別紙４

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Suzuki M, Takamisawa I, Yoshimasa Y, Harano Y	Association between insulin resistance and endothelial dysfunction in type 2 diabetes and the effects of pioglitazone.	Diabetes Research Clinical Practice	76	12-17	2007
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V. 研究成果の刊行物・別刷

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Association between insulin resistance and endothelial dysfunction in type 2 diabetes and the effects of pioglitazone

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Abstract

Endothelial dysfunction is regarded as an early stage of atherosclerosis, and plays a role in the development of atherosclerotic diseases. Insulin resistance is related to the atherosclerotic process. In this study, we examined the association between endothelial function and insulin resistance in 48 subjects with type 2 diabetes. In addition, the effects of pioglitazone treatment on endothelial function and insulin resistance were investigated in a subgroup of subjects. Endothelial function of the brachial artery was non-invasively assessed using ultrasound technique. We measured flow-mediated endothelium-dependent vasodilation (FMD) and glyceryl trinitrate-induced endothelium-independent vasodilation (GTN). Insulin sensitivity was measured by the steady-state plasma glucose (SSPG) method. High SSPG levels indicate insulin resistance. There was a significant inverse correlation ($r = -0.462$, $p < 0.001$) between SSPG and FMD. Systolic blood pressure was inversely correlated with FMD ($r = -0.360$, $p < 0.013$). By multiple regression analysis, insulin resistance was the sole predictor of FMD. The effects of chronic treatment with pioglitazone were assessed in 10 subjects with type 2 diabetes. The increase in FMD significantly correlated with the decrease in SSPG. There is a significant association between vascular endothelial dysfunction and insulin resistance in type 2 diabetes. This result was supported by the effects of the insulin sensitizer, pioglitazone.

Keywords: Endothelial dysfunction; Insulin resistance; Pioglitazone

1. Introduction

Endothelial dysfunction is thought to be an important early feature in the development of atherosclerosis and occurs in subjects with type 2 diabetes mellitus [1–4]. Insulin resistance is also associated with atherosclerosis and is observed in subjects with type 2 diabetes [5,6]. We previously reported the association between endothelial dysfunction and insulin resistance in patients with essential hypertension [7]. However, the mechanisms responsible for endothelial dysfunction and insulin resistance in hypertension might be different from those of type 2 diabetes. Therefore, we evaluated the relationship between endothelial dysfunction and insulin resistance in patients with type 2 diabetes. Thiazolidinediones, an agonist for the peroxisome proliferator-activated receptor γ (PPARγ), improve insulin resistance. If there is a significant relationship

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between endothelial dysfunction and insulin resistance, thiazolidinediones might influence endothelial function. Therefore, we examined the effects of pioglitazone on endothelial dysfunction and insulin resistance in a subgroup of subjects with type 2 diabetes to verify the relationship between endothelial dysfunction and insulin resistance.

The main purpose of this study was to investigate the relation between vascular endothelial dysfunction and insulin resistance in type 2 diabetes. In addition, the influence of pioglitazone treatment was examined.

2. Subjects and methods

2.1. Subjects

Forty-eight (30 males and 18 females) patients with type 2 diabetes were recruited in the Department of Diabetes and Atherosclerosis of the National Cardiovascular Center. The subjects did not have diabetic retinopathy or nephropathy. Subjects were included on the basis of the following criteria: age between 40 and 79 years, body mass index (BMI) between 17 and 35 kg/m^2, type 2 diabetes confirmed by American Diabetes Association criteria [8]. Subjects were excluded from participation if they had coronary heart, peripheral vascular, renal, hepatic or other endocrine diseases. Subjects were excluded if they had a resting seated blood pressure greater than 150 mmHg systolic or greater than 90 mmHg diastolic, or were taking anti-hypertensive drugs. Diabetes duration was 5.3 ± 1.9 years (3–7 years). Diabetes treatment regimens included diet alone (27 subjects), sulfonylures (18 subjects) and metformin (3 subjects).

The 48 subjects had an average age of 64 ± 1 years, with a mean BMI of 24.6 ± 0.3 kg/m^2, HbA_{1c} of 8.6 ± 0.2%, total cholesterol of 199 ± 5 mg/dl, HDL-cholesterol of 43 ± 2 mg/dl and triglycerides of 137 ± 14 mg/dl. Mean systolic and diastolic blood pressures were 131 ± 3 and 74 ± 2 mmHg, respectively.

