

Paraoxonase (PON1) 55 and 192 Polymorphism and Its Effects to Oxidant-Antioxidant System in Turkish Patients with Type 2 Diabetes Mellitus

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Summary

Paraoxonase (PON1) is a serum enzyme with an antioxidant function, protecting the low density lipoproteins (LDL) from oxidative modifications. Because diabetic patients are at greater risk of oxidative stress, we investigated the effect of PON1 55 methionine (M)/leucine (L) and PON1 192 glutamine (A)/arginine (B) polymorphisms on oxidant-antioxidant system in 213 individuals with type 2 diabetes mellitus and 116 non-diabetic control subjects from Turkish population were included in the study. Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and agarose gel electrophoresis techniques were used to determine the PON1 genotypes. Thiobarbituric acid reactive substances (TBARS), conjugated dienes levels in the serum and glutathione (GSH) levels in whole blood were measured spectrophotometrically. In both groups PON1 192 AA and PON1 55 MM genotypes had higher TBARS, conjugated dienes levels and lower GSH levels, whereas PON1 192 BB and PON1 55 LL genotypes had lower TBARS, conjugated diene levels and higher GSH level than other genotypes. We thus conclude that PON1 192 BB and PON1 55 LL alleles have protective effect against oxidative stress.

Key words

Paraoxonase • Polymorphism • TBARS • Conjugated dienes • GSH • Type 2 diabetes mellitus • Turkish population

Introduction

Paraoxonase (PON1, arylalkylphosphatase, EC 3.1.8.1) is a serum enzyme with an antioxidant function, protecting low density lipoproteins (LDL) from oxidative modifications (La Du 1992, Mackness *et al.* 1993b, Aviram *et al.* 1998b).

PON1 gene is apparently localized at q21-q22 on the long arm of chromosome 7 in humans (Humbert *et al.* 1993). PON1 has two genetic polymorphisms, one at

position 55 which is a methionine/leucine substitution (A→T), and one at position 192 which is a glutamine/arginine substitution (A→G) (Adkins *et al.* 1993). These polymorphisms affect the hydrolytic activity of PON1 isoenzymes with respect to lipid peroxides (Mackness *et al.* 1998a, Aviram *et al.* 1998a). The paraoxonase activity of the glutamine 192 (A allele) and methionine 55 (M allele) isoforms have been reported to be lower than that of the arginine 192 (B allele) and leucine 55 (L allele) isoforms (Mackness *et al.* 1998a, Eckerson *et al.* 1983).

Nevertheless, PON1 in AA and MM genotype subjects seems to be more effective in protecting LDLs from oxidation than that in BB and LL genotype subjects (Mackness *et al.* 1998a).

Lower serum PON1 activity constitutes a risk factor for atherosclerotic disease (Ruiz *et al.* 1995, Blatter Garin *et al.* 1997, McElveen *et al.* 1986, Mackness *et al.* 1991b). The enzyme has been identified as an independent, genetic risk factor for vascular disease, particularly in type 2 diabetes mellitus patients (Ruiz *et al.* 1995, Blatter Garin *et al.* 1997, Odawara *et al.* 1997, Pfohl *et al.* 1999, Mackness *et al.* 1991b, Sakai *et al.* 1998, Mackness *et al.* 1998b). It has also emerged as an independent risk factor in non-diabetic patients (Serrato and Marian 1995, Sanghera *et al.* 1997, Salonen *et al.* 1999), albeit less consistently (Antikainen *et al.* 1996, Herrmann *et al.* 1996, Suehiro *et al.* 1996). Although the pathophysiological mechanism has not been clarified, it is considered to be linked to the antioxidant role of PON1. This is of particular relevance to diabetic patients in which higher risk of oxidative stress is supposed to contribute to the greatly increased incidence of vascular disease and other complications (Giugliano *et al.* 1996, Baynes and Thorpe *et al.* 1999).

Given the growing importance of paraoxonase as a cardiovascular disease risk factor and its potential involvement in the protection against oxidative stress, we examined the polymorphism at position 55 and 192 and its effects on lipid peroxidation markers, which are thiobarbituric acid reactive substances (TBARS), conjugated dienes and glutathione (GSH) levels in type 2 diabetes mellitus patients and controls from Turkish population.

Methods

Three hundred and sixteen unrelated individuals were included in the study: 207 patients with type 2 diabetes mellitus (89 males (43 %), 118 females (57 %), mean age 59.9 ± 11.6 years) and 109 healthy subjects. Patients were selected from Taksim State Hospital, Istanbul. WHO definitions and criteria for diabetes were used for the classification (Report of WHO Consultation 1999). The patients received a standard questionnaire regarding the age in type 2 diabetes mellitus diagnosis, family history, the treatment methods and other medical issues. Only patients with a clinical diagnosis of type 2 diabetes mellitus and a history of at least 2 years of treatment without insulin were recruited. The study

individuals underwent a basic physical examination that included the measurement of height, weight, and blood pressure. The control group (55 males (50.5 %), 54 females (49.5 %), mean age 58.6 ± 16.0 years) comprised individuals with normal fasting glucose and negative family history of type 2 diabetes mellitus among first degree relatives.

