Polymorphisms 1704G/T and 2184A/G in the *RAGE* Gene Are Associated With Antioxidant Status

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The formation of advanced glycation end products (AGEs) and oxidative stress are supposed to play an important role in the development of diabetic late complications. AGEs can bind to several binding sites including receptor of advanced glycation end products (RAGE). AGE-RAGE interaction results in free radical generation. The aim of the present study was to investigate the impact of previously described polymorphisms in the *RAGE* gene (G82S, 1704G/T, 2184A/G, and 2245G/A) on the glycoxidation status in non–insulin-dependent diabetes mellitus (NIDDM). A total of 371 unrelated caucasian subjects were enrolled in the study. The NIDDM group consisted of 202 subjects, and the presence of late diabetic complications in 5 particular localizations was expressed as an index (I_{compl}). The nondiabetic group included 169 subjects. Glycated hemoglobin (HbA_{1c}), glycated stratum corneum proteins (Amadori, AGE), total carotenoids, α- and β -carotene, γ-tocopherol, lutein, lycopene, and α-tocopherol were measured in each subject. Statistically significant differences in allele frequencies between the NIDDM and the nondiabetic groups were observed for the G82S and 2245G/A polymorphisms ($P = .047$ and .032, respectively. HbA_{1c}, Amadori, and AGE did not reveal any significant association with any of the polymorphisms analyzed. However, significant differences between subjects bearing "wild-type majority" genotypes 1704GG+2184AA and subjects with "mutated" genotypes were found for total carotenoids ($P = .001$), α-carotene ($P = .046$), β-carotene ($P = .028$), lutein ($P = .001$), lycopene ($P = .006$), and α-tocopherol ($P = .047$). I_{compl} significantly correlated with the plasma levels of all antioxidants (all $P < .01$), while no correlation of I_{compl} with glycation variables was observed. The newly identified intron polymorphisms in the *RAGE* gene were proved to be associated with the antioxidant status in NIDDM subjects. The extent of diabetic vascular disease is related to the plasma levels of antioxidants.

INCREASED MORBIDITY and mortality accompanying both insulin-dependent and non–insulin-dependent diabetes mellitus (NIDDM) result from late complications due to prolonged hyperglycemia associated with these diseases. Common consequences of hyperglycemia-dependent cellular changes leading to the development of diabetic late complications are the generation of reactive oxygen intermediates and the presence of elevated oxidative stress.[1,2] Proteins exposed to aldose sugars undergo a nonenzymatic glycation (NEG). The early products of NEG, such as Schiff's bases and Amadori adducts, result from the covalent addition of reducing sugars to protein amino groups, preferentially to α-amino groups. Early glycation products on proteins with a long half-life undergo a slow complex series of chemical rearrangements to form irreversible compounds, collectively termed advanced glycation end products (AGEs). The formation of AGEs in tissues is proportional to time-integrated blood glucose levels and leads to irreversible morphologic rearrangement of such tissues.[3] The formation of AGEs is accompanied by oxidative, radical-generating reactions.[4] Reactive oxygen species, if not inactivated by antioxidative enzymes and nonenzymatic antioxidants, cause cellular dysfunction. Oxidative stress and AGE formation are largely intertwined and a combination of both processes—called glycoxidation—is supposed to be substantially involved in the pathogenesis of diabetic late complications.[1-3]

AGEs can bind to several binding sites, including receptor of advanced glycation end products (RAGE), a member of the immunoglobulin superfamily of cell-surface molecules.[5] *RAGE* is regarded as one of the logical candidate genes involved in the familial predisposition to certain types of diabetic long-term complications,[6] since it is expressed by critical cell types, including endothelial cells, mononuclear phagocytes, smooth muscle cells, mesangial cells and neurons, the dysfunction of which is thought to underlie the pathogenesis of diabetic complications.[7] Endothelial processing of AGEs in the intravascular space starts with cellular uptake and transcytosis with the delivery and release of the glycated ligand to the subendothelial space.[8] Such an AGE-RAGE interaction results in intracellular activation through multiple pathways (including the NADPH-oxidase/NFκ-B and perhaps the $p21^{ras}$/MAP-kinase/AP-1 pathways) leading to the production of reactive oxygen species (ROS) as documented by the generation of thiobarbituric acid-reactive substances, induction of heme oxygenase type I, and activation of the redox-sensitive transcription factor NFκ-B.[8,9]

Diabetes was repeatedly shown to be accompanied by a suboptimal function of protective antioxidant mechanisms.[10-14] A complex set of interactive antioxidant systems, both enzymatic and nonenzymatic, evolved as a protection against excessive oxidation. Nonenzymatic biologic antioxidants, components of natural food sources, include tocopherols and tocotrienols, carotenoids (α-carotene, β-carotene, lutein, lycopene, cryptoxanthine, astaxanthine), quinones, ascorbic acid, and metal-binding proteins. Individual carotenoids have different solubility characteristics, as well as different free radical scavenging activities.[13,15] These substances do not act in isolation, but as a part of a complex system. Alpha-tocopherol and

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ascorbate, as well carotenoids, act synergistically. Various nonenzymatic antioxidants, both lipid- and water-soluble, are present in all tissues, although each specific agent often shows a characteristic distribution pattern. Beta-carotene and lycopene were shown to be the predominant carotenoids in thyroid gland, kidneys, spleen, liver, heart, and pancreas. In the adrenals and testes, however, lycopene clearly predominated. Lutein and β-carotene were most abundant in fat, ovaries, and skin. The retina, especially the macula, contains mainly lutein but no β-carotene. Analysis of tissue antioxidant concentrations could be highly informative but it is difficult to obtain samples and for practical use it is not feasible. The measurement of total radical-trapping antioxidant capacity has been recently proposed as a good estimate of an oxidant stress and antioxidant turnover.

