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Genetic Variations in Six Candidate Genes for Insulin Resistance in Korean Essential Hypertensives

Joon-Seol Bae, Byung-Yong Kang, Ki-Tae Kim*, Jung-Hee Shin[1], and Chung-Choo Lee[1]

Seoulin Bioscience Institute, Seoulin Bioscience, Co. Ltd., Seoul 134-030, Korea;
[1]School of Biological Sciences, Seoul National University, Seoul 151-742, Korea

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Hypertension is a complex disease with strong genetic influences. Essential hypertension has been shown to be associated with insulin resistance. To clarify the genetic basis of insulin resistance in hypertension, case-control association studies were performed to examine candidate genes for insulin resistance in hypertension. Polymorphisms investigated were the *BstO* I polymorphism of the β_3-adrenergic receptor (*ADRB3*) gene, the *Xba* I polymorphism of the glycogen synthase (*GSY*) gene, the *Dde* I polymorphism of the protein phosphatase 1 G subuit (*PP1G*) gene, the *Bst*E II polymorphism of the glucagon receptor (*GCG-R*) gene, the *Pst* I polymorphism of the insulin (*INS*) gene and the *Acc* I polymorphism of the glucokinase (*GCK*) gene. No significant differences were observed in the distribution of alleles and genotypes of the *ADRB3*, *GSY*, *PP1G*, *GCG-R*, *INS*, and *GCK* genes between hypertensive and normotensive groups. Although the frequencies in each of these polymorphisms were not significantly different between essential hypertensive and normotensive individuals, our results may provide additional information for linkage analysis and associative studies of disorders in carbohydrate metabolism or in cardiovascular disease.

Essential hypertension is a complex disease with multiple environmental and strong genetic influences (Lander and Schork, 1994). Hypertensive patients have been reported to be resistant to insulin-mediated glucose uptake when compared with normotensive control subjects (Ferrannini et al., 1987). Recently, several reports have disclosed the relationship between essential hypertension and insulin resistance (Ravean et al., 1996). Subjects with essential hypertension are more insulin resistant than normotensives (Shen et al., 1988). The mechanisms of insulin resistance are multifactorial. Most of the studies concentrate on glucose metabolism. The epidemiological studies have suggested a link between hypertension and non-insulin-dependent diabetes mellitus (NIDDM) (Tai et al., 1991). According to many studies, the significant association between essential hypertension and insulin resistance genes has been reported in Caucasians (Chiu et al., 1994; Walston et al., 1995; Gulja et al., 1997) and Japanese (Ikegami et al., 1996; Shen et al., 1997). However, such study is scanty in Korean population (Kang et al., 2000). To clarify the genetic basis of insulin resistance in hypertension, we studied association of candidate genes with insulin resistance in hypertension. Since the site of insulin resistance in hypertension is reported to be the non-oxidative pathway (i.e. glycogen synthsis) in skeletal muscle (Ferrannini et al., 1987; Ravean et al., 1996; Kang et al., 2000), the genes that encode molecules involved in this pathway are candidate genes for insulin resistance in hypertension (Fig. 1). Therefore, the genes for glycogen synthase (*GSY*), protein phosphatase 1 G subunit (*PP1G*) and glucokinase (*GCK*) were studied. In addition, since recent studies suggest the contribution of the β_3-adrenergic receptor (*ADRB3*), insulin (*INS*), and glucagon receptor (*GCG-R*) to the insulin resistance syndrome (Leann et al., 1992; Hager et al., 1995; Widen et al., 1995), the genes encoding β_3-adrenergic receptor (*ADRB3*), insulin (*INS*) and glucagon receptor (*GCG-R*) were also studied.

* To whom correspondence should be addressed.
Tel: 82-2-478-8488, Fax: 82-2-479-0397
E-mail: ktkim@seoulin.co.kr

Materials and Methods

Study subjects

One hundred and eighty subjects were recruited from outpatients of Seoul Hygiene Hospital, Seoul, Korea. The essential hypertensives consisted of 90 subjects with blood pressure value higher than 140/90 mmHg, whereas the normotensives consisted of 90 individuals with blood pressure value lower than 140/90 mmHg. Male/female (M/F) ratio between the two groups was statistically similar. The M/F ratio for control group was

