Paraoxonase 192 Gln/Arg Gene Polymorphism, Coronary Artery Disease, and Myocardial Infarction in Type 2 Diabetes

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Paraoxonase is an HDL-associated enzyme implicated in the pathogenesis of atherosclerosis by protecting lipoproteins against peroxidation. Its biallelic gene polymorphism at codon 192 (glutamine/arginine) has been associated with coronary artery disease (CAD). To further evaluate the role of this paraoxonase gene polymorphism for CAD in type 2 diabetes, we determined the paraoxonase genotype in 288 type 2 diabetic patients (170 with and 118 without angiographically documented CAD). The paraoxonase 192 Gln/Arg genotype was assessed using polymerase chain reaction followed by *Alw*I digestion. The frequency of the Gln allele was 0.656 in the CAD patients and 0.746 in the controls (χ^2 = 5.36, P = 0.02). Compared with the Gln/Gln genotypes, the age-adjusted odds ratio for CAD was 1.78 (95% CI 1.08–2.96, P = 0.02) in subjects carrying at least one Arg allele. In the multivariate analysis, this association was even stronger after correction for the possible confounders age, sex, smoking history, and hypertension. Among current and former smokers, the odds ratio (OR) for having CAD among patients with at least one Arg allele was 3.58 (1.45–9.53, P < 0.01). The paraoxonase Arg allele was not associated with the history of myocardial infarction (OR 1.20 [0.73–1.99, NS]), but was with the extent of CAD (OR for three-vessel disease 1.92 [1.15–3.27, P = 0.01]). Our data indicate that the 192 Arg allele of the human paraoxonase gene is a risk factor for CAD but not myocardial infarction in type 2 diabetic patients, a risk factor further modified by cigarette smoking. This risk could possibly be explained by a reduced ability of the paraoxonase Arg isoform to protect lipoproteins against peroxidation. *Diabetes* 48:623–627, 1999

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CAD, coronary artery disease; MI, myocardial infarction; OR, odds ratio; PCR, polymerase chain reaction.

Diabetes, hyperlipidemia, hypertension, and insulin resistance are tightly related to the development of coronary artery disease (CAD) and myocardial infarction (MI). In addition to these metabolic cardiovascular risk factors, genetic predisposition is known to be important in the development of CAD and subsequent MI (1,2). Although the levels of lipoproteins such as LDL and HDL cholesterol may not be abnormal in patients with diabetes, the lipoproteins may be glycated, resulting in an abnormal function (3). Therefore, the genes involved in lipoprotein metabolism and modification may be especially important in the development of CAD and MI in patients with diabetes.

Paraoxonase (EC 3.1.8.1) is an HDL-associated arylesterase that hydrolyzes paraoxon, the active toxic metabolite of the organophosphate parathion (4). Paraoxonase purified from native human HDL is able to protect LDL from oxidative modification by destroying lipid peroxides (5,6). Human serum paraoxonase activity toward paraoxon shows large interindividual variation and underlies tight genetic control. The molecular basis of this variation is a polymorphism in the coding region of the gene, resulting in an amino acid substitution (Gln→Arg) in position 192. Subjects homozygous for Gln have significantly lower serum paraoxonase activity toward paraoxon than subjects homozygous for Arg, and heterozygous subjects have intermediate levels (7,8). An association between the paraoxonase 192 Arg allele and CAD has recently been described in French (9) and Japanese (10) patients with type 2 diabetes and in a North American general population (11) but could not be confirmed in Finnish (12), Japanese (13), and Chinese (14) CAD patients, mostly without diabetes, or in European patients with MI (15). The purpose of this study was to analyze further the relationship between the paraoxonase 192 Gln/Arg gene polymorphism, presence and extent of CAD, and MI in Caucasian patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Study population. We prospectively studied 288 unrelated Caucasian patients (188 men, 100 women) who were at the University Hospital of Tübingen, Germany, because of suspected CAD or diabetes and who fulfilled the World Health Organization criteria for type 2 diabetes (16). The patients' relevant history, cardiovascular risk factors, and current treatment were obtained using a standard questionnaire, and the data were validated with reference to hospital case records. Two hundred six patients underwent diagnostic coronary angiography, performed by

