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Association of the Gly82Ser polymorphism in the receptor for advanced glycation end products (*RAGE*) gene with circulating levels of soluble RAGE and inflammatory markers in nondiabetic and nonobese Koreans

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Abstract

We investigated the association between the Gly82Ser (G82S) polymorphism in the receptor for advanced glycation end products (*RAGE*) gene and circulating levels of soluble RAGE (sRAGE), advanced glycation end products (AGEs), and inflammatory markers in nondiabetic/nonobese Koreans. A total of 1096 men and 580 women aged 30 to 69 years and with body mass index of 18.5 to 29.9 kg/m^2 were recruited. Anthropometrics, lipid profiles, glucose, insulin, insulin resistance (IR), *RAGE* G82S polymorphism, sRAGE, AGEs, and inflammatory markers were measured. There was a significant association between G82S genotypes and plasma sRAGE concentrations ($P < .001$). sRAGE concentrations were significantly higher in subjects with the G/G genotype (1038 ± 33 pg/mL) than in those with the G/S (809 ± 19 pg/mL) or the S/S (428 ± 43 pg/mL) genotype. Furthermore, the G82S genotypes in the *RAGE* gene were associated with serum AGE ($P = .033$), homeostasis model assessment for insulin resistance (HOMA-IR) ($P < .001$), plasma tumor necrosis factor α (TNF-α) ($P = .033$), serum C-reactive protein (CRP) ($P = .002$), and urinary excretion of 8-*epi*-prostaglandin $F_{2\alpha}$ ($P = .028$) after adjusting for sex, age, body mass index, cigarette smoking, and alcohol drinking. Subjects with the S/S genotype showed higher levels of serum AGE, HOMA-IR, plasma TNF-α, serum CRP, and 8-*epi*-prostaglandin $F_{2\alpha}$ than those with the G/G or G/S combination. The sRAGE levels showed a negative relation with high-sensitivity CRP ($r = -0.250$; $P < .001$). The AGE concentrations showed a positive relation with TNF-α levels ($r = 0.398$; $P < .001$). Subjects with homozygosity for the minor S allele (S/S) of the G82S polymorphism had higher risk factors for cardiovascular disease, such as low sRAGE levels, inflammation, oxidative stress, and IR, compared with those bearing at least one G allele.

1. Introduction

The receptor for advanced glycation end products (RAGE) is a multiligand member of the immunoglobulin superfamily of cell surface molecules [1] and engages diverse ligands relevant to distinct pathologic processes [2]. Irreversible advanced glycation end products (AGEs) are one class of RAGE ligands and occur at an increased level under conditions of hyperglycemia and in inflammatory environments [3,4]. Sustained interaction with higher levels of AGEs increases receptor expression and activation of proinflammatory and procoagulant pathways [5-7], which may be the key factors linking the RAGE system with atherosclerosis [8,9].

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RAGE has a C-truncated secretory isoform of the receptor protein, termed soluble RAGE (sRAGE) that may neutralize AGE-mediated damage by acting as a decoy [10-12]. In humans, sRAGE is produced by alternative splicing of RAGE messenger RNA and is abundantly present in the circulation [10,12,13]. Because sRAGE has been shown to successfully bind to AGEs [14], it has been postulated that this soluble isoform could play an antagonistic role by competing with the cell surface receptor, thus preventing the adverse effects of RAGE signaling [15]. The proportion and production of sRAGE may therefore influence the regulation of RAGE-mediated functions in various tissues and inflammatory conditions [16].

