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ASSOCIATION OF A RFLP FOR THE INSULIN RECEPTOR GENE, BUT NOT INSULIN, WITH ESSENTIAL HYPERTENSION

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SUMMARY: **Insulin has cardiovascular actions and patients with essential hypertension display insulin resistance. A cross-sectional study of the *R1* RFLP of the insulin receptor gene (*INSR*) was carried out in 67 hypertensive (HT) and 75 normotensive (NT) subjects whose parents had a similar blood pressure status at age ≥50. The frequency of the minor (+) allele was 0.31 in HTs and 0.44 in NTs, and the difference between observed alleles in all subjects in each group was significant (χ^2 = 4.8, P<0.05). Allele frequencies of a *BglI* RFLP of the insulin gene, however, did not differ between the HT and NT groups. The data thus provide evidence in favour of an association of HT with a polymorphism at the *INSR* locus (19p13.3-13.2), so implicating this locus, and possibly a genetic variant of the insulin receptor itself, in HT.**

Genotype is considered to be the primary underlying factor responsible for essential (primary) hypertension (HT), a disorder which affects ~20% of the adult population [1,2]. In the face of such a primary genetic 'defect', environmental factors, such as a diet high in NaCl, trigger a chronic rise in blood pressure [1,2]. To date the few molecular genetic studies that have been carried out using candidate gene probes have failed to demonstrate a significant association or linkage with HT [3-6].

Patients with HT often display insulin resistance and it has been suggested that this may play a role in the clinical course of the disease [7-11]. Under normal conditions insulin appears to have cardiovascular actions. These include stimulation of renal sodium reabsorption, vascular smooth muscle contraction and proliferation, and sympathetic activity [10,11]. Moreover, such changes, elicited by mechanisms that are not yet completely understood, have also been implicated in the development of HT [12]. It was therefore of interest to examine whether genetic variants of the insulin receptor gene (*INSR*) and insulin gene (*INS*) co-segregate with HT.

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The present study examined HT and normotensive (NT) subjects for known restriction fragment length polymorphisms (RFLPs) of *INSR* and *INS*. In order to reduce the environmental component of HT and to help overcome problems of penetrance we tested two genetically predisposed populations, viz. the HT offspring of HT parents and, as control, the NT offspring of NT parents.

METHODS

Study design: Adult caucasian subjects were used for the study. Those in the HT group had been diagnosed according to conventional criteria, which included a diastolic pressure of >90 mmHg and a systolic pressure of >140 mmHg on 3 occasions spanning 2 months. Since the study was directed at molecular analysis of genomic DNA structure rather than physiological parameters, the only criteria for selection of patients was that they had been correctly diagnosed as having essential HT and that they were the offspring of two HT parents. NT subjects were selected, with the aid of the Sydney Red Cross Blood Bank, on the basis of both parents being NT at age ≥50. Blood samples of ~20 ml were drawn from the antecubetal fossa, placed in heparinized tubes, and white cells were separated and stored at -70°C prior to DNA extraction. The age of the HT patients was 51 ± 14 S.D. (range 22-82 years) and of NT subjects was 40 ± 11 (range 23-64 years); although different, this was not important, as age is an independent variable having no influence on genotype.

DNA probes: DNA probes were obtained from the American Type Culture Collection, Rockville, MD. For *INSR* this was a 1.3 kb *Bgl*I fragment of the 4.3 kb HIR12.1 *INSR* cDNA [13] and for *INS* was a 1.6 kb *Bam*HI genomic subclone, phIns 214, spanning the entire coding region and 58 bp of 5'-flanking DNA [14]. DNA probes were labelled by nick translation using [^{32}P]dCTP (Amersham, Buckinghamshire, U.K.)

RFLP analysis: DNA was isolated from peripheral blood leukocytes and was used for Southern blot analysis as described previously [15]. In brief, 15 μg of DNA was restricted with either *Rsa*I or *Bgl*I, electrophoresed on 1% agarose gels, transferred to Gene Screen Plus membranes and hybridized with ^{32}P-labelled *INSR* or *INS* probe. Filters were incubated for 16 h at 42°C in 50% formamide, 0.9 M NaCl, 50 mM sodium phosphate buffer, pH 6.8, 200 μg/ml salmon sperm DNA, 10% dextran sulfate, 3.5% SDS, then washed twice for 15 min at 22°C in 2 x standard saline citrate (SSC) (1 x SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) and once for 30 min at 65°C in 2 x SSC/1% SDS. After drying in air the filters were exposed to Kodak XAR-5 film with a Lightning Plus intensifying screen (Dupont) for 2-3 days at -70°C.