The analysis of genetic polymorphism in the complete region of the *RAGE* gene revealed 15 polymorphisms.[16] Exon polymorphism G82S and several intron polymorphisms (1704G/T, 21894A/G, and 2245G/A) proved to be rather common. The allele frequencies of these polymorphisms did not differ between diabetics and nondiabetics, but differences were found between subjects with and without a particular type of diabetic complication; a significant association with skin microvascular complications was found for exon mutation 82S and a marginal one for intron variants 1704T and 2184A/G.[16] No similar association with proliferative diabetic retinopathy was proved (unpublished). These results suggest that a predisposition to certain types of diabetic complications could be genetically modified and this particular susceptibility might be independent of diabetes itself. Genetic polymorphisms in the *RAGE* gene could eventually influence the cellular processing of AGEs or the reactions following AGE binding to *RAGE*, and thereby accelerate the development and severity of glucose-mediated tissue damage. Alternatively, variable intensity of ROS production during the processing of AGEs in the presence of polymorphisms could lead to changes in the total antioxidant capacity.

The aim of the present study was to investigate the relationships among several previously described common genetic polymorphisms in the *RAGE* gene (G82S, 1704G/T, 2184A/G, and 2245G/A), the glycoxidation status, and the extent of late diabetic microvascular and macrovascular complications in NIDDM. This complex task could be solved in diabetic subjects only, but at least relationships between common genetic polymorphisms and the glycoxidation status could be analyzed also in nondiabetics. For that reason, a group of nondiabetic subjects was also included.

MATERIALS AND METHODS

Subjects

A total of 371 unrelated caucasian subjects were enrolled in the study. The NIDDM group consisted of 202 subjects (95 men, 107 women) aged 63.9 $\pm$ 11.4 years (mean $\pm$ SD; range, 34 to 90 years). Diabetes was diagnosed previously according to World Health Organization criteria. All patients regularly attended a diabetic outpatient clinic and an antidiabetic treatment was established. The mean known duration of diabetes was 6.1 $\pm$ 6.8 years (mean $\pm$ SD), and the median was 4.0. The prevalence of diabetic retinopathy in this set was 49.8%, nephropathy 24.6%, neuropathy 28.9%, dermopathy 68.7%, coronary artery disease 30.3%, cerebrovascular stroke 6.2%, chronic occlusive peripheral arterial disease 33.2%, and arterial hypertension 66.8%. The extent of diabetic microvascular and macrovascular disease in each individual diabetic subject was assessed approximately as a presence of at least minimal signs of diabetic late complications in 5 particular organ localizations (retinopathy, nephropathy, neuropathy, macroangiopathy, and dermopathy). This extent was expressed as an index (I_{compl}) ranging from 0 to 5, in which the involvement of each organ system contributed by one to the sum.

The nondiabetic group consisted of 169 subjects (48 men, 121 women) aged 61.7 $\pm$ 15.5 years (range, 31 to 95). Diabetes was excluded by the examination of fasting plasma glucose and the absence of any clinical or anamnestic signs of diabetes. Informed consent was obtained from each patient prior to being included in the study. The study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Medical Faculty, Masaryk University Brno.

Biochemical Examinations

Blood. Glycated hemoglobin (HbA_{1c}) was determined colorimetrically using the Glycated Hemoglobin Diagnostic kit (Lachema, Brno, Czech Republic). Plasma levels of γ- and α-tocopherols, as well as the plasma levels of total carotenoids, lutein, lycopene, α-carotene, and β-carotene, were determined by reverse-phase high-performance liquid chrmoatography (HPLC) with spectrophotometrical detection.[15] Serum samples (2 mL) deproteinized with absolute ethanol (10 mL), were extracted twice with 25 mL of petroleum ether. After evaporation, the residue was dissolved in 500 μL of methanol. Equal amounts of the extract from each sample (injection volume, 100 μL) were used for the chromatographic analysis of both plasma tocopherols and carotenoids. The chromatographic system (ECOM, Prague, Czech Republic) consisted of high-pressure analytical pump LCP 4020, analytical injection loop valve type D, on-line UV-VIS variable wavelength detector LCD 2084, and the LCO 101 column oven. Operating conditions included a C18 Nucleosil 100 stainless steel column (5 mm) 4.6 $\times$ 150 mm, and the guard column 4.6 $\times$ 30 mm (Lachema) along with a solvent system of methanol:water (95:5) pumped at a flow rate of 1 mL/min. The isocratic elution was carried out at 45°C. Before injection, all samples were filtered using polytetrafluoroethane (PTFE) filters. After filtering, α- and γ-tocopherol content was determined at 289 nm, while all carotenoids (lutein, lycopene, α-carotene, β-carotene, total carotenoids) were detected at 450 nm. Integration was assessed using a CSW Integrator v.1.7 (DataApex, Prague, Czech Republic). Data were evaluated using external standards. Calibration curves based on the peak area were established for each carotenoid, γ- and α-tocopherol. The amount of total carotenoids was expressed as a total peak area of HPLC-chromatogram (mV $\cdot$ s) obtained in the course of 20 minutes at 450 nm.

Skin. The stratum corneum specimens were obtained from the sole and heel surface area using a dermatologic shaver. On average, a specimen of 300 mg was taken from each subject. The specimens were dried to a constant weight (2 hours, 60°C) and homogenized. The levels of glycated skin proteins (Amadori) were determined colorimetrically by a modified photometric method using thiobarbituric acid.[17] The amount of Amadori-skin proteins (Am_{skin}) in a sample was calculated on the basis of calibration using fructose, and expressed as micromoles fructose per 1 g dry weight of the stratum corneum. The evaluation of AGE levels in the stratum corneum samples was based on the measurement of absorbance at the absorption maximum of the yellow-brown product (365 nm) formed during the glycation of corneal proteins and peptides. The absorption maximum was determined from the absorption spectrum of the stratum corneum hydrolysate measured on spectrophotometer Helios Alpha (Unicam, Cambridge, UK). The hydrolysate was prepared using 100 mg of the stratum corneum homogenate, containing approximately 28 mg of proteins, suspended in 0.2

Table 1. Allele and Genotype Frequencies of the Four Polymorphisms Studied in the NIDDM and Nondiabetic Groups