Polymorphisms in *RAGE* may also alter AGE processing in tissues or reactions after the binding of AGEs to RAGE. The gene encoding *RAGE* is located on chromosome 6 in the major histocompatibility complex, a region of the genome containing a number of inflammatory genes [1]. The *RAGE* gene contains 11 exons and a 3′ untranslated region. Within the exons, a common variant (Gly82Ser [G82S]) and 3 rare changes (Thr18Pro, Gly329Ala, Ala389Gln) have been identified. The present study focuses on the *RAGE* G82S polymorphism because of its location in the ligand-binding V domain of *RAGE* [17]. Based on the recent observation that cells expressing the S82 isoform of RAGE displayed enhanced ligand-binding affinity and increased generation of inflammatory mediators [17], we tested for a possible association between the *RAGE* G82S polymorphism and circulating concentrations of sRAGE and AGE in a group of healthy Koreans who genetically and environmentally differ from previously reported studies in Caucasians [18]. In addition, we examined the association of G82S with other inflammatory markers including tumor necrosis factor α (TNF-α) and C-reactive protein (CRP).

2. Subjects and methods

2.1. Study subjects

Participants were recruited from the Health Service Center in the course of a routine checkup visit or by a newspaper announcement. The newspaper announcement briefly described the study design and invited healthy Koreans aged 30 years or older to participate in the Korean Cardiovascular Genome Center study as control subjects. Of the individuals who presented, only those without any clinical or anamnestic evidence of a history of atherosclerosis and without evidence of any pathologic electrocardiogram pattern were selected. The age range was 30 to 69 years, and the body mass index (BMI) range was 18.5 to 29.9 kg/m^2. Individuals identified as having diabetes were not recruited. Diabetes was ascertained according to the American Diabetes Association criteria in which diabetes is defined as a fasting plasma glucose concentration of 7 mmol/L or greater or current treatment with antidiabetic agents. Finally, a total of 1676 nondiabetic and nonobese subjects (1096 men and 580 women) were recruited. To the authors' knowledge, all the study participants were unrelated. All individuals who presented received the results of their evaluations for classical and treatable risk factors for personal use later. No individuals included were taking any medication or had diagnoses of cancer. Written informed consent was obtained from all subjects and the protocol was approved by the institutional review board of Yonsei University.

After signing an informed consent, participants were administered a standard questionnaire, and physical measurements were taken by a registered dietitian. Current cigarette smokers were defined as subjects reporting smoking at least 1 cigarette per day. Total alcohol intake was expressed as the sum of milliliters of alcohol per day.

2.2. Anthropometrics, blood pressure measurements, and blood collection

Anthropometric measurements included height, body weight, and waist and hip circumferences. Body height and weight were measured in the morning with the subject unclothed and without shoes. Body mass index was calculated as body weight in kilograms divided by height in square meters. Circumferences of waist and hip were measured with the subject in the standing position after normal expiration, and waist-hip ratio (WHR) was computed. Blood pressure was read from the left arm of seated subjects with an automatic blood pressure monitor (TM-2654, A&D, Tokyo, Japan) after 20 minutes of rest. The average of 3 measurements was recorded for each subject.

Venous blood specimens were collected in EDTA-treated and plain tubes after a 12-hour fast. The tubes were immediately placed on ice until they arrived at the laboratory (within 1-3 hours) and were stored at −70°C until analysis after plasma and serum were separated.

2.3. Genotyping of RAGE G82S

Genomic DNA was prepared from peripheral blood samples by using a Puregene DNA purification kit (Gentra, Minneapolis, MN), following the manufacturer's protocol. A *RAGE* single nucleotide polymorphism (SNP) (rs17846805, Gly82→Ser [G82S]) was genotyped in 1676 subjects. G82S genotyping was performed by SNP-IT assays using SNPstream 25K System (Orchid Biosciences, Princeton, NJ). Briefly, the genomic DNA region spanning the polymorphic site was amplified by polymerase chain reaction (PCR) using 1 phosphothiolated primer and 1 regular PCR primer. The amplified PCR products were then digested with exonuclease (Amersham Biosciences, Uppsala, Sweden). The 5′ phosphothiolates were used in this study to protect 1 strand of the PCR product from exonuclease digestion. The single-stranded PCR template generated from exonuclease digestion was overlaid onto a 384-well plate that was precoated covalently with SNP-IT primers (Integrated DNA Technologies, Coralville, IA).