Biochemical determinations

The extent of plasma lipid peroxidation was assessed by measuring TBARS, the end-product of lipid peroxidation, according to the method of Yagi (1987) with minor modifications. Conjugated dienes were determined by the method of Dormandy and Wickens (1987). Antioxidant status was determined by measuring GSH levels in the whole blood by the method of Fairbanks and Klee (1991).

Isolation of DNA

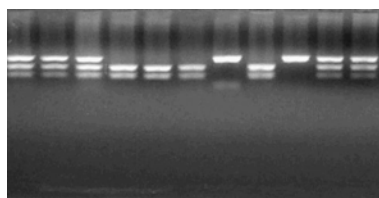
Blood specimens were collected in tubes containing ethylene diamine tetraacetate (EDTA), and DNA was prepared from leukocyte pellets by sodium dodecyl sulphate (SDS) lysis by ammonium acetate extraction and ethanol precipitation (Miller *et al.* 1988).

Paraoxonase genotype determination

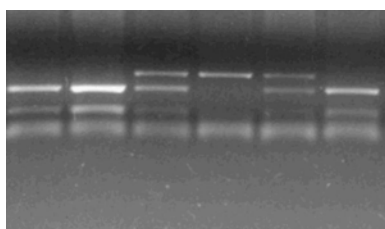
Paraoxonase genotypes were determined following PCR according to previously published protocols (Adkins *et al.* 1993, Humbert *et al.* 1993). For the 192 polymorphism sense primer 5' TAT TGT TGC TGT GGG ACC TGA G 3' and antisense primer 5' CAC GCT AAA CCC AAA TAC ATC TC 3' which encompass the 192 polymorphic region of the human PON1 gene were used. For the 55 polymorphism sense primer 5' GAA GAG TGA TGT ATA GCC CCA G 3' and antisense primer 5' TTT AAT CCA GAG CTA ATG AAA GCC 3' were used. The PCR reaction mixture contained 100 ng DNA template, 0.5 μ M of each primer, 1.5 mM $MgCl_2$, 200 μ M 4dNTP's and 1 U Taq DNA polymerase (MBI Fermentas). After denaturing the DNA for 5 min at 94 °C, the reaction mixture was subject to 35 cycles of denaturing for 1 min at 95 °C, 1 min annealing at 60 °C and 1 min extension at 72 °C for the 192. The 99 bp PCR product was digested with 8 U BspI restriction endonuclease (MBI Fermentas, Lithuania) overnight at 55 °C and the digested products separated by electrophoresis on 4 % metaphore agarose gel and visualized using ethidium bromide. The B-genotype (arginine) contains a unique BspI restriction site which

results in 66 and 33 bp products, whereas the A-genotype (glutamine) will not be cleaved by this restriction enzyme allowing thus to determine the PON1 192 genotype (Adkins *et al.* 1993).

For the PON1 55 polymorphism, PCR reaction and cycling was the same as above. The PCR product (170 bp) was digested with Hsp192II (Promega, USA) in



A. 99 bp A allele, 66+33 bp B allele



B. 170 bp L allele, 126+44 bp M allele

Fig. 1A Direct visualization of PCR products by ethidium bromide staining. PON1 192 amplified fragment was cleaved by BspI.

Fig. 1B PON1 55 amplified fragment was cleaved by Hsp192II (B) and separated by electrophoresis on 4 % metaphore agarose gel, respectively.

the presence of BSA (0.1 µg/µl final concentration) (37 °C, overnight) and the digested products were separated and identified as above. Allele L (leucine) did not contain the Hsp192II site, whereas M (methionine) contained the Hsp192II site giving rise to 126 and 44 bp products (Humbert *et al.* 1993) (Fig. 1).

Statistical analyses

Statistical analyses were performed using the SPSS software package, revision 10.0. The laboratory data are expressed as means ± S.D. Mean values were compared between patients with type 2 diabetes mellitus and control subjects by the unpaired Student's t test.

Statistical analysis for effects of PON1 alleles on glutathione and serum TBARS, conjugated dienes in type 2 diabetes mellitus and control subjects were made by one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered to be statistically significant.

