Thyrotropin-Releasing Hormone Receptor (TRHR) Gene Is Associated With Essential Hypertension
Silvia I. García, Patricia I. Porto, Guillermo Dieuzeide, María S. Landa, Tobias Kirszner, Yanquel Plotquin, Claudio Gonzalez, Carlos J. Pirola

Abstract—In essential hypertension, a polygenic and multifactorial syndrome, several genes interact with the environment to produce high blood pressure. Thyrotropin-releasing hormone (TRH) plays an important role in central cardiovascular regulation. We have described that TRH overexpression induces hypertension in a normal rat, which was reversed by TRH antisense treatment. This treatment also reduces the central TRH hyperactivity in spontaneously hypertensive rats and normalizes blood pressure. Human TRH receptor (TRHR) belongs to the G protein–coupled seven-transmembrane domain receptor superfamily. Mutations of these receptors may result in constitutive activation. As it has been demonstrated that hypertensive patients have a blunted TSH response to TRH injection, suggesting a defect in the TRHR, we postulate that the TRHR gene is involved in human hypertension. We studied 2 independent populations from different geographic regions of our country: a sample of adult subjects from a referral clinic and a population-based sample of high school students. In search of molecular variants of TRHR, we disclosed that a polymorphic TG dinucleotide repeat (STR) at −68 bp and a novel single nucleotide polymorphism, a G→C conversion at −221 located in the promoter of the TRHR are associated with essential hypertension. As STRs detected in gene promoters are potential Z-DNA–forming sequences and seem to affect gene expression, we studied the potentially different transcriptional activity of these TRHR promoter variants and found that the S/−221C allele has a higher affinity than does the L/G−221 allele to nuclear protein factor(s). Our findings support the hypothesis that the TRHR gene participates in the etiopathogenesis of essential hypertension. (Hypertension. 2001;38[part 2]:683-687.)

Key Words: epidemiology ■ genes ■ hypertension, essential ■ hormone ■ thyroliberin ■ receptor

Thyrotropin-releasing hormone (TRH), a small neuropeptide (p-Glu-His-Pro-NH₂) initially identified in the hypothalamus, which stimulates the synthesis and secretion of thyroid-stimulating hormone (TSH) and prolactin, was shown to be amply distributed in the central nervous system. TRH participates in central cardiovascular regulation, producing dose-dependent pressor effects. We have reported that central overexpression of the TRH precursor in normal rats induces a long-lasting elevation of arterial blood pressure along with an increase in the diencephalic TRH content in a dose-dependent manner. These effects were specifically reversed by an antisense treatment. In addition, spontaneously hypertensive rats (SHR) show a supersensitivity to the hypertensive effects of exogenous TRH and present a pre- and postsynaptic hyperactivity of the extrahypothalamic TRH system. Accordingly, we also recently reported that a specific intracerebroventricular antisense treatment against the initiation translation codon region of the TRH precursor gene diminished both effects, the augmented TRH content and the increased systolic blood pressure, in a dose-dependent manner, independent of the thyroid status. These results indicated that the central TRH system participates in the development or maintenance of experimental hypertension.

The human TRH receptor (TRHR) has been cloned and shown to belong to the G protein–coupled seven-transmembrane domain receptor superfamily. Many endocrine disorders arise from mutations of these receptors, which result in changes of their basal or ligand-induced activity. In this regard, Lupi et al have demonstrated that hypertensive patients have a blunted TSH response to intravenous TRH bolus injection, suggesting a defect in the TRHR activation–mediated TSH release. In summary, we postulated that the TRHR gene is involved in human hypertension.