These SNP-IT primers were designed to hybridize immediately adjacent to the polymorphic site. After hybridization of template strands, SNP-IT primers were then extended by a single base with DNA polymerase at the polymorphic site of interest. The SNP-IT primer is extended for a single base with DNA polymerase and a mixture of an appropriate acycloterminator, which is labeled with either fluorescein isothiocyanate) or biotin (Perkin-Elmer Asia, Singapore) and complementary to the polymorphic nucleotide. The final single base incorporated was identified with serial colorimetric reactions with anti-fluorescein-AP (Roche, Basel, Switzerland) and streptavidin-horseradish peroxidase (Pierce, Rockford, IL), respectively. The results of blue and/or yellow color developments were analyzed with an enzyme-linked immunosorbent assay (ELISA) reader; the final genotyping (allele) calls were made with the QCReview program.

2.4. Serum lipid profiles

Fasting serum concentrations of total cholesterol and triglycerides were measured with commercially available kits on a Hitachi 7150 Autoanalyzer (Hitachi, Tokyo, Japan). After using dextran sulfate magnesium to precipitate serum chylomicron, low-density lipoprotein (LDL), and very low-density lipoprotein, the remaining high-density lipoprotein (HDL) cholesterol from the supernatant was measured by an enzymatic method [19]. LDL cholesterol was indirectly estimated in subjects with serum triglyceride concentrations less than 4.52 mol/L (400 mg/mL) by using the Friedewald formula [20]. In subjects with serum triglyceride concentrations of 4.52 mol/L or higher, LDL cholesterol was measured directly.

2.5. Glucose, insulin, and HOMA-IR

Fasting glucose was measured by the glucose oxidase method using a Beckman Glucose Analyzer (Beckman Instruments, Irvine, CA). Insulin was measured by radioimmunoassays with commercial kits from Immuno-Nucleo (Stillwater, MN). Insulin resistance (IR) was calculated with the homeostasis model assessment (HOMA), using the following equation: HOMA-IR = [fasting insulin (μIU/mL) $\times$ fasting glucose (mmol/L)]/22.5 [21].

2.6. sRAGE and AGE

The plasma full sRAGE level was measured with a commercially available ELISA kit (R&D Systems, Minneapolis, MN). The resultant color reaction was read at 450 nm with a Victor^2 (Perkin Elmer Life Sciences, Turku, Finland), and wavelength correction was set to 540 nm.

Measurement of AGEs in serum was performed by a noncompetitive ELISA according to the method of Horiuchi et al [22]. A 96-well microplate was coated at 4°C overnight with serially diluted AGE–bovine serum albumin (BSA) as standards or diluted serum samples. Unbound sites were blocked with 2% skim milk, 0.5% BSA, and 0.5% gelatin in 0.05 mol/L carbonate buffer for 1 hour and washed with washing buffer (phosphate-buffered saline [PBS], 0.05% Tween 20). Each well was incubated for 2 hours with anti-AGE monoclonal antibody (dilution 1:1000; Transgenic, Kumamoto, Japan). The plate was washed again and incubated with horseradish peroxidase–labeled goat antimouse IgG antibody (dilution 1:2000, Zymed, San Francisco, CA) for 1 hour. The unbound antibodies were removed by washing and bound antibodies were detected by incubation with 3,3′ ,5,5′ -tetramethylbenzidine substrate for 30 minutes. After the reaction was stopped by addition of 1 mol/L sulfuric acid, the resultant color reaction was read at 450 nm with a Victor^2 (Perkin Elmer Life Sciences).