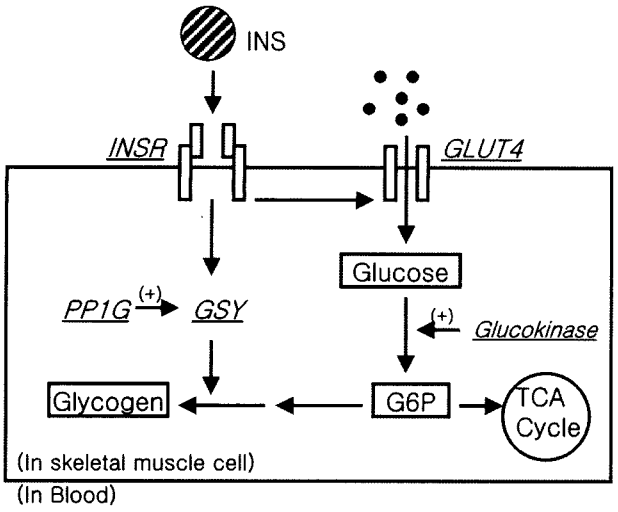

Fig. 1. Non-oxidative pathway of glucose utilization, which primarily reflects glycogen synthesis, in skeletal muscle. Genes that encode molecules involved in this pathway are shown. INS, insulin; INSR, insulin receptor; GLUT4, glucose transporter 4; PP1G, protein phosphatase 1 G; GSY, glycogen synthase; G6P, glucose 6-phosphate.

52/49 and for essential hyperensive group was 33/43, $X^2 = 2.676$, df = 2 and P = 0.262. Subjects with secondary forms of hypertension and those taking antihypertensive drugs were excluded from the study. Informed consent was obtained from all subjects.

Biochemical assay

Plasma glucose, total cholesterol (TC), triglyceride (TG), apolipoprotein AI (ApoA1), lipoprotein(a) (LP(a)) and high-density lipoprotein (HDL)-cholesterol level were determined by enzymatic method, and LDL-cholesterol level was calculated by Friedwald's equation (Friedwald et al., 1972).

Genotyping

Genomic DNA was prepared from buffy coats of 5 ml blood after lysis of red blood cell (Sambrook et al., 1989). Polymorphisms investigated were obtained from *Xba* I RFLP in glycogen synthase (*GSY*); nucleotide substitution G2713T in the protein phosphatase 1 G subunit (*PP1G*), nucleotide substitution Trp64Arg in the β_3-adrenergic receptor (*ADRB3*), nucleotide substitution Gly40Ser in the glucagon receptor (*GCG-R*), 3'-UTR/*Pst* I RFLP in the insulin (*INS*) and nucleotide substitution G-258A in the glucokinase (*GCK*) gene. Polymerase chain reactions (PCR) were performed in a final volume of 50 ul (100 ng of genomic DNA, 20 pmol of each primer, 200 μM of each dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.4 and 2.5 unit of *Taq* DNA polymerase). Primer sequences for PCR reaction and references were shown in Table 1. Amplified PCR products were digested by each restriction enzyme, and visualized by agarose gel with ethidium bromide staining.

Statistical analysis

All of the results for statistical anaysis were analyzed by using the MINITAB statistical software (version 13). Allele frequencies were estimated by gene counting. Hardy-Weinberg equilibrium was tested by chi-square fitness analysis. Genotype and allele frequencies were compared between Korean hypertensive and normotensive groups by chi-square independence analysis. Genotypic odds ratios (OR) for disease, assuming a dominant or a recessive model, were computed by logistic regression analysis. Dominant model was defined by the comparison between MM genotype and (Mm + mm) genotypes (M, normal allele; m, disease allele). Recessive model was defined by the compariosn between (MM + Mm) genotypes and mm genotype. A p value < 0.05 was considered statistically significant.

Results and Discussion

Selection of the candidate genes and the polymorphisms examined in the present study was based on pathophysiological consideration and the results previously reported in published data.