	G82S polymorphism (*Alu*I restriction site)					
	GG	GS	SS	%G	%S	*P*
NIDDM (n = 202)	187	15	0	96.3	3.7	.047
Nondiabetics (n = 169)	164	5	0	98.5	1.5	
	1704 G/T polymorphism (*Bfa*I restriction site)					
	GG	GT	TT	%G	%T	*P*
NIDDM (n = 202)	177	24	1	93.6	6.4	NS
Nondiabetics (n = 169)	154	14	1	95.3	4.7	
	2184A/G polymorphism (*Bsm*FI restriction site)					
	AA	AG	GG	%A	%G	*P*
NIDDM (n = 202)	139	57	6	82.9	17.1	NS
Nondiabetics (n = 169)	125	39	5	85.5	14.5	
	2245G/A polymorphism (*Pst*I restriction site)					
	GG	AG	AA	%G	%A	*P*
NIDDM (n = 202)	139	59	4	83.4	16.6	.032
Nondiabetics (n = 169)	134	31	4	88.5	11.5	

mol/L phosphate buffer, pH 7.3. After acid hydrolysis (100°C, 5 hours, 3 mol/L H_3PO_4) the samples were cooled and then treated with 1 mol/L cool trichloracetic acid. After 10 minutes, the samples were centrifuged at 1,000 × *g* for 20 minutes. The supernatant was used for further analysis. With regard to the character of the biological material analyzed (containing both water-soluble and insoluble proteins modified by a number of heterogenous AGE structures), no accessible reference material was available. Thus, the amount of AGE (non-specific) was expressed as an absorbance at 365 nm.

Chemicals. DL-α-tocopherol, γ-tocopherol, α-carotene, β-carotene, lycopene, and lutein (xanthophyll) were purchased from Sigma Chemical (Sigma-Aldrich, St Louis, MO). HPLC-grade solvents (methanol, Fluka, Riedel de Häen; water, Sigma-Aldrich) were used without further purification. The mobile phases were degassed by sonication for 10 minutes prior to use. Other chemicals were mostly from Merck (Darmstadt, Germany).

Genotyping

DNA was isolated from peripheral blood leukocytes (obtained from 3 mL of EDTA-anticoagulated blood) by a standard extraction method.[18] Polymerase chain reactions (PCR) with subsequent restriction with specific endonucleases were performed to detect the G82S (*Alu*I restriction site), 1704G/T (*Bfa*I), and 2184A/G (*Bsm*FI) polymorphisms as described previously.[16,19]

Detection of 2245G/A. This polymorphism lies in a highly homologous region, hence the 2-step nested PCR with a large dilution of the first PCR product used as a template for the second PCR was necessary. The first-step PCR using external sense 5′-GCCCCATTCTGGCCTTATCCCTAA-3′ and antisense 5′-CCACCATGCCTGGCTAATTTTGT-3′ primers was performed in a final volume of 15 μL to amplify a 294-bp product. After the initial denaturation (95°C for 3 minutes), each cycle (of an additional 30 cycles) consisted of a 94°C denaturation for 15 seconds, 70°C annealing plus elongation step for 30 seconds, with the final extension lasting 8 minutes at 72°C. The product (10 μL) was diluted with 500 μL of water and used as a template (1 μL) for the second PCR reaction. The second-step PCR was performed in a final volume of 15 μL using the former antisense primer plus specific amplification-created restriction site (ACRS) primer 5′-ACACTTTGGGAGGCTGCTGC-3′ to provide a 116-bp product. After the initial denaturation step (95°C for 3 minutes), each cycle (of an additional 40 cycles) consisted of a 94°C denaturation for 10 seconds, 53°C annealing for 20 seconds and 72°C elongation for 20 seconds, with the final extension lasting 8 minutes at 72°C. Each PCR contained 9.6 μL of water, 1.5 μL of 10x PCR-buffer, 1.3 μL of $MgCl_2$ (25 mmol/L), 30 pmol of each primer, 0.5 μL of dNTP (10 mmol/μL), and 1U of Taq polymerase (all Perkin-Elmer, Boston, MA). Subsequent digestion with *Pst*I (MBI Fermentas, Vilnius, Lithuania) at 37°C overnight provided fragments of 95 and 21 bp in length for the mutated allele; the wild-type allele lacks the restriction site. Fragments were separated in 3% agarose and visualized under a UV transluminator.

Statistical Analysis

The statistical significances of differences in allele frequencies between the NIDDM and the nondiabetic groups were tested by Fisher's exact test. Mann-Whitney tests were used to compare biochemical parameters between both groups. Kruskal-Wallis analysis of variance (ANOVA) (for the 1704G/T, 2184A/G, and 2245G/A polymorphisms) was performed to ascertain relationships among genotypes and the biochemical parameters. The Mann-Whitney test was used for the G82S polymorphism and for comparison of the "wild-type" versus combined "mutated" genotype groups. Spearman rank-order correlation coefficients for the NEG measures and antioxidants and for biochemical parameters and the extent of diabetic microvasuclar and macrovascular disease (I_{compl}) were calculated. The relationship between the frequencies of the RAGE genotypes and I_{compl} was evaluated by the chi-square test. For all statistical analyses, CSS Statistics (Statsoft, Tulsa, OK), version 3, was used.

RESULTS

The frequencies of the particular alleles and genotypes of the 4 polymorphisms studied in the NIDDM and the nondiabetic subjects are listed in Table 1. The allele frequencies of the 2245G/A and G82S polymorphisms differed significantly between the NIDDM and the nondiabetic group ($P = .032$ and .047, respectively). A complete set of the studied biochemical parameters along with descriptive statistics is given in Table 2. A comparison between the NIDDM and the nondiabetic groups revealed significant differences in HbA_{1c} ($P < 1 \cdot 10^{-6}$), α-carotene ($P = .019$), and β-carotene ($P = .036$). Frequencies of the I_{compl} values in the NIDDM group are shown in Table 3.