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the Judkins technique, because of symptoms or electrocardiographic changes possibly related to CAD. Each angiogram was classified as either revealing (n = 170) or excluding (n = 36) CAD. The CAD patients were then subclassified as having no coronary lesion with ≥50% luminal stenosis or one, two, or three major epicardial coronary arteries with ≥50% luminal obstruction. The additional 82 patients with type 2 diabetes had neither clinical symptoms nor electrocardiographic changes indicative of CAD and therefore did not undergo coronary angiography. History of MI was confirmed according to standard criteria, that is, two or more of the following: history of chest pain indicative of MI, increase in creatine kinase levels of at least three times the upper level of the normal range in our institution (80 U/l for men, 70 U/l for women) during follow-up, and characteristic electrocardiographic changes at the time of diagnosis (ST-segment elevation >0.1 mV in at least two leads and development of a Q during follow-up). Smokers were defined as current smokers and patients who had a smoking history of at least five pack-years, and nonsmokers were defined as patients with no history of prior or current smoking. Hypertension was defined as blood pressure >140/90 mmHg at repeated measurement or current use of antihypertensive drugs due to a previous history of arterial hypertension. Hyperlipidemia was defined as plasma total cholesterol >5.2 mmol/l, plasma triglycerides >1.7 mmol/l, or current use of lipid-lowering drugs with an established diagnosis of hyperlipidemia. The study was approved by the local ethics committee, and informed consent was obtained from each patient before the procedure.

Laboratory methods. Peripheral venous blood samples were drawn from all patients after overnight fasting to analyze the lipid profile and to extract genomic DNA. Plasma total cholesterol and triglycerides were measured by automated enzymatic methods (Boehringer Mannheim, Mannheim, Germany), and HDL cholesterol was determined after sodium phosphotungstate/magnesium chloride precipitation (17). In all patients with plasma triglycerides ≤4.5 mmol/l, LDL cholesterol was calculated according to Friedewald's formula (18). HbA_{1c} was measured by high-performance liquid chromatography (DIAMAT analyzer; Biorad, Richmond, CA).

Determination of the paraoxonase 192 genotype. Leukocyte DNA was isolated and purified from whole blood (EDTA) using QIAamp-spin-columns according to the protocol provided by the manufacturer (QIAamp Blood Kit; Qiagen GmbH, Hilden, Germany). The 99-bp target region in the paraoxonase gene was amplified by polymerase chain reaction (PCR) using forward 5′-TAT TGT TGC TGT GGG ACC TGA G-3′ and reverse 5′-CAC GCT AAA CCC AAA TAC ATC TC-3′ primers. The PCR reaction mix contained 10 µg genomic DNA, 0.5 µmol/l of each primer, 200 µmol/l of each dNTP, 5 µl of 10× reaction buffer, and 1.25 U Taq DNA polymerase. After the DNA was denatured at 95°C for 3 min, the reaction mixture was subject to 30 cycles, each cycle comprising denaturation at 94°C for 60 s, annealing at 61°C for 30 s, and extension at 72°C for 60 s, with a final extension time of 5 min. The PCR products were digested with 2.5 U *Alw*I restriction endonuclease for 1 h, and the digested products were separated by electrophoresis on a horizontal 5% polyacrylamide gel and visualized by staining with silver solution. The 192 Gln/Arg transition creates a unique *Alw*I site in the amplified fragment. Individuals homozygous for the 192 Gln allele present a 99-bp PCR product, those homozygous for the 192 Arg allele present 69- and 30-bp products, and those heterozygous present 90-, 69-, and 30-bp products. Each genotype was read by two independent observers (M.P. and J.F.) without knowledge of the clinical status of the patients or the results of the coronary angiography.