2.7. TNF-α and hs-CRP

Plasma TNF-α levels were assayed with a Quantikine ELISA kit (Human TNF-α, R&D Systems, Inc, USA) and the results measured by a Victor^2 (Perkin Elmer Life Sciences, Turku, Finland) at 450 nm with wavelength correction set to 540 nm. Quantification of TNF-α was performed by using the peak area ratio. Serum high-sensitivity CRP (hs-CRP) levels were measured with an Express$^+$ autoanalyzer (Chiron Diagnostics, Walpole, MA) using a commercially available, high-sensitivity CRP-Latex (II) $\times$2 kit (Seiken Laboratories, Tokyo, Japan) that allowed detection of CRP levels as low as 0.001 mg/dL and as high as 32 mg/dL.

2.8. Urine collection and 8-epi-prostaglandin $F_{2\alpha}$

Urine was collected after a 12-hour fast in polyethylene bottles containing 1% butylated hydroxytoluene before blood collection. The tubes were immediately covered with aluminum foil and stored at −70°C until analysis. 8-*epi*-Prostaglandin $F_{2\alpha}$ (8-*epi*-$PGF_{2\alpha}$) was measured with the use of an enzyme immunoassay (BIOXYTECH urinary 8-*epi*-$PGF_{2\alpha}$ assay kit, OXIS International, Portland, OR). The resulting color reaction was read with the use of a Victor ^{2} (Perkin Elmer Life Sciences) at 650 nm. Urinary creatinine level was determined by using the alkaline picrate (Jaffe) reaction [23], and urinary 8-*epi*-$PGF_{2\alpha}$ concentrations were expressed as picomoles per millimole creatinine.

2.9. Statistical analysis

Statistical analyses were performed with Statistical Package for Social Sciences (SPSS) version 12.0 for Windows (SPSS, Chicago, IL). Hardy-Weinberg equilibrium was examined by using the Executive SNP Analyzer 1.0 (http://www.istech21.com/en/index.html). One-way analysis of covariance followed by a Bonferroni test and a general linear model for adjustment of potential covariates such as age, BMI, cigarette smoking, and alcohol drinking were performed to compare the differences in biomarkers among genotype groups. Pearson correlation analysis was also examined among variables. Each variable was examined for normal distribution patterns, and significantly skewed variables were log transformed. For descriptive purposes,

Table 1
Anthropometrical parameters, lipid profiles, serum glucose and insulin levels according to RAGE G82S genotypes

	G/G (n = 1180)	G/S (n = 449)	S/S (n = 47)
Male/female (n)	769/411	301/148	26/21
Age (y)	49.6 ± 0.29	49.8 ± 0.48	47.2 ± 1.58
BMI (kg/m^2)	24.5 ± 0.07	24.3 ± 0.11	25.0 ± 0.31
WHR	0.89 ± 0.00	0.88 ± 0.00	0.89 ± 0.01
Current smokers (%)	20.9	24.6	23.4
Tobacco (cigarettes per day)	12.8 ± 0.54	12.3 ± 0.98	14.2 ± 2.28
Current drinkers (%)	64.5	64.1	66.7
Alcohol intake (g/d)	18.8 ± 1.25	15.9 ± 1.79	29.0 ± 15.4
Systolic blood pressure (mm Hg)	119.9 ± 0.45	120.7 ± 0.77	124.7 ± 2.33
Diastolic blood pressure (mm Hg)	77.6 ± 0.30	77.8 ± 0.53	80.4 ± 1.64
Triglyceride (mg/dL)[a]	148.9 ± 2.68	141.4 ± 4.14	146.4 ± 12.5
Total cholesterol (mg/dL)	203.4 ± 1.04	199.5 ± 1.75	192.9 ± 3.95
HDL cholesterol (mg/dL)	48.7 ± 0.36	47.7 ± 0.55	46.5 ± 1.72
LDL cholesterol (mg/dL)	124.4 ± 1.07	122.2 ± 1.75	118.4 ± 3.88
Glucose (mg/dL)	88.5 ± 0.33	87.7 ± 0.53	89.3 ± 1.95

Data are presented as mean ± S.E and or percentage. Determined by one-way analysis of covariance followed by a Bonferroni test and a general linear model for adjustment of sex, age, BMI, cigarette smoking, and alcohol drinking.