Table 2. Complete Set of Biochemical Parameters Measured in NIDDM and Nondiabetic Subjects

parameter	NIDDM (n = 202)	Nondiabetics (n = 169)	*P*
HbA_{1c} (μmol F/g)	6.30 (±1.68)	4.77 (±0.87)	$<1 \cdot 10^{-6}$
Am_{skin} (μmol F/g)	3.20 (±1.92)	3.40 (±2.42)	NS
AGE_{skin} (A_{365})	0.367 (±0.107)	0.357 (±0.109)	NS
Total carotenoids (P/mV · s)	468.5 (±258.5)	498.4 (±263.7)	NS
γ-tocopherol (μmol/L)	7.69 (±1.37)	7.58 (±1.15)	NS
α-carotene (μmol/L)	18.10 (±14.33)	23.56 (±21.36)	.019
β-carotene (μmol/L)	51.07 (±44.69)	76.39 (±108.22)	.036
Lutein (μmol/L)	262.6 (±171.0)	251.0 (±145.6)	NS
Lycopene (μmol/L)	47.78 (±36.06)	52.56 (±39.42)	NS
α-tocopherol (μmol/L)	26.62 (±14.78)	28.88 (±15.32)	NS

NOTE. Data expressed as a mean ± SD.

Abbreviations: Am_{skin}, amount of Amadori products bound to the stratum corneum proteins; AGE_{skin}, amount of advanced glycation end products bound to the stratum corneum proteins.

Units: μmol F/g, μmol fructose per 1 g dry weight of the protein; A_{365}, absorbance at the absorption maximum of the yellow-brown product (365 nm); P/mV · s, amount of total carotenoids expressed as a total peak area of the HPLC-chromatogram (mV · s) obtained as a result of plasma carotenoid separation carried out for 20 minutes (450 nm).

Correlations of NEG measures (HbA_{1c}, Am_{skin}, and AGE_{skin}) with the whole set of antioxidants were calculated as the NEG and oxidant stress are in a close reciprocal relationship. Any correlation was found in the total and NIDDM groups ($P > .05$). In the nondiabetic group, HbA_{1c} , Am_{skin}, and AGE_{skin} correlated inversly with lutein ($r = -0.31$, $P = .014$; $r = -0.30$, $P = .019$; and $r = -0.25$, $P = .059$, respectively). Moreover, HbA_{1c} correlated also with total carotenoids ($r = -0.35$; $P = .006$) and lycopene ($r = -0.31$; $P = .016$), and Am_{skin} correlated also with α-tocopherol ($r = -0.29$; $P = .018$) in this group.

Relationship Between the RAGE Genotypes and Glycoxidation Parameters

Parameters characterizing intermediate- and long-term compensation of diabetes, such as HbA_{1c} , Am_{skin}, and AGE_{skin}, did not reveal any significant relationship to any of the 4 polymorphisms analyzed ($P > .05$) in the total sample or in either group (data not shown). Mean plasma concentrations of antioxidants corresponding to the particular genotypes of the 4 polymorphisms in the NIDDM and the nondiabetic groups are given in Tables 4 through 7. In the total sample, the G82S revealed significant genotype differences for β-carotene ($P = .009$); the 1704G/T for total carotenoids ($P = .046$), lutein ($P = .038$), and lycopene ($P = .009$) and marginally for α-tocopherol ($P = .061$). The 2184A/G revealed genotype differences for lutein ($P = .016$) and the 2245G/A revealed no difference (see Tables 4 through 7 bottom lines). When analyzed separately in the NIDDM and the nondiabetic groups, the exon polymorphism G82S revealed significant genotype differences for α-carotene ($P = .029$) and β-carotene ($P = .019$) in the nondiabetic group. In the case of the intron 1704G/T polymorphism, significant genotype differences were found for lutein ($P = .037$) and lycopene ($P = .038$) in the NIDDM group. The 2184A/G polymorphism revealed a significant genotype difference for lutein ($P = .022$) in the NIDDM group. Finally, the 2245G/A polymorphism failed to produce any genotype difference in either group.

In view of the fact that the frequencies of genotypes containing "minority" intron alleles are much lower in all the 3 cases than those of the "wild-type majority" homozygotes, the intron polymorphisms 1704G/T, 2184A/G a,nd 2245G/A were also evaluated by comparing combined genotype groups (heterozygotes and mutated homozygotes) versus the wild-type majority homozygotes. We assume the effect of the mutated allele in the heterozygote to be even more expressed in the mutated homozygote (additive or multiplicative effect), and therefore, it should not introduce a gross error to considere both genotypes containing at least 1 copy of the minority allele as being functionally identical. This comparison was performed using Mann-Whitney test, and resulting values and significance levels designated as $P_{wt/mut}$ are listed on the corresponding lines in Tables 5 through 7. Most of the results copied those obtained when comparing all 3 genotypes, although the significances were somewhat higher.

As shown in Tables 5 and 6 for the 1704G/T and 2184A/G polymorphisms, the genotype differences mentioned above (whether significant or not) exhibit a similar tendency in the NIDDM and the nondiabetic groups—the wild-type majority genotypes (eg, 1704GG or 2184AA) associate with higher mean plasma levels of antioxidants than the combined groups of heterozygotes and mutated homozygotes (eg, 1704GT+TT or 2184AG+GG). The G82S polymorphism showed an opposite tendency—heterozygotes had (with one exception) higher mean plasma levels of antioxidants, but because of rather disparate numbers of the homozygotes and heterozygotes and a complete lack of mutated homozygotes, this situation might not be confirmative (Table 4). In the case of the 2245G/A polymorphism, the tendencies were entirely opposite in the NIDDM and the nondiabetic groups (Table 7). Therefore, we compared the antioxidant parameters between a group of subjects bearing concurrently both wild-type majority genotypes in positions 1704 and 2184 (eg, GG+AA, n = 231) and a group of subjects with any substitution in these 2 positions (n = 140) irrespective of the genotypes of the G82S and 2245G/A. Particular combinations found in the group of subjects with substitutions in position 1704 and 2184 were as follows: 89 GG+AG, 11 GG+GG, 31 GT+AA, 7 GT+AG, and 2 TT+AA. Comparisons were made in the total sample as well as in the NIDDM