Of the 48 diabetic subjects, 10 subjects were started on a single 15 or 30 mg-tablet of pioglitazone (Actos, Takeda Pharmaceuticals, Tokyo, Japan) by mouth each day. Inclusion criteria of the pioglitazone treatment were male, non-smoker, diet alone treatment and mild to severe insulin resistance (SSPG > 160 mg/dl). They received a mean dose of 25.5 ± 2.3 mg/day (30 mg/day: seven subjects and 15 mg/day: three subjects) of pioglitazone for 16.3 ± 1.6 weeks (10–20 weeks). The secondary assessments of endothelial function and insulin sensitivity were performed after the pioglitazone treatments.

The study protocol was approved by the ethics committee of the National Cardiovascular Center. The experiments were conducted with the understanding and the consent of each participant.

2.2. Methods

2.2.1. Assessment of endothelial function

Using the ultrasound method, arterial endothelium and smooth muscle function were measured by examining brachial artery responses to endothelium-dependent and endothelium-independent stimuli. Ultrasound measurements were carried out based on the method described by Celermajer et al. [9] and our method was reported previously [7]. The assessments were performed after an overnight fast in a quiet air-conditioned room (22–23 °C). The diameter of the brachial artery was measured on B-mode ultrasound images, with the use of a 10-MHz linear array transducer (ProSound SSD-5500, ALOKA, Tokyo, Japan). The right brachial artery was scanned in longitudinal sections 1–10 cm above the elbow, after at least 15 min of rest in the supine position. After the detection of the right transducer position, the skin surface was marked and the arm was kept in the same position during the study. All scans were recorded using a super-VHS videocassette recorder (SONY, SVO-9500MD), and analyzed later.

At first, baseline measurements of the diameter were carried out. Endothelium-dependent vasodilation (flow-mediated dilation) was determined by the scans during reactive hyperemia. Because flow-mediated vasodilation was mainly blocked by *N*-monomethyl-L-arginine (an inhibitor of endothelial nitric oxide synthase) this dilation was regarded as endothelium dependent [10]. A pneumatic cuff placed around the forearm was inflated to 220 mmHg and was deflated after 4.5 min. The diameter of the brachial artery was scanned and recorded after deflation. After 10–15 min rest, the second control scan of the diameter and the flow velocity was recorded. Then, sublingual glyceryl trinitrate spray (300 μg) was administered and 3.5–4 min later a final scan of the diameter was recorded.

Measurements of the vessel diameter were taken from the anterior to the posterior 'm' line (interface between the media and adventitia) at endo-diastole, coincident with the R wave on a continuously recorded electrocardiogram. The diameters at four cardiac cycles were measured for each scan, and these results were averaged. Determinations of the flow-mediated dilation were carried out 45–60 s after the cuff release to measure a maximum diameter. Vasodilation by reactive hyperemia (flow-mediated dilation, FMD) or glyceryl trinitrate (GTN) was expressed as the percent change in diameter compared to the baseline values.

2.2.2. Insulin sensitivity test

Glucose utilization in response to insulin was evaluated by a modified steady state plasma glucose (SSPG) method [6,7,11] using Sandostatin (octreotide acetate; Novartis, Basel, Switzerland) after an overnight fasting for at least 12 h. Sandostatin (9.8 pmol in bolus followed by a constant infusion of 73.5 pmol/h) and Novolin R insulin (Novo Nordisk S/A, Tokyo, Japan, 45 pmol/kg [7.5 mU/kg] in a bolus followed by a constant infusion at a rate of 4.62 pmol/kg/min [0.77 mU/kg/min]) were infused intravenously for 120 min.

Glucose in a final 12% solution containing KCl (0.5 μmol/kg/min) were infused at a rate of 0.033 mmol/kg/min [6 mg/kg/min] through an antecubital vein via a constant infusion pump. Blood samples were drawn routinely at 0 and 120 min (9:00 and 11:00 a.m.) for determination of glucose and insulin. Value of glucose at 120 min (SSPG) was used as a marker of insulin sensitivity to glucose utilization. High SSPG levels indicate peripheral insulin resistance. At 120 min SSPG was rapidly measured using a Glucometer (Bayer Corporation, Osaka, Japan) separate from the usual measurement of glucose and insulin. When rapidly measured, if SSPG was found to be lower than 250 mg/dl, oral glucose intake was necessary to prevent hypoglycemia after the insulin sensitivity test. The subjects should have lunch within 30 min after the insulin sensitivity test to prevent hypoglycemia. Homeostasis model assessment (HOMA-IR) was calculated from fasting glucose and insulin concentrations during insulin sensitivity test as follows: HOMA-IR = fasting glucose (mg/dl) × fasting insulin (μU/ml)/405.