[a] Log transformed.

mean values are presented using untransformed and unadjusted values. Results are expressed as mean ± SE. A 2-tailed value of P less than .05 was considered statistically significant.

3. Results

3.1. Frequency of the RAGE G82S polymorphism

The *RAGE* G82S genotype distribution among the 1676 subjects examined was as follows: 1180 subjects examined were homozygous for the G allele (G/G), 449 were heterozygous for the S allele (G/S), and 47 were homozygous for the S allele (S/S). These frequencies did not deviate significantly from Hardy-Weinberg equilibrium. The S allele frequency was 0.16, much greater than that reported in Caucasians (≈0.05) [1,17,24,25].

3.2. Anthropometric and clinical characteristics according to the RAGE G82S genotype

The general characteristics of the subjects according to the *RAGE* G82S genotype are shown in Table 1. There were no significant G82S genotype–related differences with respect to age, BMI, WHR, sex distribution, consumption of cigarettes and alcohol, blood pressure, lipid profiles, or serum glucose (Table 1). However, the G82S genotypes in the *RAGE* gene were associated with serum insulin (P < .001) (Table 1) and HOMA-IR (P < .001) (Fig. 1). Both before ($P0$) and after ($P1$) adjusting for potential covariates of sex, age, BMI, cigarette smoking and alcohol drinking, the insulin concentration ($P0$ = .007, $P1$ = .013) and HOMA-IR ($P0$ < .001, $P1$ < .001) were significantly higher in subjects with the S/S genotype than in those with the G/G or the G/S genotype (Fig. 1).

3.3. sRAGE, AGE, inflammatory markers, adiponectin, and lipid peroxides

There was a significant association between G82S genotypes and plasma sRAGE concentrations ($P0$ < .001, $P1$ < .001) (Fig. 1). sRAGE concentration was significantly higher in subjects with the G/G genotype (1038 ± 33 pg/mL) than in those with the G/S (809 ± 19 pg/mL) or the S/S (428 ± 43 pg/mL) genotype, and significantly higher in individuals with the G/S genotype than in those with the S/S genotype. The G82S genotypes in the *RAGE* gene were also associated with serum AGE ($P0$ = .013, $P1$ = .033), plasma TNF-α ($P0$ = .016, $P1$ = .033), serum hs-CRP ($P0$ = .003, P = .002), and urinary excretion of 8-*epi*-PGF$_{2\alpha}$ ($P0$ = .033, $P1$ = .028). Subjects with the S/S genotype showed higher levels of serum AGE (Fig. 1), plasma TNF-α (Fig. 2), serum CRP (Fig. 2), and urinary excretion of 8-*epi*-PGF$_{2\alpha}$ (Fig. 2) than those with the G/G or G/S combination.