Table 3. Distribution of I_{compl} Values in the NIDDM Group

I_{compl} Value	n	Age (mean ± SD), yr	Median Duration (range)
0	28	64.3 ± 8.7	3 (1–17)
1	26	57.1 ± 9.7	3 (0–18)
2	40	59.9 ± 12.5	2 (0–20)
3	47	66.0 ± 10.4	5 (0–40)
4	40	69.2 ± 9.3	6 (0–40)
5	15	68.7 ± 12.5	9 (0–25)
Ambiguous	6		
Total	202	$P = 4.10^{-5}$	$P = 2 \cdot 10^{-5}$

Table 4. The G82S Polymorphism: Mean Plasma Concentrations of Antioxidants Corresponding to Individual Genotypes in the NIDDM and Nondiabetic Groups

Genotype	Total Carotenoids	γ-Tocopherol	α-Carotene	β-Carotene	Lutein	Lycopene	α-Tocopherol
NIDDM (n = 202)							
GG	465.8 (±260.1)	7.67 (±1.38)	17.85 (±13.99)	49.24 (±41.36)	259.9 (±170.8)	46.93 (±35.18)	26.45 (±14.99)
GS	503.6 (±245.0)	8.08 (±1.18)	21.46 (±18.77)	75.30 (±74.96)	296.8 (±177.1)	58.54 (±46.24)	28.77 (±12.07)
P	NS	NS	NS	NS	NS	NS	NS
Nondiabetics (n = 169)							
GG	479.8 (±254.3)	7.56 (±1.14)	20.76 (±12.75)	62.10 (±54.15)	246.2 (±146.2)	50.39 (±36.02)	29.22 (±15.69)
GS	763.7 (±287.5)	7.94 (±1.42)	62.05 (±61.47)	272.85 (±344.13)	319.0 (±136.7)	83.50 (±74.13)	23.13 (±3.89)
P	NS	NS	.029	.019	NS	NS	NS
Total sample (N = 371)							
P	NS	NS	NS	.009	NS	NS	NS

NOTE. All data expressed as a mean ± SD. Genotype numbers equal to those listed in Table 1.

and the nondiabetic groups separately. The respective differences found for the studied parameters in the total, NIDDM, and nondiabetic groups were as follows: total carotenoids (P = .001, .016, and .021), γ-tocopherol (P = not significant [NS], NS, and NS), α-carotene (P = .046, NS, and NS), β-carotene (P = .028, NS, and .061), lutein (P = .001, .009, and .051), lycopene (P = .006, .025, and NS), and α-tocopherol (P = .047, NS, and NS). Figure 1 shows the distributions of the antioxidant parameters in the group of concurrent wild-type majority genotypes 1704GG+2184AA and the mutated group. Finally, when similar comparisons were made with respect to all 4 polymorpisms studied, eg, subjects bearing the wild-type majority genotypes in all 4 positions (82GG+1704GG+2184AA+2245GG) were compared with subjects with any type of substitution, a significant difference was found again for total carotenoids (P = .009) and marginally significant differences for lutein (P = .064) and lycopene (P = .074).

Relationship Between the Extent of Vascular Complications (I_{compl}) and the Glycoxidation Parameters

The relationships between I_{compl} values and the biochemical parameters characterizing glycoxidation were expressed by means of Spearman rank-order correlation coefficients. I_{compl} did not correlate with the levels of $HbA_{1c,}$ Am_{skin}, and AGE_{skin} ($P > .05$). On the contrary, significant inverse correlations between I_{compl} and the whole set of antioxidants, total carotenoids (r = -0.42; $P < 1 \cdot 10^{-6}$), γ-tocopherol (r = -0.25; P = .001), lutein (r = -0.39; $P < 1 \cdot 10^{-6}$), lycopene (r = -0.33; $P = 1 \cdot 10^{-5}$), α-carotene (r = -0.30; $P = 1 \cdot 10^{-4}$), β-carotene (r = -0.32; $P = 3 \cdot 10^{-5}$), and α-tocopherol (r = -0.27; $P = 2 \cdot 10^{-4}$), were found. The mean plasma levels of all antioxidants decreased with increasing values of I_{compl}. The mean plasma concentrations of antioxidants as a function of I_{compl} are shown in Fig 2.

Table 5. The 1704G/T Polymorphism: Mean Plasma Concentrations of Antioxidants Corresponding to Individual Genotypes in the NIDDM and Nondiabetic Groups

Genotype	Total Carotenoids	γ-Tocopherol	α-Carotene	β-Carotene	Lutein	Lycopene	α-Tocopherol
NIDDM (n = 202)							
GG	482.7 (±258.0)	7.75 (±1.36)	18.84 (±14.89)	53.32 (±46.78)	270.5 (±168.8)	50.21 (±36.87)	27.46 (±14.85)
GT	368.9 (±250.6)	7.33 (±1.50)	12.45 (±7.82)	34.04 (±19.19)	212.8 (±181.8)	30.81 (±24.57)	20.85 (±13.49)
TT	332.0	8.20	19.40	54.90	68.0	26.00	24.30
GT + TT	367.2 (±244.7)	7.37 (±1.47)	12.79 (±7.77)	35.03 (±19.25)	206.2 (±180.1)	30.59 (±24.00)	20.99 (±13.21)
P ($P_{wt/mut}$)	.097 (.031)	NS	NS (.052)	NS	.037 (.018)	.038 (.011)	.082 (.028)
Nondiabetics (n = 169)							
GG	507.4 (±266.2)	7.61 (±1.19)	24.05 (±21.91)	79.57 (±111.44)	255.3 (±147.1)	54.32 (±39.93)	29.23 (±15.67)
GT	389.9 (±252.0)	7.28 (±0.43)	18.40 (±12.47)	37.43 (±6.80)	183.0 (±149.0)	32.67 (±21.36)	25.2 (±4.16)
TT	309.1	7.10	11.80	18.40	212.0	12.0	16.50
GT + TT	369.7 (±209.6)	7.24 (±0.36)	16.75 (±10.71)	32.68 (±11.02)	190.3 (±122.5)	27.50 (±20.27)	23.03 (±5.52)
P ($P_{wt/mut}$)	NS	NS	NS	NS	NS	NS	NS
Total sample (N = 371)							
P ($P_{wt/mut}$)	.046 (.013)	NS	NS (.040)	NS (.041)	0.038 (.012)	.009 (.003)	0.061 (.018)

NOTE. Genotype numbers equal to those listed in Table 1.