Statistical analysis. The results for continuous variables are expressed as means ± SD. The means of the three genotype groups were compared in a one-way analysis of variance. We determined whether the distribution of the paraoxonase 192 genotypes was in Hardy-Weinberg equilibrium using χ^2 analysis as described by Emery (19). Categoric data and the frequencies of the alleles and genotypes among different subgroups were compared by the likelihood ratio test. In addition, the odds ratios (ORs) and 95% CIs were calculated as a measure of the association of the paraoxonase 192 genotypes with CAD, extent of CAD, and MI. The analysis was also carried out by means of a logistic linear regression analysis to assess the independent role of the paraoxonase genotype and other CAD or MI risk factors. In this analysis, CAD, extent of CAD, or MI was regarded as the dependent variable, and age, paraoxonase 192 Arg allele, sex, smoking history, BMI, hypertension, hyperlipidemia, diabetes duration, and HbA_{1c} level as independent variables. In addition, the interaction between paraoxonase 192 genotype and smoking history was calculated using the Cochran-Mantel-Haenszel test. All analyses were done using a personal computer with JMP 3.2.2 (SAS Institute, Cary, NC). A P value <0.05 after Bonferroni correction was considered to be statistically significant.

RESULTS

The clinical characteristics of the 288 study patients are given in Table 1. The overall paraoxonase 192 genotype distribution (Gln/Gln, 48.3; Gln/Arg, 42.0; Arg/Arg, 9.7%) was consistent with Hardy-Weinberg equilibrium, and the allele frequencies (Gln 0.693, Arg 0.307) were similar to those in other Caucasian populations (9,12). The three genotype groups were well matched for age, BMI, diabetes duration, and HbA_{1c} level, but the group homozygous for the 192 Arg allele comprised more men and a higher prevalence of smokers (Table 1). Systolic or diastolic blood pressure and plasma lipid and lipoprotein levels were not different between the three genotype groups.

Paraoxonase 192 genotype and CAD. The paraoxonase 192 Arg allele was significantly associated with both the presence and the extent of CAD, comparing the patients with angiographically proven CAD and those without angiographic, clinical, or electrocardiographic evidence of CAD (Table 2). For the presence of CAD, the age-adjusted ORs were indicative of an additive effect of the 192 Arg allele, with an OR (95% CI) of 2.42 (1.01–6.58, P = 0.05) for the subjects homozygous for the 192 Arg allele compared with those homozygous for the 192 Gln allele, and 1.78 (1.08–2.96) for those carrying at least one Arg allele compared with the 192 Gln/Gln genotypes. There was also a significant association between the 192 Arg allele and the extent of CAD, represented by the increased frequency of three-vessel disease (Table 2). Among the current and former smokers, the association between the paraoxonase 192 genotype and CAD was even more pronounced (n = 126, OR 3.58 [1.45–9.53, P = 0.001]). Using the Cochran-Mantel-Haenszel test, the relationship between the paraoxonase 192 genotype and the presence or extent of CAD clearly persisted after stratification for the smoking history (χ^2 = 4.98, P = 0.025 for presence of CAD; χ^2 = 6.19, P = 0.013 for extent of CAD). In the multiple logistic regression analysis, the factors age, paraoxonase 192 Arg allele, history of smoking, hypertension, and hyperlipidemia were significantly associated with CAD (Table 3). Factors significantly associated with three-vessel disease were age, paraoxonase 192 Arg allele, BMI, and hypertension.