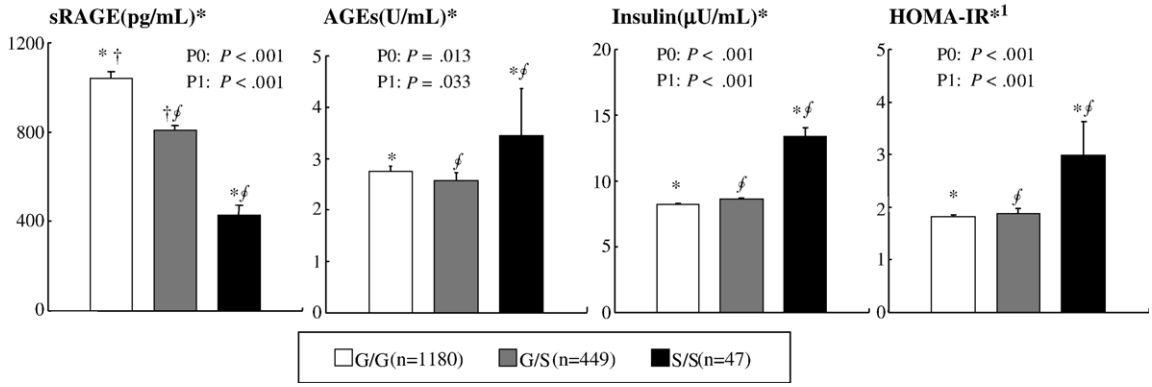

Fig. 1. Influence of RAGE G82S genotypes on sRAGE, AGEs and HOMA-IR in healthy Koreans. Mean ± S.E. *Log transformed. *P < .05 between G/G and S/S; †P < .05 between G/G and G/S; ϕP < .05 between G/S and S/S based on 1-way analysis of covariance followed by a Bonferroni test and a general linear model for adjustment of sex, age, BMI, cigarette smoking, and alcohol drinking. [1]Insulin resistance = [fasting insulin (μU/mL) × fasting glucose (mmol/L)]/22.5.

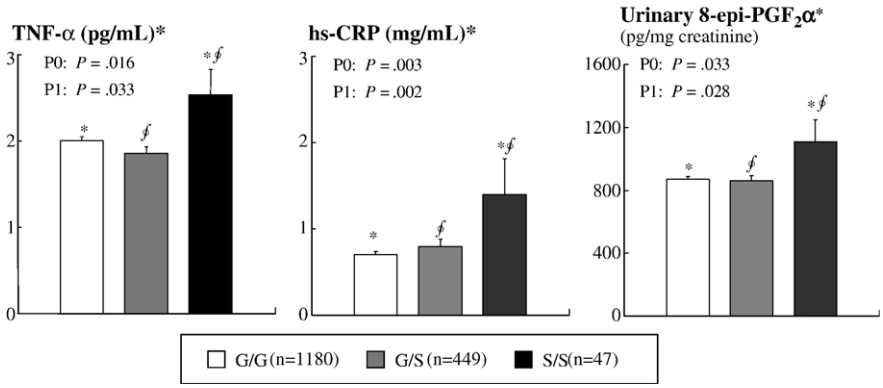

Fig. 2. Influence of RAGE G82S genotypes on TNF-α, hs-CRP, and urinary PGF2 α in healthy Koreans. Mean ± S.E. *Log transformed. *$P < .05$ between G/G and S/S; †$P < .05$ between G/G and G/S; ϕ$P < .05$ between G/S and S/S based on 1-way analysis of covariance followed by a Bonferroni test and a general linear model for adjustment of sex, age, BMI, cigarette smoking, and alcohol drinking.

3.4. Relation of sRAGE and AGE with inflammatory markers

Circulating sRAGE levels showed a highly significant negative relation with serum hs-CRP ($r = -0.250$, $P < .001$) in healthy subjects. Serum AGE concentrations showed a highly significant positive relation with plasma TNF-α levels ($r = 0.398$, $P < .001$). These correlations were maintained even after adjusting for sex, age, BMI, cigarette smoking, and alcohol drinking (sRAGE and hs-CRP: $r = -0.247$, $P < .001$; AGE and TNF-α: $r = 0.266$, $P < .001$) (data not shown).

4. Discussion

The current study shows a strong association ($P < .001$) between *RAGE* G82S genotypes and circulating sRAGE concentrations in healthy Korean subjects after adjustment for potential confounders, including age, sex, BMI, and smoking and alcohol consumption. sRAGE, a scavenger that prevents ligand binding to RAGE [9], is a soluble receptor produced by alternative splicing of RAGE messenger RNA [11]. sRAGE is known to contribute to the removal/detoxication of AGE by acting as a decoy [26]; thus, the highly circulating levels of AGE detected in S/S subjects of this study might partly relate to low levels of sRAGE. In addition, concentrations of AGE were similar between G/G and G/S subjects, although concentrations of sRAGE were lower in G/S compared with G/G subjects, indicating the possibility of the presence of the threshold concentration of sRAGE to increase in circulating AGE concentration. This should be elucidated by further study.

