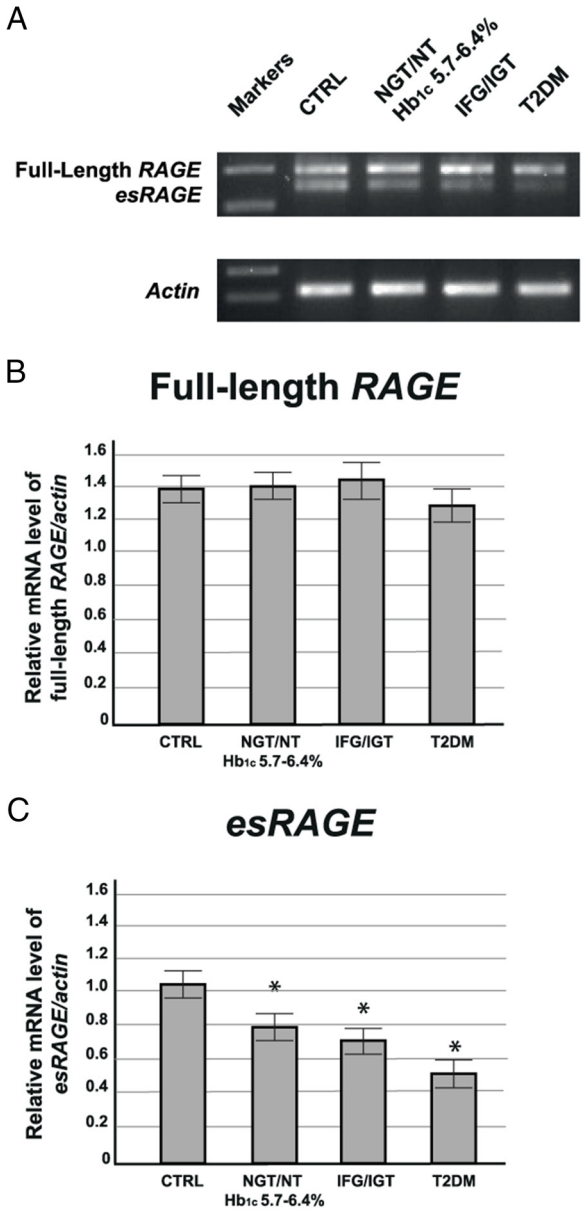

Figure 1. Differential expression of full-length *RAGE* and *esRAGE* in peripheral blood mononuclear cells (PBMC) by RT-PCR. A, Representative agarose gel image for RT-PCR analysis of full-length *RAGE* (282 bp) and *esRAGE* (216 bp) splicing variants in PBMC from controls; $HbA1_c$, 5.7–6.4% with NFG/NT, IFG/IGT, and T2DM subjects. Statistical data showing the mRNA ratio of full-length *RAGE*/*β-actin* (B) and *esRAGE*/*β-actin* (C). The data are expressed as the mean ± SD from ten different experiments; *, $P < .05$.

$P < .01$), systolic BP (r = 0.27; $P < .0001$), diastolic BP (r = 0.22; $P < .0001$), triglycerides (r = 0.16; $P < .01$), HDL cholesterol (r = 0.16; $P < .01$), CML (r = 0.17; $P < .05$), and S100A12 (r = 0.14; $P < .01$) in the simple regression analysis.

Subsequently, we performed multiple regression analysis using two models (see Statistical Analysis). The first model exhibited a significant correlation between PWV and age ($P < .001$) and systolic BP ($P = .01$). In the second model, the variables that remained significantly associated with PWV were S100A12 ($P = .02$) and systolic BP ($P = .001$) (Table 3). In the subgroup analysis including only subjects with prediabetes the variables that were significantly associated with PWV was S100A12 ($P = .02$) (Table 4).

Circulating levels of sRAGE, esRAGE splice variant, and inflammatory profile in subjects with prediabetes according only to HbA_{1c} with normal fasting glycemia and normal glucose tolerance after OGTT

As a new approach for prediabetes diagnosis, it is unclear whether HbA_{1c} could provide additional information regarding cardiovascular risk compared with fasting glycemia and glucose tolerance. In line with this question, we focused on individuals with prediabetes identified only by HbA_{1c} (5.7–6.4%) who had NFG and were NT after OGTT. Thus, we re-examined the prediabetic population separating the patients with prediabetes identified only by HbA_{1c} from all those with IFG and/or IGT after the OGTT. As shown in Figure 2, the subgroup with prediabetes identified only by HbA_{1c} (n = 127) showed significantly lower plasma esRAGE levels compared with controls (0.29 ± 0.18 vs 0.45 ± 0.26 ng/mL; $P < .05$) but similar to those of patients with IFG and/or IGT (n = 93) and T2DM (n = 61). Circulating plasma levels of S100A12 were higher in subjects with prediabetes than in controls according only to HbA_{1c} and IFG and/or IGT. sRAGE and CML plasma levels were similar among the four groups (Figure 2). The mRNA expression level of the *esRAGE* splice variant progressively decreased in patients with prediabetes with respect to controls according only to HbA_{1c}, IFG/IGT, and T2DM (Figure 1).