Statistical analyses: Data were compiled according to genotype, and allele frequencies were calculated. Statistical analysis required calculation of observed number of alleles from genotype data in each group. The difference between groups was then tested by χ^2 analysis with 1 degree of freedom.

RESULTS

The three possible patterns of hybridization of the *INSR* probe to Southern blots of *Rsa*I-restricted human leukocyte DNA is shown in Fig. 1 (a). Homozygotes for the *R1 Rsa*I RFLP have either a 7.0- or a 6.2-kb band and

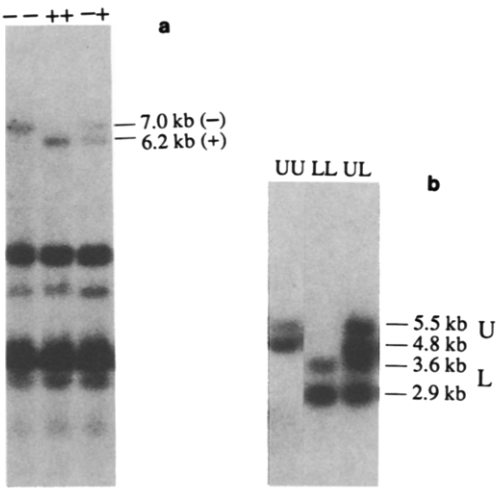

Fig. 1. (a) RFLP of insulin receptor gene, showing the 3 patterns of hybridization of ^{32}P-labelled *INSR* cDNA on Southern blots of *Rsa*I-digested DNA from different subjects; these represent the 3 genotypes for the *R1 INSR* RFLP examined, viz. '– –', '+ +', and '– +' for the 7.0-kb homozygote, the 6.2-kb homozygote, and the 7.0-kb/6.2-kb heterozygote, respectively, where sizes are indicated only for the relevant bands. (b) *Bgl*I *INSR* RFLP, where 'U U' homozygote has bands of 5.5- and 4.8-kb, 'L L' homozygote has 3.6- and 2.9-kb bands, and 'U L' heterozygote has all 4 bands.

heterozygotes have both the 7.0- and 6.2-kb bands. The genotypes have been abbreviated in Fig. 1 to '– –', '+ +' and '– +', where, the 7.0-kb allele of the *R1 INSR Rsa*I RFLP has been designated *R1*(-) and the 6.2-kb allele *R1*(+) [13].

Genotype and derived allele frequencies are shown in Table 1. Most notable in the case of genotype data was a 61% reduction in '+ +' homozygote frequency in the HT group compared with NTs. The NT '–' and '+' allele frequencies were similar to values of 0.52 and 0.48, respectively, reported in a previous study of 92 caucasian subjects [13]. In examining data for NT and HT groups the correct data for analysis was the observed numbers of alleles; i.e., the sum of the number of alleles on each chromosome of all of the subjects in each group (e.g., for HT '–' allele was (2 x 32) + 28 = 92) (Table

Table 1. Genotype and allele frequencies for the *R1*(–) and *R1*(+) alleles of a *Rsa*I RFLP of the insulin receptor gene in essential hypertensive (HT) and normotensive (NT) groups

		Frequencies						
		Genotypes			Alleles		Total alleles on all chromosomes	
	n	– –	– +	+ +	–	+	+	–
HT	67	32	28	7	0.69	0.31	92	42
NT	75	27	30	18	0.56	0.44	84	66
							χ^2 = 4.8 (P<0.05)	

Table 2. Genotype and allele frequencies for the U and L alleles of a *BglI* RFLP of the insulin gene in HT and NT groups

		Frequencies						
		Genotypes			Alleles		Total alleles on all chromosomes	
	n	U U	U L	L L	U	L	U	L
HT	68	11	27	30	0.36	0.64	49	87
NT	75	12	41	22	0.43	0.57	65	85
							χ^2 = 1.6 (P>0.2)	

1). Values for the HT group differed significantly from those for the NT group (χ^2 = 4.8; P<0.05).

The *BglI INS* RFLP has alleles U and L [16], where UU-homozygotes display bands of 5.5- and 4.8-kb, and LL-homozygotes give bands of 3.6- and 2.9-kb (Fig. 1 (b)). Genotype and allele frequencies are shown in Table 2 and were similar to values reported previously for non-diabetic caucasian subjects [16]. By χ^2 analysis of observed alleles no significant difference was apparent between the HT and NT groups in our study.