Glycogen synthase

We have investigated *Xba* I polymorphism of glycogen synthase gene in Korean hypertensive and normotensive groups. Both groups only showed a X1/X1 genotype. The glycogen synthase (*GSY*) is a key enzyme of the non-oxidative pathway in glucose meta-

Table 1. Polymorphic sites and primer sequence of each candidate gene

Genes	Polymorphic sites	Primer sequences	Reference
Glycogen synthase	*Xba* I RFLP	5'-CTCCTTCCTCTACAGTTTCTG-3' 5'-GTGAGTCTCCTCTTTGGCCA-3'	Camilla *et al.*, 1996
Protein phosphatase1 G subunit	*Dde* I RFLP	5'-CTGGATTTACAGTTGGGAATGT-3' 5'-CGTAGAAATAGGTTGGCTAGC-3'	Hansen *et al.*, 1995
β_3-adrenergic receptor	*Bst*O I RFLP	5'-CGCCCAATACCGCCAACAC-3' 5'-CCACCAGGAGTCCCATCACC-3'	Kristi *et al.*, 1997
Glucagen receptor	*Bst*E II RFLP	5'-TGTCTGGTTGCTTGTGCATG-3' 5'-GAAGAGAACTCAGGAAGTGC-3'	Hager *et al.*, 1995
Insulin	*Pst* I RFLP	5'-AAGCGTGGCATTGTGGAACAAT-3' 5'-CTGGGAGGGGCTCACAACAGT-3'	Paul *et al.*, 1991
Glucokinase	*Acc* I RFLP	5'-CAGACCCTGGATATGAAATG-3' 5'-GGCTGCCTTGGCCACAGTA-3'	Chiang *et al.*, 1997

bolism. It seems likely that this enzyme is a candidate gene for contributing to the pathogenesis of NIDDM with marked insulin resistance (Kuroyama et al., 1994). Studies in Russian population (Gulja et al., 1997), Danish (Fenger et al., 2000), and Japanese (Kuroyama et al., 1994) have shown associations with various anthropometric markers of hypertension.

Protein phosphatase 1 G subunit

We have investigated a *Dde* I polymorphism of protein phosphatase 1 gene in Korean hypertensive and normotensive groups. The observed genotype frequencies of Asp/Asp, Asp/Tyr and Tyr/Tyr were 49.4, 33.7 and 16.9% in the hypertensive group, and 49.4, 40.4 and 10.2% in the normotensive group, respectively. Frequencies of the Asp allele were 0.71 for the hypertensive group and 0.79 for the normotensive group. There were no statistically significant differences between the hypertensive and normotensive groups in either allele and genotype frequencies. The regulatory G subunit of the glycogen-associated form of protein phosphatase 1 is a key protein in the stimulation of glycogen synthesis by insulin and regulation of nonoxidative glucose disposal (Strfors et al., 1985; Hubbard and Cohen, 1993). The PP1G subunit is expressed in skeletal muscle (Tang et al., 1991) which is considered to be a major site of peripheral insulin resistance in essential hypertension (Ferrannini et al., 1987; DeFronzo et al., 1991). Both fasting and insulin-stimualted PP1 activities in skeletal muscle have been reported to be reduced in insulin-resistant Pima Indians (Kida et al., 1990; Kida et al., 1992). White subjects who carry the Tyr allele have been reported to exhibit insulin resistance and hypersecretion of insulin (Hansen et al., 1995).

β_3-adrenergic receptor gene

We have investigated *Bst*O I polymorphism of β_3-adrenergic receptor gene in Korean hypertensive and normotensive group. The observed genotype frequencies of Trp/Trp, Trp/Arg and Arg/Arg were 71.4, 28.6 and 0.03% in the hypertensive group, and 73.0, 24.7 and 1.3% in the normotensive group, respectively. The Arg/Arg genotype was only observed in the normotensive group. Frequencies of the Trp allele were 0.83 and 0.89 for the hypertensive and normotensive groups respectively. There were no statistically significant differences between the hypertensive and normotensive groups in allele and genotype frequencies. The β_3-adrenergic receptor is a seven membrane spanning protein which is expressed in visceral adipose tissue, and is thought to regulate lipolysis and energy expenditure via thermogenesis (Revelli et al., 1993). Studies in Pima Indians (Walston et al., 1995), French Caucasians (Clement et al., 1995), Finns (Widen et al., 1995), Danes (Urhammeret et al., 1996), Japanese (Kadowaki et al., 1995) and Australian Caucasians (Kurabayashi et al., 1996) have shown modest associations of the Arg allele with various anthropometric markers of hypertension, obesity and diabetes (Biery et al., 1997).