Discussion

In this study we measured sRAGE and esRAGE and examined their association with other proinflammatory factors and early markers of atherosclerosis in subjects with prediabetes.

We found that prediabetic patients exhibited lower esRAGE plasma levels than controls and that the levels were similar to those of patients with T2DM. Furthermore, we demonstrated an inverse association between low plasma esRAGE and carotid atherosclerosis in prediabetic patients. In addition, S100A12, a proinflammatory molecule strongly linked with RAGE signaling, was increased in prediabetic and diabetic patients with respect to controls.

Although diabetes imparts a 2- to 3-fold increase in the risk of developing macroangiopathy, the magnitude of the risk for cardiovascular disease associated with prediabetes

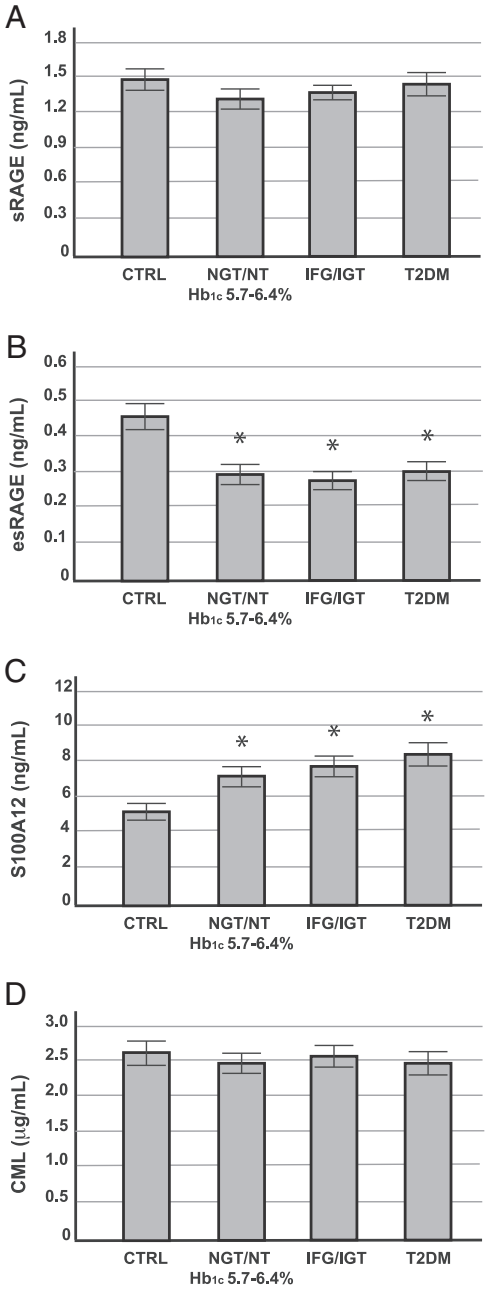

Figure 2. Circulating levels of sRAGE (A), esRAGE (B), S100A12 (C), CML (mean ± SD) (D), according to glucose tolerance and HbA_{1c} levels.

remains unclear and is dependent on diagnostic criteria. In consideration of the expected increased use of HbA_{1c} as a screening tool to identify individuals with alteration of glycemic homeostasis, it is important to evaluate the ability of HbA_{1c} to identify patients who are at increased risk for cardiovascular disease. With this aim, we specifically focused on individuals with prediabetes identified only by HbA_{1c} (5.7–6.4%) who had NFG and were NT after OGTT. As previously reported (5), we found that these subjects exhibited an alteration of early markers of cardiovascular injury. In addition, we found that NFG/NT subjects with high HbA_{1c} (≥5.7%) showed low esRAGE levels and high S100A12 concentration. These data may suggest that HbA_{1c} can identify subjects with NFG and NT with alteration of early markers of cardiovascular damage. Accordingly, in a recent prospective study, European subjects with prediabetes identified by HbA_{1c} experienced a significant increase in coronary heart and cardiovascular disease with respect to normoglycemic subjects (21). All of these observations suggest that HbA_{1c} may be a relevant marker of cardiovascular risk.