DISCUSSION

Association analysis of the *R1*(-) and *R1*(+) alleles of a *RsaI* RFLP for the insulin receptor gene in NT and HT non-diabetic caucasian subjects has demonstrated a statistically significant association of the polymorphism with HT. It is unlikely that this is a spurious association arising by random chance or is due to imperfect matching of HT and NT groups, since the frequency of the minor (+) allele in our NT group, 0.44, was similar to a value of 0.48 reported by others for a caucasian population in the U.S.A. [13]. Differences reported previously have been inter-racial, where for Blacks and Japanese, *R1*(+) allele frequencies are higher than seen in caucasians [15]. Because the probability of intragenic crossovers occurring within *INSR* has been estimated as <1% [17], a mutation in *INSR* might be in linkage disequilibrium with the *R1* RFLP. Our results suggest that possession of the '–' allele might be a marker for predisposition to HT, although, since not all '– –' genotypes were hypertensive, the '–' allele alone does not appear to be sufficient for HT. One possibility is that other loci act to oppose the development of HT in a '– –' individual.

As well as being the locus for insulin-resistant diabetes mellitus, 19p13.3-13.2 is also the locus for complement C3 deficiency [18] and, interestingly, an earlier study of the $C3^F$ allele, found no association with familial predisposition to HT [19]. An association with atherosclerosis was, however, indicated, as $C3^F$ conferred a high risk of coronary heart disease in treated

HTs [19]. This finding would be consistent with a role of the *INSR* locus in atherosclerosis, where insulin is known to be atherogenic [11]. The *INSR* locus is also close to *LDLR* (19p13.2-13.1), which is associated with familial hypercholesterolaemia [20].

The *R1 Rsa*I RFLP has been mapped to the region of the 5.3 kb *INSR* cDNA between bases 1928 and 2478 [13], i.e., occurs near the hinge region separating the α and β chains of the receptor, and where rare mutations cause severe insulin resistance [17]. The defect responsible for insulin resistance in HT most likely involves a defect in glucose transport *per se* or in coupling of the insulin receptor with the glucose transport system [11]. Neither of these are, however, likely to cause HT. The β-subunit of the insulin receptor has an integral tyrosine kinase activity that may be involved in second messenger generation [21]. A genetic variant in this region of the receptor could lead to decreased glucose transport and insulin resistance. The mechanism that might lead to HT is, however, unclear. Increased Na^+-H^+ exchange is seen in essential HT [22,23], yet a recent report failed to show linkage of the Na^+-H^+ exchanger gene locus with HT [24]. It has therefore been suggested that increased Na^+-H^+ exchanger activity might be secondary to alterations in the signalling mechanisms that regulate its activity [23]. Since insulin stimulates Na^+-H^+ exchange, it has been proposed that insulin may contribute to the development of HT [23], where increased Na^+-H^+ exchange is known to increase peripheral vascular resistance, proximal tubular sodium reabsorbtion and sympathetic activity [10,11,23]. The present findings are consistent with such hypertensive mechanisms being a consequence of an inherited defect in the insulin receptor gene itself, which is then responsible for secondary changes in Na^+-H^+ exchanger activity.

The nucleotide change responsible for the *R1* RFLP might itself alter the tyrosine kinase activity of the encoded β-chain or may be a linkage marker of change(s) elsewhere that affect enzyme activity. Alternatively the RFLP may be a marker for difference(s) in some other *INSR*-associated region that could, by various possible mechanisms [17], lead to decreased affinity of the receptor for insulin, lower receptor number or altered gene regulation, where, to date, none of these parameters have been reported in patients with essential HT [11]. The other possibility is that the genetic variant responsible for HT is another gene located not far from the insulin receptor gene.

Previous studies could not find an association of *R1* or any other *INSR* RFLP with non-insulin-dependent diabetes mellitus (NIDDM) [13,17], although, in the case of *R1*, only 10-15 patients were tested [13]. The present study, involving a much higher number of subjects, shows an association of *R1* alleles with HT, which, like NIDDM, is a state of insulin

resistance, and where the co-existence of an endemic insulin-resistance gene with HT gene(s) has been said to result in the HT phenotype [11]. Finally, it should be noted that the polygenic nature of essential HT will require studies of the interaction of a variant at the *INSR* locus with other yet-to-be identified genes in determining the aetiology of this disease.

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