Original Article

The Influence of PON1 192 Polymorphism on Endothelial Function in Diabetic Subjects with or without Hypertension

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Hypertension and type 2 diabetes mellitus (T2DM) cause endothelial dysfunction probably through increased oxidant stress. Paraoxonase (PON1) is an high-density lipoprotein (HDL)–linked anti-oxidant enzyme whose capacity is influenced by a genetic polymorphism at codon 192. In the present study we have investigated the role of PON1 polymorphism on endothelial function in subjects with T2DM with or without hypertension. Three groups of male subjects were enrolled: 65 healthy control subjects without T2DM or hypertension (CON), 51 with only T2DM (DM), and 67 with both hypertension and T2DM (HYP+DM). The PON1 Gln192Arg polymorphism was determined by polymerase chain reaction (PCR) amplification and restriction analysis. Endothelial function was evaluated as flow-mediated vasodilatation (FMD) of the brachial artery after forearm ischemia. Data were analyzed according to the presence or absence of the Arg allele. Subjects with T2DM had markedly impaired FMD, compared with those of the CON group. In the CON and HYP+DM groups no difference was observed in FMD between subjects homozygous for the Gln allele and those carrying the Arg allele. In the DM group FMD was lower among those carrying the Arg allele compared with Gln/Gln homozygotes (2.1±2.4% *vs.* 6.2±5.2%, $p=0.002$). In conclusion, the present findings demonstrated that FMD was less impaired in normotensive diabetic subjects homozygous for the Gln allele, consistent with the notion that this isoform has a more effective antioxidant action that serves to protect circulating low-density lipoprotein (LDL). Hypertension seems to abolish the protective effect of the Gln isoform. These findings, however, warrant further investigation to clarify their clinical import. (*Hypertens Res* 2008; 31: 507–513)

***Key Words*: paraoxonase, diabetes mellitus, genetic polymorphism, endothelial function**

Introduction

Endothelial dysfunction has been documented in subjects with diabetes mellitus or impaired glucose tolerance (*1–4*). The pathogenesis of diabetic vascular disease may involve increased levels of small-dense low-density lipoprotein (LDL) and its oxidative derivatives (*5–9*). A recent study reported *in vivo* evidence of increased oxidation of circulating LDL in subjects with impaired glucose tolerance (*10*), suggesting that the oxidative stress might influence endothelial function in these subjects. Hypertension is also associated with increased oxidant stress that is thought to represent a major mechanism leading to endothelial dysfunction in this disease (*11*). It is known that a high-density lipoprotein (HDL)–linked enzyme, paraoxonase (PON1), protects LDL from oxidative modification (*12*). A genetic polymorphism of PON1, Gln192Arg, has been suggested to influence its anti-

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oxidant capacity (*13–15*), with the Arg allele being associated with lesser protection of LDL against the accumulation of lipid peroxides (*16*). Recently, Yamane *et al*. reported that the PON1 polymorphism was not correlated with coronary macro- or microvasomotor responses induced by bradykinin in patients with normal coronary arteries (*17*). There is a relative dearth of data on the influence of the genetic polymorphism of PON1 on endothelial function in clinical situations known to be associated with increased oxidant stress.

In the present study we have therefore investigated the possible association between the PON1 Gln192Arg polymorphism and the brachial artery endothelial function in subjects with diabetes mellitus with or without hypertension.

Methods

Subjects

Patients were recruited from the diabetic outpatient clinic, and controls from among participants in a regional cardiovascular disease prevention campaign. All participants were informed about the aim of the study and written informed consent was obtained before examination. All subjects were examined in the morning in a room at 22°C, after overnight fasting. Because of the objective difficulty of confirming abstinence on the morning of examination, smokers were excluded. Since flow-mediated vasodilatation (FMD) is influenced by gender, only male subjects were enrolled. Blood pressure, height and weight were measured by routine methods. Body mass index (BMI) was computed as weight (in kg) divided by height (in m^2). A comprehensive questionnaire aimed at evaluating the presence of risk factors for coronary heart disease (CHD), which included a smoking habit and drug use, was administered by trained personnel. Further exclusion criteria were: use of drugs known to influence endothelium-dependent and/or -independent vasodilatation, such as statins, angiotensin converting enzyme (ACE)–inhibitors, angiotensin II type 1 (AT1)–receptor antagonists and Ca channel blockers (*18*); known inflammatory disease; cancer disease; unstable diabetes, defined as a variation of more than 1% in glycated hemoglobin within the previous 6 months. All participants, diabetic patients and control subjects, were selected consecutively and simultaneously. If they met the inclusion and exclusion criteria, they were informed about the aim and procedures of the study and invited to participate. Among eligible subjects three diabetic and two control subjects refused to participate. Blood was withdrawn from an antecubital vein, after echo-Doppler examination, following an overnight fast. Blood lipids and glucose were measured by commercially available kits. Diabetes was defined as fasting blood glucose >7.0 mmol/L (126 mg/dL) on at least two occasions and/or use of antidiabetic agents. Non-diabetic subjects received oral glucose tolerance test to exclude any alteration in carbohydrate metabolism. Hypertension was defined as systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg and/or use of antihypertensive drugs.