Glucagon receptor

We have investigated a Gly40Ser polymorphism of glucagon receptor gene in Korean hypertensive and normotensive group. Both groups showed only a Ser/Ser genotype. Glucagon secreted from the pancreatic islet α-cells in response to low blood glucose levels is a key hormone involved in the maintenance of euglycemia primarily by stimulating hepatic glucose production (Cherrington and Liljenquuist, 1981; Unger and Orci, 1990) and also by potentiating glucose-induced insulin secretion (Kawai et al., 1995). The physiological effects of glucagon are mediated by the glucagon receptor, a 480-amino-acid protein, that is a member of the superfamily of seven transmembrane-spanning receptors (Stephan et al., 1993).

Insulin

The hormone insulin regulates normal glucose homeostasis and also has other physiological effects. Insulin appears to exert its biological activities through interaction with receptor present on the membrane of most, if not all, cells (Graeme et al., 1980).

We have investigated *Pst* I polymorphism of insulin gene in Korean hypertensive and normotensive group. The observed genotype frequencies of P1/P1, P1/P2 and P2/P2 were 92.1, 7.9 and 0.0% in the hypertensive group, and 90.0, 10.0 and 0.0% in the normotensive group respectively. Frequencies of the P1 allele were 0.93 for the hypertensive group and 0.91 for the normotensive group. Both groups did not show P2/P2 genotype. There were no statistically significant differences between hypertensive and normotensive groups in allele and genotype frequencies.

Glucokinase

We have investigated *Acc* I polymorphism of glucokinase gene in Korean hypertensive and normotensive group. The observed genotype frequencies of G/G, G/A and A/A were 57.4, 33.8 and 8.8% in the hypertensive group, and 52.2, 40.3 and 7.5% in the normotensive group respectively. Frequencies of the G allele were 0.89 for the hypertensive group and 0.91 for the normotensive group. There were no statistically significant differences between the two groups in allele and genotype frequencies. The glucokinase gene has been proposed as a candidate for the inherited component of NIDDM (Meglasson and Matschinsky, 1986; Matschinsky, 1990). Glucokinase is expressed in liver and pancreatic α-cells and plays a key role in the regulation of glucose metabolism in these tissues (Meglasson and Matshinsky, 1986). It has been descri-

Table 2. Genotype and allele frequencies of candidate gene polymorphisms in hypertensive group and normotensive group

Polymorphism	Genotypes			Allele frequency
	N (%)	N (%)	N (%)	
GSY *Xba* I	X1/X1	X1/X2	X2/X2	f (X1)
HT[1]	90 (100.0)	0 (0.0)	0 (0.0)	1.00
NT[2]	90 (100.0)	0 (0.0)	0 (0.0)	1.00
PP1G Asp905Tyr	Asp/Asp	Asp/Tyr	Tyr/Tyr	f (Asp)
HT[1]	44 (49.4)	30 (33.7)	15 (16.9)	0.83
NT[2]	44 (49.4)	36 (40.4)	9 (10.2)	0.89
ADRB3 Trp64Arg	Trp/Trp	Trp/Arg	Arg/Arg	f (Trp)
HT[1]	50 (71.4)	20 (28.6)	0 (0.0)	0.71
NT[2]	54 (73.0)	19 (24.7)	1 (1.3)	0.79
GCG-R Gly40Ser	Gly/Gly	Gly/Ser	Ser/Ser	f (Ser)
HT[1]	0 (0.0)	0 (0.0)	91 (100.0)	1.00
NT[2]	0 (0.0)	0 (0.0)	89 (100.0)	1.00
Insulin 3'-UTR/*Pst* I	P1/P1	P1/P2	P2/P2	f (P1)
HT[1]	70 (92.1)	6 (7.9)	0 (0.0)	0.93
NT[2]	72 (90.0)	8 (10.0)	0 (0.0)	0.91
Glucokinase G-258A	G/G	G/A	A/A	f (G)
HT[1]	39 (57.4)	23 (33.8)	6 (8.8)	0.89
NT[2]	35 (52.2)	27 (40.3)	5 (7.5)	0.91