Table 6. The 2184A/G Polymorphism: Mean Plasma Concentrations of Antioxidants Corresponding to Individual Genotypes in the NIDDM and Nondiabetic Groups

Genotype	Total Carotenoids	γ-Tocopherol	α-Carotene	β-Carotene	Lutein	Lycopene	α-Tocopherol
NIDDM (n = 202)							
AA	492.0 (±266.7)	7.75 (±1.47)	18.21 (±13.68)	51.80 (±42.67)	277.2 (±178.4)	49.98 (±37.73)	26.97 (±14.85)
AG	410.2 (±232.3)	7.59 (±1.19)	18.25 (±16.52)	51.17 (±51.22)	214.3 (±138.2)	42.56 (±31.87)	25.52 (±15.07)
GG	484.5 (±262.4)	7.67 (±0.29)	14.12 (±6.06)	33.00 (±14.57)	397.8 (±178.9)	46.20 (±34.83)	29.28 (±11.51)
AG + GG	416.9 (±233.5)	7.59 (±1.14)	17.86 (±15.84)	49.49 (±49.19)	230.7 (±149.9)	42.89 (±31.82)	25.89 (±14.71)
P ($P_{wt/mut}$)	NS	NS	NS	NS	.022 (.029)	NS	NS
Nondiabetics (n = 169)							
AA	530.1 (±280.3)	7.55 (±1.22)	25.68 (±24.04)	87.24 (±124.18)	265.9 (±149.5)	55.16 (±42.16)	29.69 (±14.91)
AG	402.6 (±177.9)	7.76 (±0.98)	18.42 (±10.87)	50.38 (±30.55)	198.4 (±104.4)	43.14 (±24.87)	28.42 (±17.05)
GG	479.5 (±322.7)	7.20 (±0.42)	15.40 (±4.55)	33.48 (±22.40)	278.7 (±244.7)	58.33 (±59.60)	17.33 (±12.40)
AG + GG	416.2 (±199.1)	7.68 (±0.94)	17.85 (±9.94)	47.22 (±29.33)	212.5 (±131.7)	45.82 (±31.34)	26.67 (±16.63)
P ($P_{wt/mut}$)	NS	NS	NS	NS	NS	NS	NS
Total sample (N = 371)							
P ($P_{wt/mut}$)	NS (.033)	NS	NS	NS	.016 (.028)	NS	NS

NOTE. Genotype numbers equal to those listed in Table 1.

Relationship Between the RAGE Genotypes and the Extent of Vascular Complications (I_{compl})

Genotype frequencies of the four polymorphisms studied corresponding to the particular values of I_{compl} in the NIDDM group are shown in Table 8. No significant differences between the frequencies of the RAGE genotypes corresponding to the particular values of I_{compl} were found ($P > .05$).

DISCUSSION

A central role of RAGE in transmitting the oxidant signal was postulated in NIDDM.[7] Several possible mechanisms through which AGEs might induce oxidative stress after binding to RAGE can be considered. AGEs by themselves have been shown to generate ROS and, conversely, the generation of AGEs is enhanced by oxidant stress. Another possible effect resulting from the AGE-RAGE interaction is triggering a signal transduction mechanism leading to the generation of intracellular oxidants. Increased oxidant stress in NIDDM is not only supposed to be substantially involved in the pathogenesis of diabetic late complications, but also in the modulation of insulin sensitivity. Antioxidant supplementation was shown to lead to an improvement of insulin sensitivity.[20,21]

The aim of our study was to investigate novel polymorphisms in the *RAGE* gene in relation to glycoxidation parameters (in diabetic as well as in nondiabetic subjects) and in relation to the extent of diabetic microvascular and macrovascular complications. The case-control approach led to following results: (1) allele frequencies of the G82S and 2245G/A

Table 7. The 2245G/A Polymorphism: Mean Plasma Concentrations of Antioxidants Corresponding to Individual Genotypes in the NIDDM and Nondiabetic Groups

Genotype	Total Carotenoids	γ-Tocopherol	α-Carotene	β-Carotene	Lutein	Lycopene	α-Tocopherol
NIDDM (n = 202)							
GG	493.2 (±284.5)	7.63 (±1.47)	18.64 (±15.68)	55.69 (±51.05)	271.7 (±186.7)	50.17 (±39.22)	27.63 (±15.53)
GA	417.9 (±181.6)	7.92 (±1.24)	17.43 (±10.92)	42.32 (±22.76)	243.4 (±132.0)	42.82 (±27.75)	25.03 (±13.02)
AA	349.2 (±105.4)	7.14 (±0.43)	10.35 (±5.69)	21.98 (±9.52)	227.3 (±56.8)	37.50 (±23.53)	16.03 (±5.61)
GA + AA	412.9 (±177.4)	7.86 (±1.21)	16.90 (±10.74)	40.78 (±22.65)	242.2 (±127.8)	42.44 (±27.31)	24.41 (±12.83)
P ($P_{wt/mut}$)	NS	NS	NS	NS	NS	NS	NS
Nondiabetics (n = 169)							
GG	485.6 (±270.1)	7.64 (±1.78)	23.24 (±22.90)	74.37 (±114.87)	241.6 (±144.9)	50.55 (±40.39)	27.78 (±15.29)
GA	523.1 (±137.2)	7.10 (±0.95)	24.43 (±5.00)	74.97 (±32.85)	273.0 (±93.6)	62.00 (±31.38)	37.40 (±14.45)
AA	899.9 (±141.4)	7.35 (±0.21)	32.50 (±1.41)	144.7 (±63.22)	524.5 (±102.5)	91.50 (±22.12)	36.90 (±2.69)
GA + AA	582.7 (±211.1)	7.16 (±0.88)	25.56 (±5.63)	89.28 (±50.61)	313.5 (±143.6)	65.88 (±31.05)	37.28 (±13.39)
P ($P_{wt/mut}$)	NS	NS	NS	NS (.055)	NS	NS	NS (.035)
Total sample (N = 371)							
P ($P_{wt/mut}$)	NS	NS	NS	NS	NS	NS	NS

NOTE. Genotype numbers equal to those listed in Table 1.