2.3. Statistical analysis

Values are expressed as mean ± S.E. A probability value of <0.05 was considered to indicate statistical significance. The strength of the correlation between FMD and GTN with respect to risk factors was assessed by Pearson's linear correlation and multiple regression analysis. The effects of pioglitazone on each clinical parameter were assessed by paired *t*-test and Pearson's linear correlation.

3. Results

3.1. Association between endothelial dysfunction and each parameter in 48 subjects

A significant inverse correlation was observed between FMD and SSPG ($r = -0.462$, $p < 0.001$; Fig. 1). There was no relation between FMD and HbA_{1c} ($p = 0.856$). We also observed a significant inverse correlation between FMD and systolic blood pressure ($r = -0.360$, $p < 0.013$). No significant correlation was found between FMD and diabetic duration, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI. There was no relationship between FMD and HOMA-IR ($p = 0.097$).

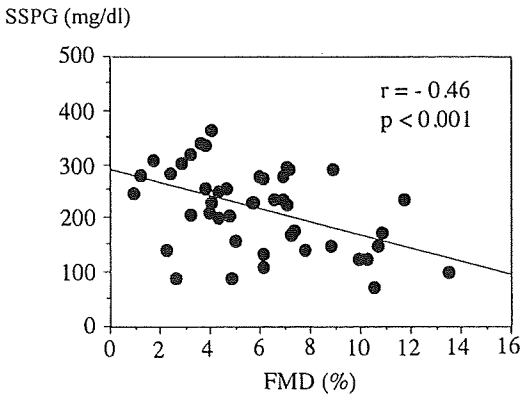

Fig. 1. Relationship between FMD and SSPG in subjects with type 2 diabetes. FMD, flow-mediated vasodilation; SSPG, steady state plasma glucose.

We performed multiple regression analysis to evaluate the independent influence of risk factors including SSPG, systolic blood pressure, HbA_{1c}, total cholesterol, BMI and age on FMD. FMD was independently related to SSPG (regression coefficient: $\beta = -0.419$, $p = 0.0086$) but not to systolic blood pressure ($\beta = -0.254$, $p = 0.0782$), HbA_{1c} ($\beta = -0.090$, $p = 0.5616$), total cholesterol ($\beta = -0.067$, $p = 0.6336$), BMI ($\beta = -0.258$, $p = 0.0863$) or age ($\beta = -0.085$, $p = 0.5650$).

With respect to GTN, no significant correlation was observed between GTN and SSPG or other parameters, including HbA_{1c}, diabetic duration, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI.

3.2. Effects of pioglitazone treatment on endothelial function and insulin resistance

The effects of treatment with pioglitazone were assessed in 10 male subjects with type 2 diabetes (a subgroup of 48 subjects). Table 1 shows the clinical parameters of the 10 subjects before and after pioglitazone treatment. SSPG, HbA_{1c} and fasting plasma glucose decreased and FMD increased significantly due to pioglitazone treatment. However, BMI, total cholesterol, HDL-cholesterol, triglyceride, systolic blood pressure and diastolic blood pressure did not

Table 1
Clinical characteristics of the subjects with type 2 diabetes treated with pioglitazone

	Before Tx	After Tx
Number	10	
Age (years)	65 ± 2	
SSPG (mg/dl)	230 ± 13	185 ± 17*
FMD (%)	4.5 ± 1.1	8.1 ± 1.5***
Body mass index (kg/m^2)	24.4 ± 0.4	24.7 ± 0.4
Fasting plasma glucose (mg/dl)	162 ± 11	133 ± 8*
HbA_{1c} (%)	8.4 ± 0.4	7.0 ± 0.3**
Total cholesterol (mg/dl)	199 ± 8	206 ± 7
HDL cholesterol (mg/dl)	47 ± 4	50 ± 4
Triglyceride (mg/dl)	120 ± 15	129 ± 13
Systolic blood pressure (mmHg)	137 ± 5	137 ± 2
Diastolic blood pressure (mmHg)	78 ± 5	79 ± 1

Values are mean ± S.E. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. before Tx. Tx, Treatments with pioglytazone.

significantly change. GTN was also not significantly altered.

The change in FMD before and after administration of pioglitazone was not significantly correlated with the change in HbA_{1c} ($p = 0.314$) or fasting plasma glucose ($p = 0.717$). The increase in FMD, that is, the improvement in endothelial function, was significantly correlated with the decrease in SSPG ($r = -0.649$, $p < 0.05$).

4. Discussion

In this study we found that vascular endothelial dysfunction was associated with insulin resistance in type 2 diabetes. This result was supported by the effects of the insulin sensitizer, pioglitazone, which improved both endothelial dysfunction and insulin resistance in patients with type 2 diabetes.