Results

Glutathione, TBARS and conjugated dienes levels of the controls and type 2 diabetes mellitus subjects are given in Table 1. The type 2 diabetes mellitus patients had significantly higher TBARS levels and low erglutathione levels compared with the control subjects ($p < 0.05$). Serum conjugated dienes levels were increased in type 2 diabetes mellitus patients compared to the control group but the difference was not significant ($P = 0.07$).

Table 1. Glutathione, TBARS and conjugated dienes levels of the controls and type 2 diabetes mellitus patients

	Controls n=109	Patients n=207
Glutathione (µmol)	157.76±33.48	104.54±23.71**
TBARS (nmol/dl)	5.47±1.76	7.13±3.87**
Conjugated dienes (nmol/ml)	13.04±5.71	9.67±7.07*

Data are means ± SD; n = number of individuals; * $p < 0.01$; ** $p < 0.001$

The relationships between the PON1 genotypes and TBARS, GSH and conjugated dienes are shown in Table 2. In the control group, GSH levels were significantly lower in PON1 55 MM genotypes than in LL and LM genotypes ($P < 0.01$) and TBARS levels were significantly higher in MM genotypes than in LL ($P < 0.001$) and LM genotypes ($P < 0.01$). GSH levels were

significantly lower in PON1 192 AA genotypes than in BB and AB genotypes ($P < 0.05$). Although TBARS and conjugated dienes levels tended to be higher in AA genotypes than in BB and AB genotypes, but the results were not significant ($p > 0.05$).

In the type 2 diabetes mellitus patients, TBARS levels were significantly higher in PON1 55 MM

genotypes than in LL and LM genotypes ($P<0.0001$). GSH levels were significantly lower in AA genotypes than in AB genotypes, TBARS and conjugated dienes levels were significantly higher in PON1 192 AA genotypes than in BB ($P<0.05$) genotypes. PON1 55 LL homozygotes had significantly higher glutathione ($P<0.001$) and conjugated dienes ($P<0.05$) levels and significantly lower TBARS ($P<0.001$) levels in the control group than in type 2 diabetes mellitus patients. LM heterozygotes had significantly higher glutathione levels in controls than in type 2 diabetes mellitus patients

($P<0.0001$).

PON1 192 AA homozygotes had higher conjugated dienes ($P<0.05$) levels and lower TBARS ($P<0.01$) levels in controls than in type 2 diabetes mellitus patients. BB homozygotes had higher glutathione level in controls than in type 2 diabetes mellitus patients ($P<0.0001$). AB heterozygotes had higher glutathione ($P<0.0001$) and conjugated dienes ($P<0.05$) levels and lower TBARS ($P<0.05$) levels in controls than in type 2 diabetes mellitus patients.

Table 2. Glutathione, TBARS and conjugated dienes levels in controls and type 2 diabetes mellitus patients according to their PON1 genotypes

	Controls			Patients		
PON1 55 Genotypes	LL (n=51)	MM (n=7)	LM (n=51)	LL (n=111)	MM (n=8)	LM (n=86)
Glutathione (μmol)	166.7 $\pm$ 23.2***	106.4 $\pm$ 18.1	166.7 $\pm$ 33.1***	103.2 $\pm$ 22.4	88.4 $\pm$ 2.5	106.8 $\pm$ 26.8
TBARS (nmol/dl)	5.09 $\pm$ 1.40***	7.85 $\pm$ 2.26	5.51 $\pm$ 1.81	6.63 $\pm$ 2.37	14.45 $\pm$ 12.48	6.98 $\pm$ 2.75
Conjugated dienes (nmol/ml)	12.99 $\pm$ 5.81*	13.57 $\pm$ 8.42	12.84 $\pm$ 5.24	9.70 $\pm$ 6.55	12.49 $\pm$ 8.47	9.74 $\pm$ 7.70
PON1 55 Genotypes	AA (n=36)	BB (n=10)	AB (n=61)	AA (n=100)	BB (n=22)	AB (n=85)
Glutathione (μmol)	111.0 $\pm$ 19.1	175.4 $\pm$ 6.0***	161.9 $\pm$ 32.0***	94.7 $\pm$ 13.6	98.5 $\pm$ 19.7	112.4 $\pm$ 28.4
TBARS (nmol/dl)	6.19 $\pm$ 1.71**	4.74 $\pm$ 0.51	5.18 $\pm$ 1.88*	7.89 $\pm$ 2.75	5.43 $\pm$ 1.87	7.19 $\pm$ 5.20
Conjugated dienes (nmol/ml)	14.73 $\pm$ 5.33*	9.87 $\pm$ 3.77	12.23 $\pm$ 6.02*	11.54 $\pm$ 6.59	9.65 $\pm$ 8.42	8.42 $\pm$ 6.87

Data are means $\pm$ SD; n = number of individuals; * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (significantly different from patients).