Table 1. Clinical and Biochemical Characteristics of CON and Diabetic Subjects

	CON	Diabetes
Number	65	118
Age (years)	55.9±6.6	56.0±8.3
SBP (mmHg)	126±13	126±24
DBP (mmHg)	82±3	78±10*
BMI (kg/m^2)	27.14±3.99	29.40±4.81*
Cholesterol (mg/dL)	222±36	201±41*
HDL-chol (mg/dL)	65±22	48±12*
Triglycerides (mg/dL)	128±113	154±72
Glucose (mg/dL)	96±12	169±52*
Brachial artery diameter (mm)	3.00±0.73	3.43±0.57*

Values are mean±SD (unless otherwise stated). *$p<0.05$. CON, control; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high-density lipoprotein.

Genetic Analysis

PON1 192 polymorphism was investigated as previously described (*19*). Genomic DNA was extracted from peripheral blood leukocytes according to standard methods. PON1 genotypes were determined by polymerase chain reaction (PCR) amplification and restriction analysis. The following set of primers was used: forward 5′-TTGAATGATATTGTTGCT GTGGGACCTGAG-3′ (nt. 499–528) and reverse 5′-CGA CCACGCTAAACCCAAATACATCTCCCAGaA-3′ (nt. 609–577). The lower case base in the PON1 192 reverse primer indicates a mismatch introducing a restriction site for *Hinf*I (G/ANTC) in the DNA amplification product in the presence of arginine (CGA) at codon 192 of the PON1 gene. The 50 μL PCR reaction contained 0.5 μg DNA template, 0.1 μmol/L of each primer, 200 μmol/L of the four dNTPs, 1 unit of Taq DNA polymerase (Promega, Madison, USA), and 1.5 mmol/L $MgCl_2$. 40 cycles of amplification (94°C 1 min, 65°C 45 s, 72°C 45 s) with a final extension of 5 min at 72°C were performed on a Perkin Elmer Gene Amp 2400 (Perkin Elmer, Norwalk, USA). Restriction analysis with 5 units of *Hinf*I (Boehringer Mannheim, Mannheim, FRG) for 3 h at 37°C yielded one undigested 111-bp band in the case of Gln and two digested products (77 and 34 bp) in the case of Arg on 3.5% agarose gel electrophoresis.

Endothelium-Dependent and -Independent Dilation of the Brachial Artery

FMD was recorded 5–10 min after carotid ultrasound, always in the non-dominant arm. The brachial artery was visualized 5–6 cm above the elbow and scanned in longitudinal sections, on the anterior side of the bicipital muscle. The probe was

Table 2. Clinical and Biochemical Characteristics of CON and Diabetic Subjects According to Absence or Presence of the Arg Allele

	CON		DM	
	Arg−	Arg+	Arg−	Arg+
Number	26	39	64	54
Age (years)	55.8±6.7	55.9±6.6	56.7±7.6	54.7±9.2
SBP (mmHg)	127±13	125±13	130±20	118±27*
DBP (mmHg)	82±3	82±7	79±9	76±8
BMI (kg/m^2)	26.90±5.16	27.30±3.02	29.93±4.98	28.71±4.46
Cholesterol (mg/dL)	231±28	215±40	200±41	208±39
HDL-chol (mg/dL)	73±23	59±20*	47±13	48±8
Triglycerides (mg/dL)	92±39	153±138	157±67	149±78
Glucose (mg/dL)	92±13	98±9	175±54	163±48
Brachial artery diameter (mm)	2.95±0.69	3.04±0.76	3.47±0.59	3.35±0.50