The close association between insulin resistance and endothelial dysfunction is our main interest. In a study by Hogikyan et al. [3], insulin resistance as measured by the insulin sensitivity index (minimal model: S_I), was not found to be correlated with endothelial dysfunction in subjects with type 2 diabetes. They measured the forearm blood flow (FABF) using venous occlusion plethysmography and used the FABF response to acetylcholine as an index of endothelial function. The narrow range of S_I values among the subjects might have led to the lack of a relationship between S_I and endothelial dysfunction. In addition, the sensitivity of the techniques using plethysmography might have been low.

Balletshofer et al. [12] reported a significant association between endothelial dysfunction and insulin resistance, as measured by the glucose clamp method, in young normotensive and normoglycemic first-degree relatives of patients with type 2 diabetes. Therefore, this association was observed in a non-diabetic population at future risk of type 2 diabetes.

Insulin causes endothelium-derived nitric oxide (NO)-dependent vasodilation [13]. It is suggested that this insulin action occurs via the phosphatidylinositol 3-kinase and Akt pathway [14,15]. As for insulin action, phosphatidylinositol 3-kinase activation is critical for insulin-mediated glucose uptake into skeletal muscle [16]. Therefore, insulin resistance due to a systemic defect in the phosphatidylinositol 3-kinase pathway might cause a combined defect in insulin-mediated glucose uptake and insulin-mediated endothelial vasodilation.

Among the risk factors for atherosclerosis, insulin resistance was found to be the sole predictor of endothelium dependent vasodilation by multiple regression analysis in the present study. We observed no relationship between FMD and HbA_{1c}. Bagg et al. found that a short-term reduction of HbA_{1c} levels did not appear to affect endothelial function in patients with type 2 diabetes [17]. Furthermore, Mather et al. reported that insulin resistance was the sole predictor of endothelial dysfunction following metformin treatment in type 2 diabetes in stepwise multivariate analysis, and HbA_{1c} and glucose levels were not significant predictors of endothelial dysfunction [18].

Treatment with HMG-CoA inhibitors (statins) has been shown to improve endothelial dysfunction [19–21]. Therefore, statin treatment may have affected the relationship between FMD and risk factors in the present study. In 48 diabetic subjects, 5 were treated with pravastatin and one with simvastatin. We performed statistical analysis in 42 subjects without statin treatment. There was a significant inverse correlation between SSPG and FMD ($r = -0.538$, $p < 0.001$). A significant inverse correlation was observed between FMD and systolic blood pressure ($r = -0.330$, $p < 0.05$). No significant correlation was found between FMD and HbA_{1c}, diabetic duration, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI. On multiple regression analysis, FMD was independently related to SSPG (regression coefficient: $\beta = -0.500$, $p = 0.0032$) but not to systolic blood pressure, HbA_{1c}, total cholesterol, BMI or age.

Smoking is associated with endothelial dysfunction [22,23]. Smoking might interfere in the relationship between FMD and risk factors. In 48 diabetic subjects, 13 were smokers in the present study. Statistical analysis was performed in 35 non-smokers. A significant correlation was found between SSPG and FMD ($r = -0.582$, $p < 0.001$). There was a significant inverse correlation between FMD and systolic blood pressure ($r = -0.357$, $p < 0.05$). No significant correlation was observed between FMD and HbA_{1c}, diabetic duration, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI. On multiple regression analysis, FMD was independently related to SSPG (regression coefficient: $\beta = -0.591$, $p = 0.0019$) but not to systolic blood pressure, HbA_{1c}, total cholesterol, BMI or age. In the present study, FMD did not correlate with HOMA-IR. SSPG is a more sensitive marker to measure insulin sensitivity than HOMA-IR.

Endothelial dysfunction and insulin resistance were improved by pioglitazone treatment in the present study. SSPG, HbA_{1c} and fasting plasma glucose were decreased and other risk factors were not changed by the treatment. It was reported that hyperglycemia itself inhibits endothelial NO synthase activity [24] and causes endothelial dysfunction [25]. On the other hand,

insulin resistance was also associated with endothelial dysfunction in 48 subjects with type 2 diabetes in this study. The change in FMD before and after treatment with pioglitazone was not significantly correlated with the change in HbA_{1c} or fasting plasma glucose, and the increase in FMD was significantly correlated with the decrease in SSPG in this study. Because of the small number of subjects ($n = 10$), we cannot exclude the possibility that the decreased plasma glucose level improved endothelial dysfunction. The decrease in plasma glucose level might be associated with improved endothelial function if the pioglitazone study was performed with more cases. It can at least be said that insulin resistance is an important factor affecting endothelial function. As previously described, a similar study [18] found that treatment with metformin improved both endothelial function and insulin resistance, and the glucose level and HbA_{1c} were not significant predictors of endothelial dysfunction. Considering generally than the above-mentioned points, it is suggested that increased insulin sensitivity plays an important role in the improvement of endothelial function by pioglitazone treatment.