Discussion

Peroxidation of low density lipoproteins (LDL) plays a pivotal role in atherogenesis (Steinberg *et al.* 1989, Berliner and Haberland 1991). High-density lipoprotein (HDL) retards the accumulation of lipid peroxides in LDL when incubated under oxidizing conditions *in vitro* (Mackness *et al.* 1993a). This effect seems to be primarily due to paraoxonase (PON1) activity in HDL. PON1 protects LDL against oxidative modifications *in vitro* by preventing the accumulation of lipid peroxides (Mackness *et al.* 1991a, 1993b). Human PON1 polymorphisms affect the hydrolytic activity of

PON1 isoenzymes with respect to lipid peroxides (Mackness *et al.* 1998a, Aviram *et al.* 1998a).

Paraoxon hydrolysis activity varies widely among individuals. Part of this variability is due to the polymorphism of PON1 gene (Humbert *et al.* 1993). A glutamine (A)/arginine (B) substitution at position 192 which is determinant for enzyme activity, the B allele coding for a protein displays several-fold higher activity towards paraoxon hydrolysis than the A allele (Adkins *et al.* 1993, Nevin *et al.* 1996). A methionine (M)/leucine (L) substitution at position 55 has a lesser effect on enzyme activity, while a strong linkage disequilibrium exists between L-55 and A-192 (Blatter Garin *et al.* 1997).

Arylesterase activity borne by the same protein is not affected by either polymorphism and can be considered as an index of actual protein concentrations, independent of PON1 variability. If, the protection by PON1 of LDL against oxidation is indeed a meaningful phenomenon, then the B allele should confer increased cardiovascular protection compared to A allele. Nevertheless, recent studies revealed that the BB genotype was more prevalent among subjects with a history of cardiovascular disease than in controls (Ruiz *et al.* 1995, Odawara *et al.* 1997, Serrato and Marian 1995, Sanghera *et al.* 1997). These results were, however, quite controversial (Antikainen *et al.* 1996, Herrmann *et al.* 1996, Suehiro *et al.* 1996) and we have shown that PON1 55 and 192 genotype distributions were similar in Turkish type 2 diabetes mellitus patients and controls (Agachan *et al.* 2003). In the present investigation, we have shown that the polymorphisms of PON1 due to amino acid substitutions at positions 55 and 192 can greatly affect lipid peroxidation. In both groups, MM and AA genotypes had higher TBARS and conjugated diene levels and lower GSH level, whereas BB and LL genotypes had lower TBARS and conjugated diene levels and higher GSH level than other genotypes. We thus think that PON1 192 BB and PON1 55 LL alleles have protective effects against oxidative stress. Nevertheless, Mackness *et al.* (1998a) suggested that PON1 55 MM / 192 AA genotypes HDL provided the greatest protection against lipid peroxide generation on LDL and the PON1 55 LL/192 BB genotypes HDL provided the least protection. We were unable to confirm their conclusion in our study of type 2 diabetes mellitus patients.

Another interesting aspect concerns the inactivation of PON1 in the presence of oxidative stress. PON1 activity has been shown to be reduced in the course of oxidative incubation with Cu²⁺-induced peroxidation of

LDL (Cao *et al.* 1999). Oxidized LDL appears to inactivate PON1 through interactions between the enzyme-free sulfhydryl group and oxidized lipids, which are formed during LDL oxidation (Aviram *et al.* 1999). There is evidence of an increase in lipid peroxidation products in diabetic patients (Kaji *et al.* 1985, Mooradian 1991, Griesmacher *et al.* 1995). There are also some observations suggesting that a low PON1 activity toward paraoxon is likely to be present in type 2 patients (Ruiz *et al.* 1995, Blatter Garin *et al.* 1997, Odawara *et al.* 1997, Pfohl *et al.* 1999, Mackness *et al.* 1991b, Sakai *et al.* 1998, Mackness *et al.* 1998b). It is probable that PON1 activity may be partially inactivated in the presence of oxidative stress as probably occurs in patients with type 2 diabetes mellitus. Nevertheless, as observed in the present study, the decrease in PON1 activity and increase in lipid production products are more evident in the low paraoxonase activity AA and MM genotypes. Assuming that serum PON1 is inactivated in the presence of a high oxidative stress, as it probably occurs in type 2 diabetes mellitus patients, it is possible that subjects with low PON1 activity and the AA and/or MM genotype have a reduced ability to preserve PON1 activity.

In conclusion, the PON1 192 BB and 55 LL genotypes, which are associated with high PON1 activity towards paraoxon, may be adequate to prevent lipid peroxidation, but AA and MM genotypes, which are associated with low PON1 activity, may be insufficient when oxidative stress is present in type 2 diabetes mellitus patients.

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