Paraoxonase 192 genotype and MI. In contrast with CAD, we found no significant associations between the paraoxonase 192 genotype and the history of MI (Table 2). There was a positive history of MI in 30.9% of the patients homozygous for the 192 Gln allele, in 33.1% of those heterozygous, and in 42.9% of those homozygous for the 192 Arg allele (n = 288, χ^2 = 1.45, P = 0.48). Restriction of the analysis to the patients with angiographically proven CAD also did not reveal an influence of the paraoxonase 192 genotype on MI. In that analysis, the prevalence of MI was 58.9% in Gln/Gln genotypes, 52.0% in Gln/Arg, and 60.0% in Arg/Arg (n = 170, χ^2 = 0.89, P = 0.64). In the multiple logistic regression analysis, a positive history of smoking and hypertension were the only factors associated with the history of MI (Table 3).

DISCUSSION

The present study further supports the hypothesis that the paraoxonase 192 Gln/Arg polymorphism is related to CAD in type 2 diabetic patients. This observation was first made by Ruiz et al. (9), who found the same association between the paraoxonase 192 Arg allele and CAD in 434 French type 2 diabetic subjects, and has recently been confirmed in a smaller cohort of Japanese origin (10). Our study also gives evidence that the paraoxonase 192 Arg allele is associated not only with the presence, but also with the extent of CAD.

The predisposition to CAD is determined by a combination of genetic and environmental factors. Reports on the rela-

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TABLE 1
Clinical characteristics and plasma lipid and lipoprotein levels of the type 2 diabetic patients according to the paraoxonase 192 Gln/Arg genotype

	Gln/Gln	Gln/Arg	Arg/Arg
n	139	121	28
Sex (M/F)	86/53	78/43	24/4
Age (years)	61.4 ± 10.5	62.4 ± 9.2	61.3 ± 5.8
BMI (kg/m^2)	28.3 ± 5.7	28.5 ± 6.2	28.8 ± 6.0
Current/former smoker (%)	43.6	44.6	66.7*
Diabetes duration (years)	9.4 ± 7.9	8.5 ± 7.5	10.9 ± 8.7
HbA_{1c} (%)	7.8 ± 1.5	7.7 ± 1.5	8.0 ± 1.7
Systolic blood pressure (mmHg)	140.8 ± 23.3	139.6 ± 27.5	138.2 ± 23.7
Diastolic blood pressure (mmHg)	71.0 ± 12.5	69.8 ± 12.2	72.3 ± 11.9
Total cholesterol (mmol/l)	4.94 ± 1.00	4.92 ± 1.06	5.02 ± 1.23
LDL cholesterol (mmol/l)	2.93 ± 0.83	2.92 ± 0.87	3.00 ± 1.01
HDL cholesterol (mmol/l)	1.02 ± 0.36	0.99 ± 0.32	1.09 ± 0.30
Triglycerides (mmol/l)	2.18 (0.4–6.8)	2.25 (0.3–6.8)	2.02 (0.5–3.8)

Data are means ± SD, means (range), or proportion (%). *$P < 0.05$. There were no further significant differences between the three genotype groups.

tionship between the paraoxonase 192 gene polymorphism and CAD in the general population are inconsistent. Significant associations have been described in North American Caucasians (11) and in Asian Indians (14), but not in Finns (12) or in Chinese living in Singapore (14). Studies in Japanese populations found a high prevalence of the paraoxonase 192 Arg allele, but the relationship with CAD remained contradictory (13,20). Although these inconsistent findings may simply reflect the imponderables of genetic association studies (21), additional gene–environment interactions are also likely to play a role. It is thus conceivable that in type 2 diabetic patients the functional role of paraoxonase is more important than in nondiabetic subjects. Chronic hyperglycemia causes considerable modification of protein structure and function due to nonenzymatic glycation of amino acid residues (3), and LDL cholesterol containing glycated apolipoprotein B100 interacts with vascular endothelium (22) and platelets, thereby increasing thromboxane production and decreasing thrombolytic prostaglandins (23). In addition, glycated LDL cholesterol is more readily oxidized (24), resulting in accelerated macrophage uptake by the scavenger receptor pathway. It can therefore be speculated that the protective effects of paraoxonase against peroxidation of LDL particles are more important in diabetic patients. The difference could explain the much clearer effect of the paraoxonase 192 genotype on CAD in type 2 diabetic patients than in general populations.