Pistrosch et al. [26] demonstrated that treatment with rosiglitazone, another PPARγ activator, ameliorated insulin resistance measured by glucose clamp method, and improved endothelial function determined by venous occlusion plethysmography in patients with recently diagnosed type 2 diabetes. They performed a double-blind cross-over trial and treated with rosiglitazone and nateglinide in random order. Glycemic control was comparable under rosiglitazone and nateglinide. Only rosiglitazone improved insulin resistance and endothelial function in the study. Thus, they also showed the relation between insulin sensitivity and endothelial function independent of glucose level in type 2 diabetes.

In conclusion, in the present study we demonstrated significant association between vascular endothelial dysfunction and insulin resistance in type 2 diabetes, and pioglitazone treatment improved both endothelial dysfunction and insulin resistance with a statistical link. These data support the concept of the important role of insulin resistance in the pathogenesis of endothelial dysfunction in type 2 diabetes mellitus.

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ORIGINAL ARTICLE

Association of single nucleotide polymorphisms in endothelin family genes with the progression of atherosclerosis in patients with essential hypertension

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Endothelin-1 (ET-1) is a potent vasoconstrictive peptide and its activity is mediated by the receptors ET type A (EDNRA) and ET type B (EDNRB). Although ET-1 is thought to play an important role in the development of atherosclerosis, it remains unclear whether polymorphisms of ET-1 family genes, including the ET-1 gene (*EDN1*), *EDNRA*, *EDNRB* and the genes for endothelin converting enzymes 1 and 2 (*ECE1* and *ECE2*), are associated with the progression of atherosclerosis. We investigated the relationship between 11 single nucleotide polymorphisms (SNPs) of ET-1 family genes (including three in *EDN1*, one in *EDNRA*, two in *EDNRB*, four in *ECE1* and one in *ECE2*) and atherosclerotic changes assessed using pulse wave velocity (PWV) and carotid ultrasonography in 630 patients with essential hypertension (EHT). In male subjects, we found significant differences in brachial-ankle PWV (baPWV) in additive and recessive models in *EDNRB*-rs5351 after Bonferroni correction. Also in male subjects, there were significant differences in mean intima-media thickness (IMT) in additive and recessive models in *EDNRA*-rs5333 after Bonferroni correction. We found no significant correlation between any SNPs in the ET family genes and baPWV, IMT and Plaque score (PS) in female subjects. Furthermore, after multiple logistic regression analysis, only *EDNRB*-rs5351 indicated as an independent risk of atherosclerosis in male hypertensive subjects. Of the endothelin-related genes, *EDNRB*-rs5351 was the most susceptible SNP associated with atherosclerosis in male hypertensives, and the genetic background may be involved in the progression of atherosclerosis in EHT patients.
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Keywords: endothelin 1 (ET-1) family genes; single nucleotide polymorphisms (SNPs); atherosclerosis

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide produced primarily by vascular endothelial cells and appearing in many other organs.[1] ET-1 is thought to play an important role in the development of atherosclerosis through endothelial dysfunction and the proliferation of vascular smooth muscle cells (VSMCs). ET-1 may be a marker for arterial vascular disease; Lerman *et al.*[2] showed a significant correlation between plasma endothelin levels and the number of vascular disease sites. Some reports have linked plasma levels of ET-1 to hypertension, while others have argued against this relationship. Hirai *et al.*[3] suggested that high ET-1 levels are not related to hypertension, but rather to subclinical renal dysfunction and smoking. The expression of ET-1 is mediated by the activation of specific receptors: ET type A (EDNRA) and ET type B (EDNRB). The former is the predominant ET receptor on VSMCs, and signalling via EDNRA causes long-lasting vasoconstriction.[4,5] EDNRB is located primarily on endothelial cells and its signalling promotes the formation of nitric oxide, as well as the clearance and reuptake of ET-1.[6–9] Endogenous ET-1, which acts via EDNRA, increases resistance-vessel tone in subjects with hypertension to a level greater than that in smokers and in subjects with hypercholesterolemia.[10] Plasma ET-1

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concentrations can be reduced by resistance training and aerobic exercise.[11]

Pulse wave velocity (PWV) is generally recognized as a surrogate marker for atherosclerosis.[12] Using sheep, McEniery *et al.*[13] showed that endogenous ET-1 production regulates large artery PWV *in vivo*. They also revealed that exogenous ET-1 increases PWV and that this increase can be blocked by ET type A receptor blockers. Vuurmans *et al.*[14] examined whether ET-1 increases central aortic systolic blood pressure, pulse pressure and PWV in healthy men, and the effect of ET-1 is prevented by ET-1 receptor blockers.