Values are mean±SD (unless otherwise stated). *$p<0.05$ (Arg− *vs*. Arg+). DM, diabetic patients without hypertension; CON, control; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high-density lipoprotein.

adjusted to obtain a straight imaging of the artery. The skin was marked to keep the transducer in the same position throughout the study. The gain setting was adjusted until wall boundaries were evident. Internal diameter, defined as the distance from the leading edge of the near wall to the leading edge of the far wall, at the R (ID_R) and T (ID_T) waves of the ECG, was measured on a freeze scan. For statistical analyses, the mean arterial diameter at the R and T waves were computed. A pneumatic tourniquet, previously placed on the forearm distal to the site of artery measurement, was then inflated to a suprasystolic level (around 200 mmHg) for 5 min. Fifty seconds after the cuff was deflated, internal brachial artery diameter was again measured. This time lag is necessary in order to have maximal arterial dilation in response to increased flow velocity.

To evaluate the endothelium-independent vasodilatation, a new baseline measurement of the brachial artery diameter was performed 15 min after ischemia, following which period an exogenous NO donor was administered as a sublingual tablet, and the brachial artery was measured 3 to 4 min later.

The reproducibility of FMD measurement has been previously reported (*20*). Briefly, the agreement between the brachial artery diameter measurements under a resting condition and after hyperemia has been evaluated in subjects studied three times at intervals of 1 week, and Kendall's *W* coefficient of concordance was calculated. For the diameter under a resting condition and after hyperemia, Kendall's *W* coefficients were highly significant: $W_{0.05,5,3}=14.16$ ($p<0.001$) and $W_{0.05,5,3}=12.49$ ($p<0.001$), respectively.

Skewness and kurtosis (mean±SEM) for FMD were 0.241±0.181 and 0.640±0.359, respectively, and did not show significant departure from the normal distribution ($p>0.2$ and $p>0.1$ by Fisher's test, respectively).

Statistical Analyses

All statistical analyses were performed using the program SPSS 13.0 (SPSS, Chicago, USA).

Allele frequencies were calculated by gene counting, and Hardy-Weinberg equilibrium was assessed by χ^2 test. Normal distribution for continuous variables was tested by the Lilliefors test. Triglycerides, not normally distributed, were log-transformed before analysis. Values are expressed as the means±SD, unless otherwise stated. Student's *t*-test was used to analyze differences in continuous variables between controls and diabetics, between diabetics with and without hypertension, and between subjects carrying the Arg allele and those homozygous for the Gln allele within each group. To allow for the independent contribution of age, SBP, DBP, BMI, blood lipids, glucose, and brachial artery diameter to FMD, stepwise multiple regression analyses were performed. The cut-off value for variables entering the model was set at $p<0.1$.

Results

Sixty-five healthy control subjects without T2DM or hypertension (CON), and 118 diabetic patients, 51 of whom were without hypertension (DM), and 67 of whom had hypertension (HYP+DM) were enrolled. The prevalence of the Gln allele was 64%, 72%, and 78% in the CON, DM and HYP+DM groups, respectively. Analysis of the distribution of the genotypes showed that, in the CON group, the frequency was 0.400 for Gln/Gln, 0.476 for Gln/Arg and 0.123 for Arg/Arg. In the DM group, the prevalences were 0.529, 0.393 and 0.078, and in the HYP+DM group they were 0.552, 0.328 and 0.119, respectively. These distributions were not significantly different among the three groups and were compatible with the Hardy-Weinberg equilibrium.

Subjects with diabetes (with or without hypertension,

Table 3. Clinical and Biochemical Characteristics of Diabetic Patients According to the Presence of Hypertension

	DM	HYP+DM
Number	51	67
Age (years)	55.2±9.2	56.6±7.5
SBP (mmHg)	117±26	133±19*
DBP (mmHg)	76±8	81±11*
BMI (kg/m^2)	28.15±4.30	30.46±4.99*
Cholesterol (mg/dL)	210±32	196±45
HDL-chol (mg/dL)	49±12	46±11
Triglycerides (mg/dL)	143±55	161±81
Glucose (mg/dL)	169±52	170±53
Diabetes duration (years)	8.3±6.8	10.2±7.5
HbA1c (%)	6.3±1.4	6.4±1.0
Brachial artery diameter (mm)	3.40±0.56	3.46±0.57

Values are mean±SD (unless otherwise stated). *$p<0.05$. DM, diabetic patients without hypertension; HYP+DM, diabetic patients who had hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high-density lipoprotein.