This hypothesis is further supported by the results of our subgroup analysis in current and former smokers, which supposed an even stronger association between the paraoxonase 192 Arg allele and CAD among smokers. In accordance with diabetes, smoking is an established risk factor for coronary artery disease in which oxidative mechanisms play an important role. Cigarette smoke contains large amounts of free radicals (25), and plasma antioxidative capacity is lower in smokers (26,27), so oxidized LDL particles from smokers generate more lipid peroxidation products than LDL from nonsmokers (28). Thus it seems conceivable that the paraoxonase activity has a more important role in smokers in protecting from the lipid peroxidation process in vivo. Plasma paraoxonase activity has recently been shown to be inhibited by cigarette smoke extract (29), and in vitro stud-

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TABLE 2
Paraoxonase 192 genotypes, allele frequencies, and ORs for CAD, three-vessel disease, and history of MI in type 2 diabetic patients

	CAD	No CAD	Three-vessel disease	No three-vessel disease	History of MI	No history of MI
n	170	118	84	204	95	193
Paraoxonase 192 genotype (%)						
Gln/Gln	42.9	55.9	36.9	52.9	45.3	49.7
Gln/Arg	45.3	37.3	50.0	38.8	42.1	42.0
Arg/Arg	11.8	6.8	13.1	8.3	12.6	8.3
Allele frequency (%)						
Gln	65.6	74.6	61.9	72.3	66.3	70.7
Arg	34.4	25.4	38.1	27.7	33.7	29.3
OR* (95% CI)	1.78 (1.08–2.96)†		1.99 (1.17–3.44)‡		1.20 (0.73–1.99)	

*Age-adjusted OR in patients with at least one 192 Arg allele for having CAD, three-vessel disease, or a positive history of MI. †$P = 0.02$; ‡$P = 0.01$.

TABLE 3
ORs for presence of CAD, three-vessel disease, and MI according to paraoxonase 192 genotype and other variables by multiple logistic regression analysis

Factor	Presence of CAD		Three-vessel disease		History of MI	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Age	3.10 (1.24-8.04)	0.01	3.38 (1.43-8.33)	0.005	1.97 (0.86-4.63)	NS
Paraoxonase 192 Arg allele	2.12 (1.09-4.19)	0.02	2.54 (1.37-4.80)	0.003	1.28 (0.43-1.41)	NS
Male sex	1.52 (0.70-3.30)	NS	1.33 (0.60-2.98)	NS	1.87 (0.86-4.17)	NS
History of smoking	3.95 (1.81-8.92)	0.0005	1.67 (0.80-3.53)	NS	2.53 (1.27-5.20)	0.008
BMI	1.58 (0.65-3.91)	NS	2.35 (1.00-5.68)	0.05	1.56 (0.68-3.63)	NS
Hypertension	3.91 (2.00-7.85)	<0.0001	1.97 (1.01-3.94)	0.045	2.12 (1.12-4.14)	0.02
Hyperlipidemia	2.39 (1.19-4.89)	0.02	1.45 (0.76-2.80)	NS	1.08 (0.57-2.05)	NS
Diabetes duration	0.86 (0.29-2.57)	NS	1.19 (0.41-3.44)	NS	0.89 (0.32-2.49)	NS
HbA_{1c}	0.88 (0.30-2.61)	NS	1.04 (0.35-3.06)	NS	2.56 (0.87-8.00)	NS

Age, BMI, diabetes duration, and HbA_{1c} were each grouped in quartiles. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg at repeated measurements, or the current use of antihypertensive agents because of the confirmed diagnosis of arterial hypertension.

ies suggest that the paraoxonase 192 Arg/Arg alloenzyme is less able to protect LDL against the accumulation of lipid-peroxides than the alloenzymes containing the Gln variant (30,31). This combination of genetically determined lower paraoxonase-mediated protection from lipid peroxidation, increased nonenzymatic glycation and oxidative damage caused by hyperglycemia, and eventually further impairment of paraoxonase activity by cigarette smoking and from diabetes (6) may lead to an increased entrapment of oxidized LDL particles in the arterial wall, resulting in increased frequency and extent of CAD.