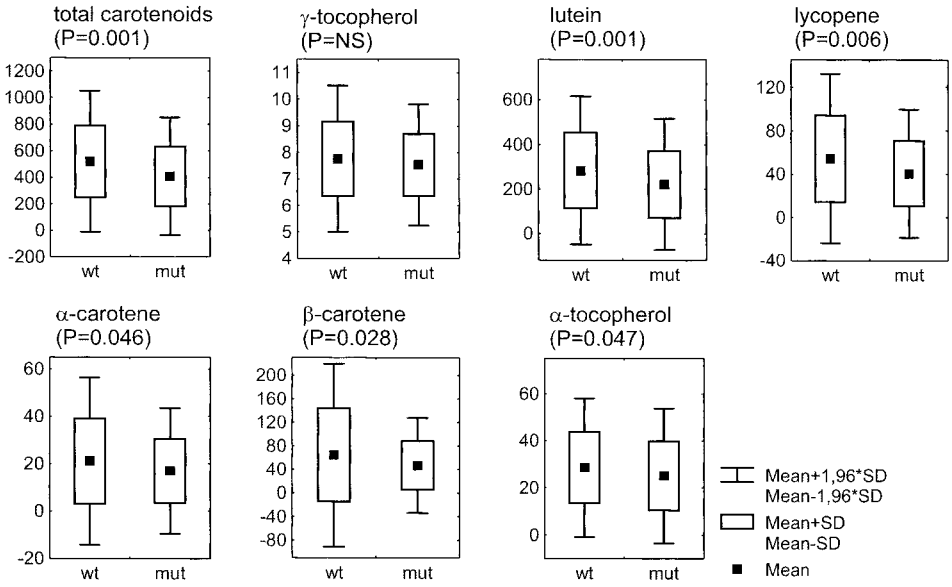

Fig 1. Distribution of antioxidant parameters in a group of subjects bearing both wild-type majority genotypes in positions 1704 and 2184 (eg, GG+AA, n = 231) and a group with any substitution in these 2 positions (n = 140); box and whisker plots.

differed between NIDDM and nondiabetic subjects, but these 2 polymorphisms exhibited only sporadic and very weak relationships with antioxidant levels (see below), and allele and genotype frequencies of the intron polymorphisms 1704G/T and 2184A/G did not differ significantly between the 2 groups; (2) the NIDDM and nondiabetic groups differed significantly in HbA_{1c}, α-carotene, and β-carotene; and (3) measures of NEG ($HbA_{1c,}$ Am_{skin}, and AGE_{skin}) inversely correlated with lutein solely in the nondiabetic group. Analysis of the relationships among the 4 polymorphisms in the *RAGE* gene (G82S, 1704G/T, 2184A/G, and 2245G/A) and parameters characterizing the long-term glycation and antioxidant status revealed interesting results. No relationship between any of the *RAGE* polymorphisms and glycation variables (HbA_{1c}, Amadori, and AGE) was found, which indicates that these common *RAGE* polymorphisms probably did not significantly influence the extent of extracellular NEG in tissues. On the contrary, plasma levels of several antioxidants were related to the genotypes of the *RAGE* polymorphisms (Kruskal-Wallis ANOVA). With respect to low frequencies of genotypes containing mutated

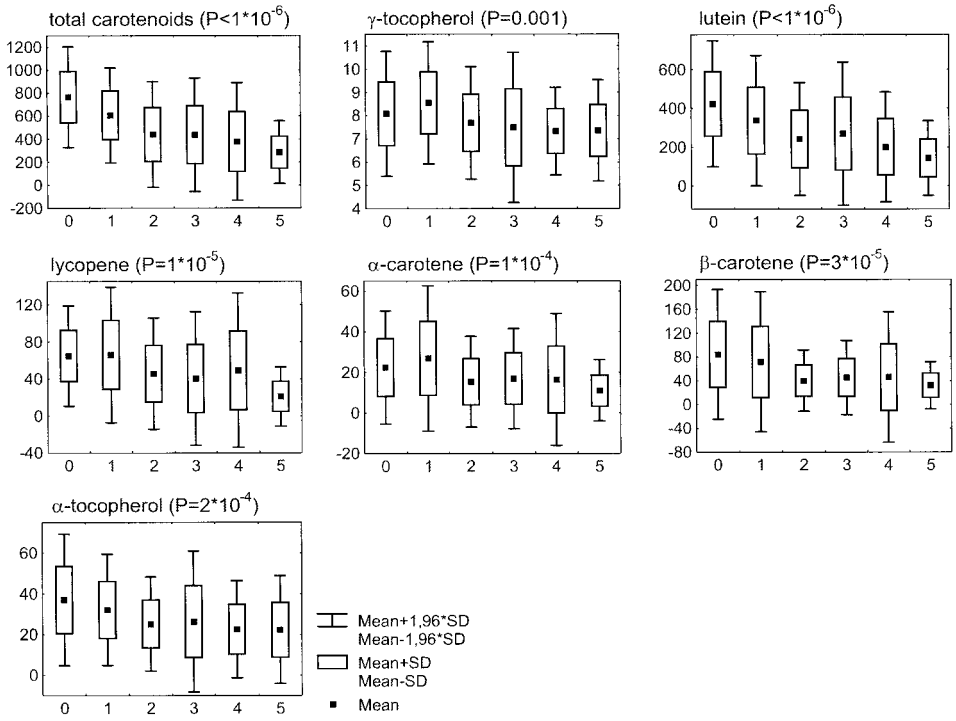

Fig 2. Mean plasma concentrations of antioxidants plotted against I_{compl} (I_{compl} values on horizontal axis).