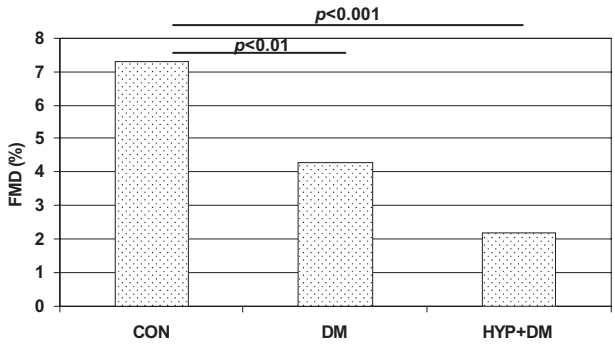

Fig. 1. *Flow-mediated vasodilation of the brachial artery in subjects without diabetes or hypertension (CON), in subjects with only diabetes (DM) and in subjects with both hypertension and diabetes (HYP+DM).*

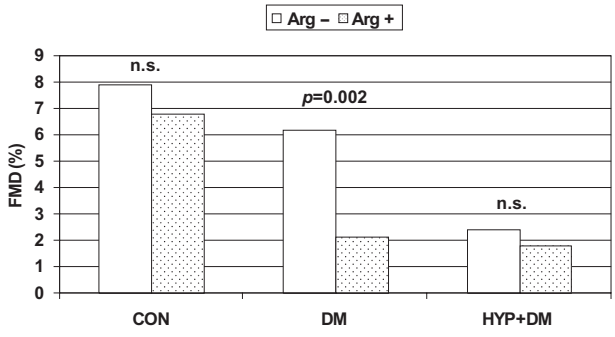

Fig. 2. *Flow-mediated vasodilation of the brachial artery in subjects without diabetes or hypertension (CON), in subjects with only diabetes (DM) and in subjects with both hypertension and diabetes (HYP+DM) according to the absence or presence of the Arg allele.*

$n=118$) had lower levels of DBP, total and HDL cholesterol, and higher values of BMI, glucose and brachial artery diameter compared to controls (Table 1). They also had markedly lower levels of FMD (3.1±3.6% *vs*. 7.3±6.1%, $p<0.001$).

Table 2 shows the clinical and biochemical characteristics of control and diabetic subjects, divided according to the absence or presence of the Arg allele. In the CON group, those carrying the Arg allele had slightly lower HDL-cholesterol concentration, while in diabetics they showed lower SBP values. The brachial artery diameter was similar between subjects carrying and those not carrying the Arg allele. In the CON group, FMD was similar between the Gln homozygotes and Arg allele carriers (7.9±7.3 *vs*. 6.8±5.1, respectively), while in diabetics it was significantly higher (4.00±4.2 *vs*. 1.93±2.8, $p<0.05$, respectively).

Diabetic subjects were then analyzed according to the presence of hypertension. HYP+DM subjects had higher blood pressure values and BMI compared with the DM group, but were similar in terms of glucose control, diabetes duration, lipid profile, and brachial artery diameter (Table 3). FMD was lower in HYP+DM subjects compared with DM (2.2±2.5 *vs*. 4.3±4.8%, $p<0.05$, respectively) (Fig. 1). When further analyzed according to PON1 genotype, Arg allele carriers in the HYP+DM group showed lower SBP levels. No other clinical and biochemical difference was appreciable between Arg allele carriers and non-carriers, either in the DM or HYP+DM group (Table 4). Figure 2 shows the results of FMD: while there was no appreciable difference between Gln homozygotes and Arg allele carriers in HYP+DM subjects (2.4±2.8 *vs*. 1.8±1.9%, respectively), in the DM group a marked difference was observed, Gln homozygotes having a much higher FMD value (6.2±5.2 *vs*. 2.1±2.4%, respectively), similar to that observed in CON subjects.