It remains unclear, however, whether gene polymorphisms of the ET-1 family (including the ET-1 gene (*EDN1*), *EDNRA*, *EDNRB* and the genes for endothelin converting enzymes 1 and 2 (*ECE1* and *ECE2*) are associated with the progression of atherosclerosis. Therefore, we investigated the relationship between single nucleotide polymorphisms (SNPs) of ET-1 family genes and atherosclerotic changes assessed by PWV and carotid echo ultrasonography in patients with essential hypertension (EHT).

Materials and methods

Subjects

This study included 630 outpatients (340 men and 290 women) with EHT at the Division of Hypertension and Nephrology of the National Cardiovascular Centre (NCVC). All subjects provided written informed consent and the protocol was approved by the ethics committee of NCVC. Hypertension was defined as a systolic blood pressure (SBP) of 140 mm Hg or greater and/or a diastolic blood pressure (DBP) of 90 mm Hg or greater, or the current use of antihypertensive medication. The blood pressure used was the average of at least three measurements made during each visit. We also measured brachial-ankle PWV (baPWV) using Form ABI (Colin Medical Technology) and examined carotid arteries using a commercially available ultrasound system (SSA-390A; Toshiba Medical, Japan).[4] We measured the mean intima-media thickness (IMT) and maximum-IMT (max-IMT) of common carotid arteries and the sum of the plaque score (PS) of bilateral common and internal carotid arteries, as reported previously.[15] Blood samples were also taken at the clinic, and diabetes mellitus was defined as a fasting blood sugar level greater than 126 mg/dl, an HbA_{1c} level greater than 6.5%, or the use of anti-hyperglycemic medications. Hyperlipidemia was defined as a total-cholesterol concentration of 220 mg/dl or greater, a triglyceride (TG) concentration of 150 mg/dl or greater, or the use of lipid-lowering medication at the time of the first examination. Subjects who had ankle-brachial indices (ABI) lower than 0.9 were excluded because their baPWV readings were unreliable.

Table 1 The entire coding region of the endothelin-1 gene

		Allele 1/Allele 2			*Allele 1*		*Allele 2*		*Allele frequency*		*Flanking sequence*	*dbSNP ID*
Gene name	*Locus*	*SNPs*	*Aa info.*	*Region*	*Homo*	*Hetero*	*Homo*	*Total*	*Allele 1*	*Allele 2*		
EDN1	6p24-p23	10bp del.(−173)		promoter	47	1	0	48	0.990	0.010	AGGTTTAGCAAA[GGTCTCTAAT/-]GGGTATTTTCTT	
		A201- (4A/3A)		exon1	1	8	39	48	0.104	0.896	TTTCTCCCGTT[A/-]AAAGGGCACTTG	
		G2087A	Gly36Arg	exon2	47	1	0	48	0.990	0.010	GGTGAGAACGGC[G/A]GGGAGAAACCCA	
		G2244T		intron2	8	18	21	47	0.362	0.638	ATTGTAACCCTA[G/T]TCATTCATTAGC	rs2070699
		T2252A		intron2	46	1	0	47	0.989	0.011	CCTAGTCATTCA[T/A]TAGCCCTGGCTC	
		T3609C		intron2	33	12	2	47	0.830	0.170	AAGACTATTAAT[T/C]ACACTAATATAG	rs1800543
		A3730G	Glu106Glu	exon3	0	1	46	47	0.011	0.989	AACAGACCGTGA[A/G]AATAGATGCCAA	rs 5369
		T5629A		intron4	47	1	0	48	0.990	0.010	GGGTGATTTTTT[T/A]AAAATAACATTT	
		G5727T	Lys198Asn	exon5	31	14	2	47	0.809	0.191	GCTGAAAGGCAA[G/T]CCCTCCAGAGAG	rs 5370

Abbreviation: SNPs, single nucleotide polymorphisms.
By Gene Cards. Version: 2.25, released 3 July, 2002.