In our study, carotid IMT, a good predictor of cardiovascular disease, was independently associated with low esRAGE plasma levels, HbA_{1c}, and age. Recent clinical reports have focused on the potential significance of circulating esRAGE and sRAGE in a variety of pathophysiological conditions. Studies that used a detection system to specifically measure human esRAGE cross sectionally reported its significant and inverse association with atherosclerosis in metabolic syndrome and T1DM and T2DM (22). In agreement with our data, these findings suggest that esRAGE may represent a potential protective factor for carotid atherosclerosis and suggest that the inverse correlation between esRAGE and cardiovascular risk is not confined to diabetes but is extended to nondiabetic populations as well.

esRAGE plasma levels were tightly and inversely correlated with HbA_{1c} and hs-CRP in multiple regression analysis after adjusting for other risk factors. The inverse association between esRAGE and glycemic control is unsurprising. Katakami et al (23) have reported an inverse and significant association between esRAGE and HbA_{1c} in patients with T1DM. Furthermore, Koyama et al (24) found that esRAGE is significantly and inversely correlated with HbA_{1c} and components of metabolic syndrome in T2DM and nondiabetic subjects. Because a number of factors, such as glycemic control, inflammation, underlying disease, renal function, and drugs seem to affect plasma esRAGE levels, we evaluated *esRAGE* mRNA expression in MNCs isolated from peripheral blood samples. We observed decreased mRNA expression of full-length *RAGE* and *esRAGE* in patients with prediabetes and T2DM with respect to controls. The regulatory mechanism for alternative splicing to generate esRAGE remains unclear, and environmental or genetic factors may be involved. Other studies have reported the ability of some oral agents such as statins to modulate AGE/RAGE axis increasing circulating esRAGE levels in patients with T2DB (12). In this study we did not exclude patients who were on lipid lowering therapy; however, we found no difference in the percentage of patients on statin therapy in each group and further statistical analysis evidenced that our results are not affected by statin therapy. Another

possibility is that esRAGE production may be down-regulated via hyperglycemia-induced oxidative stress directly or via increased cytokines.

Shemitani et al (25), in a recent molecular study on K562 cells, supported this hypothesis and revealed a significant decrease in sRAGE levels following treatments with high glucose. In Chinese patients with T2DM, an association between *RAGE* gene polymorphisms and circulating levels of esRAGE has been reported (26). Other studies are needed to explain the precise mechanism underlying the decrease in sRAGE in diabetic and nondiabetic humans.

Inflammation may play a role in linking the RAGE system with atherosclerosis because RAGE ligands include proinflammatory proteins. In the current study, esRAGE was initially associated with hs-CRP and S100A12 in an unadjusted model. After adjustment for conventional risk factors and inflammatory markers, only hs-CRP remained significantly associated with esRAGE, and the association with S100A12 disappeared.

In our study, we found no differences in sRAGE plasma levels among the three groups. To date, the data regarding circulating total sRAGE levels remain controversial (27). Falcone et al (28) reported that low levels of sRAGE in plasma are independently associated with CHD in nondiabetic men. Furthermore, Basta et al (27) reported that plasma sRAGE levels were lower in diabetic patients than in controls. Low levels of sRAGE have been indicated as being associated with the risk of diabetes, CHD, and mortality in humans, and the administration of sRAGE suppressed accelerated diabetic atherosclerosis in animal models (29). In subjects with T1DM, higher sRAGE levels are reportedly associated with cardiovascular events and all-cause mortality (10). Accordingly, Nakamura et al (22) demonstrated that serum sRAGE levels are significantly higher in T2DM patients than in nondiabetic subjects and are positively associated with the presence of coronary artery disease. The disagreement among these studies may be the result of the use of a detection system that was unable to discriminate between specific sRAGE variants.

We also found high levels of S100A12 in subjects with prediabetes and a tight correlation between S100A12 and PWV, an early marker of cardiovascular disease. Many studies have reported that elevated concentrations of acute phase reactants, such as S100A12, IL-6, and TNF-α, are found in patients with atherosclerosis-related complications. However, to date, little is known regarding the modulation of S100A12 in patients with alterations of glucose homeostasis and its potential association with cardiovascular disease. Basta et al (27) observed higher plasma S100A12 levels in a group of diabetic patients than in age-matched controls and a strong association with increased cardiovascular risk. Other studies have reported that S100A12 was independently associated with major cardiovascular events in patients with chronic heart failure, highlighting the potential role of S100A12 as a biomarker for cardiovascular disease.

There were several limitations of this study. First, this was a cross-sectional study, and a longitudinal causal relationship cannot be established between changes in plasma esRAGE and the IMT. Furthermore, *sRAGE* gene expression should be better characterized using quantitative analyses on larger groups of patients. Finally, although we did not perform an opportunistic procedure during recruitment, the group with high HbA_{1c} and NFG/NT represents, in this study, approximately 30% of the entire population and is, therefore, not a rare subset. In line with these finding, Rosella et al (30) have recently reported that the prevalence of undiagnosed prediabetes in a representative sample of Canadians was significantly greater when using screening strategies that used HbA_{1c} measures compared with plasma glucose diagnostic criteria.

In conclusion, subjects with prediabetes exhibit low esRAGE plasma levels and increased levels of markers of inflammation. These findings suggest that esRAGE may play an important role in activation and progression of atherosclerotic disease in this population.

The further examination of the molecular mechanism underlying esRAGE regulation will provide potential targets for the prevention or treatment of cardiovascular disease.

Acknowledgments

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