Among T2DM patients with hypertension, 8 did not receive drug treatment. These patients had higher SBP values compared with those who were under treatment (144±13 *vs*. 132±19 mmHg), but similar DBP (80±7 *vs*. 81±11 mmHg). The independent effect of PON1 192 polymorphism and of variables potentially influencing endothelial function on FMD was also evaluated in stepwise multiple regression analyses, performed separately for the three groups of subjects. In a model including age, blood pressure, blood lipids, BMI, glucose, arterial diameter and PON1 192 polymorphism, SBP significantly contributed to FMD only in control subjects (β standardized coefficient = −0.288, $p=0.035$), while PON1 polymorphism significantly contributed to FMD in the DM group (β standardized coefficient = −0.395, $p=0.005$), and age did so in the HYP+DM group (β standardized coefficient = −0.354, $p=0.008$).

Endothelium-independent vasodilatation was similar in the

Table 4. Clinical and Biochemical Characteristics of Diabetic Subjects According to Absence or Presence of the Arg Allele

	DM		HYP+DM	
	Arg−	Arg+	Arg−	Arg+
Number	27	24	37	30
Age (years)	55.8±7.9	54.5±10.5	57.4±7.4	54.9±7.6
SBP (mmHg)	120±18	112±33	137±19	126±15*
DBP (mmHg)	76±9	75±6	82±11	77±9
BMI (kg/m^2)	28.54±4.78	27.70±3.74	30.95±4.93	29.52±5.09
Cholesterol (mg/dL)	217±33	202±30	188±42	212±48
HDL-chol (mg/dL)	50±15	48±9	46±13	47±7
Triglycerides (mg/dL)	154±44	132±62	159±76	169±91
Glucose (mg/dL)	180±52	156±50	171±56	169±45
Diabetes duration (years)	8.4±7.8	8.1±5.6	11.1±7.3	7.7±7.6
HbA1c (%)	6.7±1.7	6.0±1.0	6.6±0.9	5.9±1.2
Brachial artery diameter (mm)	3.42±0.48	3.37±0.64	3.51±0.66	3.33±0.29

Values are mean±SD (unless otherwise stated). *$p<0.05$ (Arg− *vs*. Arg+). Abbreviations are the same as in Table 2.

CON and DM groups, but markedly reduced in HYP+DM subjects (19.1±7.6% *vs*. 22.5±8.8% *vs*. 12.2±8.0%, CON *vs*. DM *vs*. HYP+DM, respectively, $p<0.002$), and was similar, within each group, between subjects homozygous for the Gln allele and those carrying the Arg allele (21.8±6.2 *vs*. 16.4±8.2%, 23.6±8.2 *vs*. 20.8±9.9%, and 11.9±8.5 *vs*. 12.8±7.3%, in the CON, DM and HYP+DM groups, respectively, n.s.).

Discussion

In the present study we report, for the first time, that subjects with T2DM who are homozygous for the Gln allele of the PON1 192 polymorphism are protected against endothelial dysfunction. The presence of hypertension, however, seems to eliminate this protection.

FMD is widely accepted as a valid parameter of endothelial function. Though influenced by CHD risk factors it is often used as a marker of early atherosclerosis. Subjects with altered glucose metabolism and those with hypertension have been repeatedly reported to have impaired FMD. The alteration in vasomotor response of the brachial artery has been attributed, at least in part, to oxidative stress and consequent increased LDL oxidation, which is particularly high in diabetics (*9*, *21*).

The Gln→Arg polymorphism does not seem to influence FMD in non-diabetic subjects. The lack of association between PON1 polymorphism and FMD in healthy subjects has already been reported (*22*). This is probably the consequence of the relatively low oxidative stress of these subjects, which might reduce the influence of the antioxidant capacity of the PON1 system. However, when these subjects were challenged with acute hypertriglyceridemia as a prooxidant factor, those homozygous for the Arg allele showed impaired brachial artery reactivity (*22*). This finding demonstrates that an acute oxidative stress reduces FMD and that the Gln→Arg PON1 polymorphism plays a role in modulating this effect.

Subjects with T2DM have an increased oxidative stress, and an impaired FMD. It can be hypothesized that a reduction of oxidative stress in these patients might lead to an improvement of FMD. Indeed, in a recent study conducted in hypertensive patients with or without glucose intolerance, vitamin C administration was found to ameliorate FMD in the glucose intolerance group, but not in the group with normal glucose metabolism (*23*).