Table 2 Subject characteristics

	All (n = 630)	*Male (n = 340)*	*Female (n = 290)*	P-*value*
Age (years)	64.6±10.6	63.3±11.3	66.0±9.6	0.0015
Height (cm)	160.0±8.7	165.8±6.4	153.1±5.5	<0.0001
Weight (kg)	62.9±11.6	68.5±10.6	56.4±9.1	<0.0001
Heart rate (b.p.m.)	64.0±10.7	62.0±9.4	66.2±11.8	<0.0001
Systolic blood pressure (mm Hg)	138.8±17.1	137.0±15.8	140.9±18.3	0.0042
Diastolic blood pressure (mm Hg)	82.7±10.3	83.2±10.2	82.1±10.5	0.1799
Mean IMT (mm)	0.83±0.16	0.83±0.16	0.84±0.17	0.4634
Plaque score	3.13±4.76	3.57±5.18	2.61±4.17	0.0131
baPWV (cm/s)	1786.2±309.1	1755.7±297.7	1822.0±318.8	0.0071
ABI	1.12±0.08	1.13±0.09	1.11±0.07	0.0018
CRP (mg/dl)	0.15±0.28	0.17±0.20	0.14±0.30	0.1728
HbA_{1c} (%)	5.63±0.80	5.66±0.77	5.58±0.83	0.2259
Total cholesterol (mg/dl)	203.0±35.2	196.7±30.4	210.4±39.0	<0.0001
Triglyceride (mg/dl)	138.3±125.3	152.4±149.7	121.5±85.3	0.0020
HDL-cholesterol (mg/dl)	52.7±15.2	48.7±13.0	57.4±16.3	<0.0001
Smoking (current/past/never)	69/211/339	59/183/89	10/28/250	<0.0001
Anti-hypertensive medication (%)	570/630 (90.5%)	308/340 (90.6%)	262/290 (90.3%)	0.9174

Abbreviations: ABI, ankle brachial index; baPWV, brachial-ankle pulse wave velocity; CRP, C-reactive protein; HbA_{1c}, hemoglobin A_{1c}; HDL, high-density lipoprotein; Mean IMT, mean intima-media thickness.
Values are expressed as the means±s.d. *P* ; Student's *t*-test (male vs female).

Table 3a Comparison between SNPs of ET-1 genes and baPWV in male subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*baPWV (cm/s)*	P-*dominamt*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	251	1763.8±301.0	0.4250	0.6509	0.7682
			3A4A	81	1730.3±291.5			
			4A4A	5	1795.3±271.3			
	rs2070699	T/G	TT	104	1768.1±341.2	0.6029	0.3509	0.2902
			TG	158	1731.8±265.6			
			GG	76	1787.3±297.1			
	rs5370	G(Lys)/T(Asn)	GG	182	1759.6±308.5	0.8425	0.8318	0.5438
			GT	134	1758.8±286.2			
			TT	23	1720.2±284.5			
EDNRA	rs5333	T/C	TT	182	1746.8±307.0	0.5958	0.4479	0.2086
			TC	130	1752.5±269.4			
			CC	23	1830.1±369.9			
EDNRB	rs 5351	A/G	AA	107	1706.6±285.1	0.0409 (0.4499)*	0.0004 (0.0044)*	0.0001 (0.0011)*
			AG	162	1736.1±277.7			
			GG	65	1882.2±332.7			
	rs3818416	G/T	GG	305	1759.9±301.1	0.2393	0.3593	0.2593
			GT	28	1708.0±260.2			
			TT	3	1560.5±241.2			
ECE1	rs212526	C/T	CC	247	1746.9±294.9	0.4798	0.7583	0.9557
			CT	82	1775.1±298.4			
			TT	7	1747.6±415.2			
	rs212528	T/C	TT	198	1724.6±292.4	0.0311 (0.3421)*	0.0246	0.3099
			TC	122	1810.8±298.3			
			CC	16	1679.9±308.2			
	rs213045	G/T	GG	102	1732.0±282.7	0.3865	0.3293	0.3737
			GT	174	1776.5±305.3			
			TT	59	1722.0±301.3			
	rs2038089	A/G	AA	153	1773.4±300.4	0.3051	0.0821	0.0262 (0.2882)*
			AG	138	1764.3±304.3			
			GG	43	1661.1±253.9			
ECE2	rs2272471	C/T	CC	94	1778.0±303.1	0.3573	0.6116	0.9717
			CT	164	1739.8±282.3			
			TT	76	1755.1±324.4			

Abbreviations: baPWV, brachial-ankle pulse wave velocity; SNPs, single nucleotide polymorphisms.
P-value (dominant), major vs hetero+minor; *P*-value (additive), major vs heterozygote vs minor; *P*-value (recessive), minor+hetero vs major.
*Bonferroni correction (× 11).