Table 8. Genotype Frequencies Corresponding to Particular Values of I_{comp1} in the NIDDM Group

	G82S Polymorphism			1704G/T Polymorphism			2184A/G Polymorphism			2245G/A Polymorphism		
I_{compl} Value	GG	GS	SS	GG	GT	TT	AA	AG	GG	GG	GA	AA
0	26	2	0	26	2	0	22	6	0	18	9	1
1	24	2	0	24	2	0	18	6	2	19	7	0
2	38	2	0	33	7	0	26	13	1	27	11	2
3	43	4	0	42	5	0	33	12	2	32	15	0
4	36	4	0	34	6	0	25	14	1	29	10	1
5	15	0	0	12	2	1	9	6	0	10	5	0
P		NS			NS			NS			NS	

NOTE. Comparison made by χ^2 test.

alleles, a comparison of the wild-type majority genotypes with the mutated minority genotypes (eg, heterozygotes and mutated homozygotes) seemed to be more appropriate than post hoc ANOVA testing. This comparison revealed several significant genotype-based differences in antioxidant parameters in the total sample, the NIDDM, and the nondiabetic groups. Polymorphisms 1704G/T and 2184A/G showed a simmilar tendency in both groups—genotypes consisting of majority alleles in these positions were associated with higher levels of antioxidants than those with any substitution. Comparisons between the"wild-type majority genotypes in positions 1704 and 2184 and the mutated combinations revealed significant differences in most antioxidant parameters. Finally, none of the glycation variables, but all the antioxidant ones, were significantly related to the extent of vascular complications. No relationship between the distribution of the genotypes of the 4 polymorphisms and the overall extent of vascular complications was proved.

The explanation of the relationship between the particular *RAGE* intron polymorphisms and the plasma levels of nonenzymatic antioxidative micronutrients is not possible as yet. The 1704G/T and 2184A/G polymorphisms probably do not influence the extent of the NEG of structural long-lived proteins, such as keratin or collagen. NEG in various tissues is evidently largely dependent on local conditions (protein content and turnover) and glucose content. However, intracellular processing of circulating "second-generation" AGEs on the endothelial surface could be substantially influenced by these polymorphisms. While the functional impact of the intron polymorphisms studied is not known as yet, their quantitative impact on *RAGE* expression could be supposed. Alterations in auxiliary transcription sites (enhancers or silencers), alternative splicing or different mRNA stability might be considered. The AGE-RAGE interaction is followed by multiple effects; thereby the interpretation of the relation of the particular genotypes to the plasma antioxidant levels is difficult. We suppose the effect could be indirect through an increased consumption of a variety of antioxidants in the situation of augmented oxidative stress. We can speculate that the identified polymorphisms may have an effect on the tissue processing of circulating AGEs, and the receptor-mediated pathway in the presence of some alleles may lead to aggravated oxidative stress. The highly significant relationship of the plasma levels of antioxidants to the extent of microvascular and macrovascular disease (roughly estimated by the I_{compl}) is a clear evidence of a direct involvement of oxidative stress in the pathogenesis of diabetic late complications. Measures of NEG and most of antioxidants did not differ between diabetics and nondiabetics on average; however, differences in antioxidants were apparent inside the NIDDM group. While glycation variables remained almost constant in particular I_{compl} levels, antioxidants decreased with increasing I_{compl}. This could explain the lack of correlation between antioxidants and AGEs in NIDDM found in healthy subjects. Diabetes (especially with advanced complications) could lead to enhanced oxidant stress not corresponding to formation of AGEs. The lack of a relationship between the *RAGE* genotypes and the I_{compl} could reflect the fact that the development of late complications (with most decidedly multifactorial etiopathogenesis) is influenced by many factors other than just *RAGE* polymorphisms and, thereby, a significant relationship need not be proved. Moreover, a heterogeneity in organ and tissue susceptibility to long-term consequences of prolonged hyperglycemia is supposed.[2] Different hyperglycemia-driven metabolic pathways are probably critical to different organs and tissues. A significant association with cutaneous type of complications was already proved by our group, while a similar association with diabetic retinopathy was not.[16] The first identified G82S polymorphism was specifically associated with skin complications,[16] but not with retinopathy,[19] nephropathy,[22] or macrovascular disease in NIDDM.[23] The same genetic factor could be critical for one particular tissue while not for another; the final clinical picture then depends on the whole cluster of molecular variants.

Oxidative mechanisms may play an important role in NIDDM as well as in aging processes.[24] Accelerated aging seen in NIDDM may be related to a deficit in the intake of antioxidant vitamins (tocopherols, carotenoids, and vitamin C) and trace elements (selenium and zinc), as well as to impaired adaptive enzymatic mechanisms against oxidative stress. Physiologic modifications occurring during the lifetime and environmental influences are significant factors contributing to the impairment of the micronutrient status. There is no evidence for the benefit of supplementation in healthy subjects, but in the present state of knowledge a combined supplementation including vitamins and trace elements could be the best way to prevent accelerated aging and reduce the risk of several common age-related diseases including NIDDM. In vitro studies on carotenoids—including β-carotene, lycopene, and lutein have identified these as effective quenchers of singlet oxygen with considerable radical-trapping properties, or to be effective peroxyl radical scavengers. NIDDM together with advanced age

leads to increased oxidative damage, and *RAGE* polymorphisms can further contribute to the striking interindividual variability in the rate of development and severity of the late complications seen in diabetics.

In conclusion, newly identified common intron polymorphisms in the *RAGE* gene were associated with nonenzymatic antioxidant status in NIDDM, as well in nondiabetic subjects. *RAGE* appeared to be one of the genetic factors influencing interindividual susceptibility to oxidant damage, which might play an important role, especially in diabetes.

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