A recent meta-analysis of 43 published studies has confirmed the role of the Gln192Arg polymorphism as a predictor of CHD, while the results have been inconsistent for three other often investigated polymorphisms, *i.e.*, the Leu55Met, the T−107C at the promoter level of PON1 and the Ser311Cys of the PON2 gene (*24*).

In our study, we observed that FMD was less impaired in diabetic subjects homozygous for the Gln allele, consistent with a more effective antioxidant action and, presumably, greater protection against the circulating LDL of this isoform. The Arg allele has consistently emerged as a risk factor in diabetic populations (*25*, *26*). In one study on T2DM patients, the odds ratio for CHD in subjects with Arg/Arg was 2.5 (95% confidence interval: 1.2–5.3). However, no data on functional arterial parameters have so far been reported. Intriguingly, in the present study the difference in FMD between Gln homozygous and Arg carriers was evident only in hypertensive patients with T2DM. In our opinion, this finding has several possible explanations. First, subjects with diabetes and hypertension might have extremely high oxidative stress, which could blunt the antioxidant capacity of the paraoxonase. It has been hypothesized that inactivation of NO by reactive oxygen species might be an important common mechanism by which endothelial dysfunction occurs both in hypertensive and diabetic vascular disease (*27*). Second, the mechanisms responsible for endothelial dysfunction might differ between hypertension and diabetes. HYP+DM subjects

seem to have a more advanced endothelium-dependent and endothelium-independent vascular dysfunction, and this cannot be accounted for merely by the worsening clinical conditions in terms of diabetes duration, glucose control, lipid profile, *etc*. This finding suggests that the endothelial dysfunction of diabetic hypertensive subjects is at least in part independent of oxidative modifications. A clear-cut answer to this question will be given by planning a study with a primary objective of comparing selected groups of diabetics, hypertensives without diabetes, diabetics with hypertension, and healthy subjects, while bearing in mind the finding that blood pressure values appear to influence FMD in healthy subjects. Indeed, the lack of a group of subjects with hypertension but without diabetes represents probably the main limitation of the present study. In light of this shortcoming, while the present findings are worth documenting, they are also rather speculative and warrant further investigation for clarification of their clinical impact.

Endothelium-independent vasodilatation was similar in controls and in diabetics but markedly impaired in patients who also had hypertension. Since it is well known that endothelium-dependent vasodilatation is strictly dependent upon NO released locally in response to diverse stimuli, such as ischemia in the model used herein, our results might indicate that hypertension causes damage to vascular smooth muscle cells, which then become unresponsive to any source of NO. The fact that PON1 polymorphism was not associated with endothelium-independent vasodilatation suggests that the effect of paraoxonase is limited to specific endothelial operative oxidative mechanisms.

Given the large interindividual variability in the amount of PON1 protein in serum, its assessment would have been important in addition to the genotype-associated information (*28*). It has been reported that PON1 activity and concentration might also be relevant in predicting the presence of CHD (*29*). On the other hand, PON1 activity and concentration are probably influenced by environmental variables such as dietary modifications or clinical evidence of CHD and genetic polymorphism might play a modulating role (*30*). Patients and controls enrolled in the present study were all coming from the same geographical area, with deep-rooted traditions and dietary habits, and are likely to represent a homogeneous group of individuals.

T2DM subjects, with or without hypertension, have larger arterial diameter than controls. While this finding has been previously reported in community-based cohort studies and case-control studies (*31*, *32*), the reasons for it are not clear. It is possible that endothelial dysfunction causes changes in vascular tone leading to arterial enlargement. Another possible explanation is an alteration of collagen and elastin metabolism of the arterial wall, caused by nonenzymatic glycosylation and accumulation of advanced glycation end products (*33*, *34*).

In conclusion, the present study demonstrated that Gln→Arg polymorphism of PON1 influenced endothelial function in normotensive T2DM subjects. These results are consistent with a major physiopathological role of oxidative stress in the determinism of endothelial dysfunction in diabetic subjects. The Gln→Arg polymorphism does not influence vasodilatation in patients with T2DM with elevated blood pressure, suggesting that hypertension can cause endothelial damage at least in part through mechanisms distinct from oxidative stress. If this were to be confirmed in larger studies, the Gln→Arg polymorphism of PON1 could help to improve the definition of CHD risk, and might also influence the therapeutic approach in these patients.

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