Besides its protective effects against LDL peroxidation, HDL-associated paraoxonase has recently been demonstrated to inhibit the oxidative damage of HDL as well (32). This effect could be crucial in states that favor oxidation such as hyperglycemia or cigarette smoking, because the oxidation of HDL not only reduces its capability to prevent the oxidative modification of LDL, but also diminishes the ability of HDL to function as a potent acceptor for cholesterol efflux (33). In addition to the 192 Gln/Arg polymorphism, the serum paraoxonase concentrations and its activity toward paraoxon—which is an exogenous substrate and should therefore be interpreted with caution—is influenced by the 55 Leu/Met polymorphism (34,35). Interestingly, the paraoxonase 55 Leu/Met and the 192 Gln/Arg polymorphisms are in linkage disequilibrium, and homozygosity for the 55 Leu allele has been identified as a risk factor for CAD (34) in the sample of type 2 diabetic patients in whom the association of the 192 Gln/Arg polymorphism with CAD was first described (9). Furthermore, a common polymorphism in the paraoxonase-2 gene (cystein → serine at codon 311) has been associated with CAD in a recent study of 318 Asian Indians, including 60 diabetic subjects (36). This association of the paraoxonase-2 311-Ser allele with CAD was confined to paraoxonase-1 192-Arg allele carriers, however, indicating a possible linkage disequilibrium of the two gene polymorphisms (37).

In contrast with CAD, we found no association between the paraoxonase 192 genotype with the history of MI in either the whole patient group or the patients with angiographically documented CAD. This is in accordance with the results in patients with MI from the ECTIM study (15). Since the development of myocardial infarction is nowadays regarded as a consequence of plaque disruption, acute coronary thrombosis, and vasoconstriction (38), a pathophysiologic role of paraoxonase in the onset of MI seems rather unlikely. The observed lack of association between diabetes duration or actual HbA_{1c} level and CAD or myocardial infarction must be interpreted with caution, because it could possibly result from a selection bias toward type 2 diabetic patients with CAD but relatively short diabetes duration. In addition, the actual HbA_{1c} level cannot be regarded as representative for the patients' long-term glycemic control. Two recent studies have shown that both diabetes duration and poor glycemic control are important predictors of the occurrence of CAD (39,40). In the large NIDDM Patient Outcome Research Team study (41), however, glycemic control as assessed by glycohemoglobin was also not associated with cardiovascular disease. Another limitation of our study is that CAD has not been excluded angiographically in all patients. Clinical and electrocardiographic signs of CAD may be even less reliable in diabetic patients, because many diabetic patients might not develop angina pectoris despite coronary atherosclerosis. This is highlighted by the recent observation that the mortality from MI in type 2 diabetic patients without known CAD is as high as in nondiabetic subjects with a history of previous MI (42).

In conclusion, this study gives further evidence that the 192 Arg allele of paraoxonase is associated with an increased risk of presence and extent of CAD, but not with MI, in patients with type 2 diabetes. The additional influence of cigarette smoking is compatible with the hypothesis that these associations are related to the lipid peroxidation process and indicates a strong gene–environment interaction of the paraoxonase 192 Gln/Arg polymorphism. Whether other paraoxonase polymorphisms, such as the paraoxonase-1 gene 55 Met/Leu and the paraoxonase-2 gene 311 Cys/Ser polymorphism, or the paraoxonase-2 gene 148 Ala/Gly variant, which is associated with elevated fasting plasma glucose in type 2 diabetic patients (43), are also involved in this gene–environment interaction will have to be further clarified.

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