[1] Hypertensives, [2] Normotensives

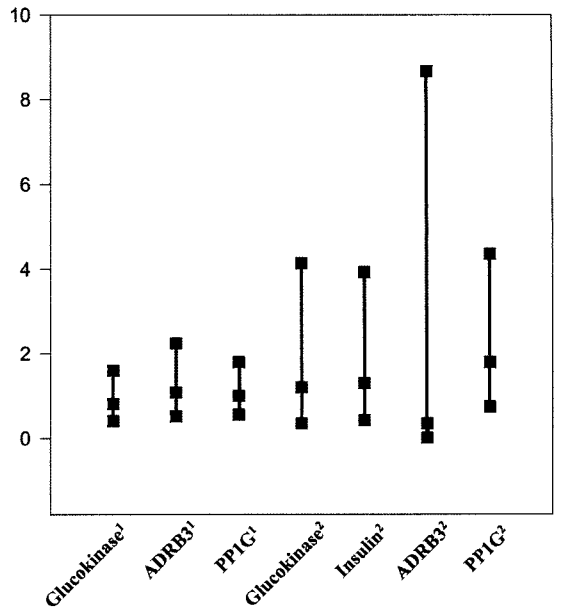

Fig. 2. Genotypic odds ratio for hypertension and 95% confidence intervals assuming a dominant[1] and recessive[2] genetic model. For GSY *Xba* I, GCG-R Gly40Ser, and INS 3 -UTR/*Pst* I, the dominant model was not considered because of the low frequency of the minor allele. For GSY *Xba* I and GCG-R Gly40Ser, the recessive model was not considered because of the same reason.

bed as the pancreatic glucose sensor because of its role in glucose recognition and stimulation of insulin synthesis and secretion (Meglasson and Matschinsky, 1984, 1986). Glucokinase phosphorylates glucose to produce glucose-6-phosphate in the first metabolic step for glucose within the cell. Expression of the enzyme is increased by glucose in the pancreas and by insulin in the liver (Magnuson, 1990). A G-258A polymorphism of the β-cell specific promoter of the glucokinase gene has been observed in Japanese- American (Stone et al., 1994), French (Zouali et al., 1993), African-American (Chiu et al., 1994), and white American (Elbein et al., 1994) subjects. Recently, this polymorphism was observed to increase in the frequency of the A allele among subjects with impaired glucose tolerance in a small study of Japanese-American subjects with a family history of NIDDM (Stone et al., 1994). Japanese-American are at high risk of developing abnormal glucose tolerance (Fujimoto et al., 1987) which is believed to be an important predictor of risk for the development of NIDDM (Yudkin et al., 1990).

In conclusion, we performed case-control association study for insulin resistance in hypertension. None of the polymorphisms exhibited significant difference in genotype or allele frequencies between cases and controls (Table 2). Although the frequencies in each of these polymorphisms were not significantly different between essential hypertensive and normotensive individuals, our results may provide additional information for linkage analysis and associative studies of disorders in carbohydrate metabolism or cardiovascular disease. There was no deviation from Hardy-Weinberg equilibrium for any of the polymorphisms considered. No significant association was observed when considering either a dominant or recessive models for each polymorphism (Fig. 2; Table 3). We suggest several reasons which might explain why the polymorphisms showed association with hypertension. The first one is that the investigated genes, despite being strong candidates a priori, do not play any significant role in the pathogenesis of hypertension. The second reason might be that the polymorphisms selected in each gene were not appropriate and that there exist other unmeasured polymorphisms of these genes whose effect on disease could not be detected through linkage disequilibrium with the polymorphisms studied. The third explanation might be related to the heterogeneity of patients with respect to progression of the disease. This question would have to be addressed in longitudinal studies.

In addition, the results of our studies were not consistant with those performed on various ethnic groups. The reason for these differences may be due to various genetic or environmental backgrounds. Therefore, further studies will be required to clarify the association between essential hypertension and insulin resistance genes.

Table 3. Odds ratio risk of polymorphism in six candidate genes

Polymorphism	Causative allele	OR (95% CI)	
		Dominant	Recessive
PP1G Asp905Tyr	Tyr	1.00 (0.56-1.80)	1.80 (0.74-4.36)
ADRB3 Trp64Arg	Trp	1.08 (0.52-2.24)	0.35 (0.01-8.67)
Insulin 3'-UTR/*Pst* I	P1	–	1.30 (0.43-3.93)
Glucokinase G-258Al	A	0.81 (0.41-1.60)	1.20 (0.35-4.14)

* Because GSY *Xba* I and GCG-R Gly40Ser is monomorphic, the OR (95% CI) value could not calculated in this study.

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