Recently, genetic variants of the full-length receptor or its novel splice forms have been suggested to possibly contribute to the risk for atherosclerosis, distinct from environmental factors [27]. Falcone et al [8] have found that low circulating levels of sRAGE are independently associated with the presence of CAD in nondiabetic men and suggested that sRAGE is one of the clinically important molecules associated with atherosclerosis. Lower levels of sRAGE have been suggested to increase the propensity toward inflammation because RAGE ligands have better access to RAGE, the binding of which leads to the activation of inflammatory pathways [16]. The production of sRAGE may therefore influence the regulation of RAGE-mediated functions in inflammatory conditions [16]. Interestingly, in this study we observed a negative correlation between the sRAGE level and CRP concentration, which is known to be a strong independent predictor of future coronary events in healthy subjects [28]. Furthermore, we also found a positive correlation between AGE levels and TNF-α concentrations.

Subjects bearing the S/S genotype had significantly higher levels of serum CRP and plasma TNF-α than those bearing G/G or G/S, supporting a previous suggestion relating to the G82S polymorphism in the AGE-binding domain of the *RAGE* gene on the inflammatory response [1,11]. Although the S allele at the *RAGE* G82S polymorphism has displayed increased ligand-stimulated generation of proinflammatory proteins [29], RAGE ligands such as AGEs are thought to be more important than RAGE itself in the propagation of inflammatory response [5] and oxidative stress, not only contributing to vascular dysfunction but also potentially amplifying the insulin-resistant state [30-32].

Formation of AGEs is known to occur naturally with aging and at an accelerated level under hyperglycemia and during inflammation [1]. In this study, higher concentrations of inflammatory markers may have resulted in higher circulating AGE concentrations in subjects with the S/S genotype, as serum glucose levels, age, BMI, and macronutrient intake levels (data not shown) were not significantly different between the genotype groups. Our results of S/S subjects having higher HOMA-IR and urinary excretion of $PGF_{2\alpha}$ than those with G/G or G/S further support the fact that both this variant and high levels of AGEs could lead to a cascade of proinflammatory responses that are important in oxidative stress and the pathogenesis of insulin resistance [25].

In summary, the association of G82S genotypes of the *RAGE* gene with circulating sRAGE concentration might be established in the current study in healthy Korean subjects. In particular, subjects with homozygosity for the minor S allele of the G82S polymorphism had higher risk factors for cardiovascular disease, such as low sRAGE levels, inflammation, oxidative stress, and insulin resistance, compared with those bearing at least 1 G allele. Our study suggests that the genotype effects on sRAGE levels might determine AGE concentrations and AGE engagement of the cell surface RAGE, which result in increased expression of cytokines and induction of oxidative stress and insulin resistance. However, we could not definitely conclude that the *RAGE* gene is involved in the proinflammatory responses that could potentially lead to insulin resistance, based on analyzing only 1 polymorphism, G82S. To clarify the association between *RAGE* SNPs, sRAGE, AGE, and proinflammatory markers, further study on other SNPs in the *RAGE* gene as well as an intervention study are needed to confirm these data.

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All authors were involved in the development of the study protocol and the experimental design. Yangsoo Jang, Seok-Min Kang, Jung-Sun Kim, Hyun Chul Lee, Chul Woo Ahn, and Young Duk Song managed the recruitment and schedule of the subjects. Jeysook Chae, Soo Jeong Koh, and Ji Young Kim performed sample collection and experiments. Ji Young Kim performed DNA analysis. Soo Jeong Koh, and Oh Yoen Kim analyzed data. Jong Ho Lee wrote the draft manuscript with contribution from Yangsoo Jang. All the authors read, commented on, and contributed to the submitted and revised manuscripts.

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