Table 3b Comparisons between ET-1 gene SNPs and baPWV in female subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*baPWV (cm/s)*	P-*dominant*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	198	1831.0±329.4	0.4510	0.7278	0.9152
			3A4A	84	1798.0±305.9			
			4A4A	5	1836.6±199.9			
	rs2070699	T/G	TT	80	1845.0±367.1	0.4673	0.7631	0.7183
			TG	139	1816.2±305.3			
			GG	69	1810.8±289.1			
	rs5370	G (Lys)/T(Asn)	GG	147	1843.9±321.8	0.2519	0.4711	0.5298
			GT	116	1795.2±316.4			
			TT	26	1825.8±319.9			
EDNRA	rs5333	T/C	TT	153	1796.2±306.1	0.1163	0.1601	0.5298
			TC	116	1867.4±331.3			
			CC	17	1776.6±342.9			
EDNRB	rs 5351	A/G	AA	85	1859.2±315.1	0.2257	0.3921	0.3211
			AG	145	1817.9±339.8			
			GG	56	1785.8±268.3			
	rs3818416	G/T	GG	255	1822.4±325.2	0.8168	0.3676	(–)
			GT	29	1821.6±270.6			
			TT	1	2277.0			
ECE1	rs212526	C/T	CC	208	1827.0±308.5	0.7909	0.4074	0.1873
			CT	67	1835.0±360.3			
			TT	11	1698.1±257.9			
	rs212528	T/C	TT	184	1833.1±320.8	0.5150	0.4206	0.3855
			TC	86	1791.7±307.6			
			CC	16	1891.4±371.4			
	rs213045	G/T	GG	93	1834.6±369.1	0.6899	0.4138	0.2837
			GT	142	1801.0±281.4			
			TT	50	1867.8±326.9			
	rs2038089	A/G	AA	124	1821.2±322.9	0.8902	0.9691	0.8109
			AG	131	1824.3±321.9			
			GG	24	1839.2±323.7			
ECE2	rs2272471	C/T	CC	73	1795.8±343.4	0.3612	0.5926	0.4611
			CT	144	1828.5±314.6			
			TT	68	1850.3±304.4			

Abbreviations: baPWV, brachial-ankle pulse wave velocity; SNPs, single nucleotide polymorphisms.
P-value (dominant); major vs hetero+minor, *P*-value (additive); major vs heterozygote vs minor, *P*-value (recessive); minor+hetero vs major.

Screening of genetic variations in EDN1 EDNRA, EDNRB, ECE1 and ECE2

We isolated genomic DNA from the peripheral blood leukocytes of 630 subjects and directly sequenced the entire coding region of the endothelin-1 gene (*EDN1*). The results of the *EDN1* screening are shown in Table 1. Finally, we selected three SNPs in the *EDN1*. We selected SNPs of the endothelin type A receptor gene (*EDNRA* rs5333), endothelin type B receptor gene (*EDNRB* rs5351, rs3818416), endothelin converting enzyme-1 gene (*ECE1* rs212526, rs212528, rs213045, rs2038089) and endothelin converting enzyme-2 gene (*ECE2* rs2272471) from a public database (dbSNP http://www.ncbi.nlm.nih.gov/SNP/). SNPs with a minor allele frequency of greater than 5% were genotyped using the TaqMan-PCR method described previously.[16] The representative SNPs were genotyped when they were linkage disequilibrium (LD: r^2 over 0.5). The LD was calculated between each SNP. The primers and probes used in the TaqMan-PCR system are available upon request.

Statistical analysis

Values are expressed as means ±s.d. and were analyzed using a Student's *t*-test and a χ^2-test where appropriate. Hardy–Weinberg equilibrium was assessed by χ^2analysis, and we considered *P*-values less than 0.05 to be statistically significant. The levels of the *P*-values were adjusted by Bonferroni correction). The LD between each SNP was checked using Haploview version 4 (http://www.broad.mit.edu/mpg/haploview/). The association of genotypes with blood pressure, IMT and PS of carotid arteries and baPWV was examined by simple regression analysis and then investigated using a logistic regression model that adjusted for confounding factors. The distribution of plaque score (PS) was not normal, so we compared the prevalence of severe PS ($\geqslant$10.1)[17] for each allele. All statistical analyses were performed using Stat-View version 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Patient Characteristics and the Correlation between baPWV and Clinical Parameters

The characteristics of the subjects at baseline are summarized in Table 2. Significant differences were apparent between men and women in age, height, weight, heart rate (HR), systolic blood pressure (SBP), plaque score (PS), baPWV and ABI and lipid