We first analyzed the presence of polymorphic variants of a TG dinucleotide repeat (STR) and disclosed a novel single nucleotide polymorphism (SNP) (G for C) in the promoter of the TRHR gene. Then we studied the association of these markers to essential hypertension in 2 independent population samples, showing for the first time that carriers of specific TRHR haplotypes have an almost 2-fold higher age- and
sex-adjusted risk of hypertension per dose of the allele. We proposed that these TRHR gene promoter variants might represent forms with different transcriptional activity and performed mobility gel shift analysis, finding that the S→221C allele has a higher affinity than does the L/G→221 allele to nuclear protein factor(s). Our results support the hypothesis that the TRHR gene participates in the etiopathogenesis of essential hypertension.

Methods

Subjects
We recruited 129 hypertensive patients (mean±SD; age, 59±7 years; 91 women; systolic blood pressure, 148±14 mm Hg; diastolic blood pressure, 94±8 mm Hg) attending a hypertension clinic at Buenos Aires. The ethical committee of the hospital approved the study. The subjects provided a complete medical history and underwent physical and laboratory examinations. Subjects were considered to have hypertension if they documented untreated systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg, or both on ≥3 occasions or if they had been treated for hypertension for at least 6 months.

The control group consisted of 64 normotensive adults (age, 53±12 years; 44 women; diastolic blood pressure, 74±6 mm Hg; systolic blood pressure, 129±6 mm Hg) from the same community. Subjects with diabetes mellitus, endocrine diseases, or significant renal or hepatic disease and those receiving oral contraceptives or systemic corticosteroids were excluded.

In addition, we interviewed 934 adolescents from a high school student population at an inner city who agreed to participate and provided a signed informed consent from their parents (Chacabuco, Province of Buenos Aires); they also underwent physical and laboratory examinations. Anthropometric variables were measured. Based on resting blood pressure normalized as a z score by sex and age using the US task force tables (average of 6 measurements taken on 3 different days in a 6-month period), we selected 54 essential hypertensives having an average of systolic or diastolic blood pressure >95% percentile and no causes of secondary hypertension (age, 15±2 years; 22 females) and 121 normotensive age-matched controls (age, 15±2; 73 females; 77.2% of them without parental history of hypertension). All the included subjects were descendants of Spanish and/or Italian people.

TRHR TG Dinucleotide of the Promoter Region Amplification
Genomic DNA was conventionally extracted from peripheral blood leukocytes by proteinase K digestion, phenol extraction, and ethanol precipitation of the DNA.

To analyze the TG dinucleotide repeats (STRs) located in the position −68 from the transcription initiation site in the 5′ region of the TRHR gene, we used a [α-32P]dCTP (3000 Ci/mmol, NEN Life Science Products) radioactive polymerase chain reaction (PCR) designed to amplify a microsatellite-containing fragment of 123 bp according to the D85375-1 GenBank sequence (locus ID, 7201). We constructed 5′ and 3′ primers as follows: upper, 5′-AGA GCA GAA GAC TCT GAA TC-3′ (from 343 to 362); and lower, 5′-CCC CTA ATG AGA ACA TAC AC-3′ (from 446 to 465). PCR conditions were as follows: 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute.

Single-Strand Conformational Polymorphism
We searched for TRHR gene variants along the promoter region and exon 1 and 2, including intron-exon boundaries. PCR products were run in a 6%, 8%, and 10% polyacrylamide gel at 4°C and at room temperature and were detected by AgNO3 staining. We found mobility differences in the 8% polyacrylamide at room temperature condition (data not shown). For the sake of simplicity, we described only the common SNP corresponding to the promoter region because other SNPs were very rare or not associated with essential hypertension.

PCR Products Sequencing
We used the Sequenase Version 2.0 DNA Sequencing kit for PCR products (Amersham). In both fragments, we used [α-32P]dCTP.

Analysis of the SNP (the G→C Transition) Located at −221 of Promoter Region of the TRHR Gene
We used PCR to amplify a fragment of 345 bp by the same sequence listed above, using as an upper primer, 5′-ATG AGA GGA GGA AGC CAA TT-3′ (from 23 to 43); and as a lower primer, 5′-TCA CAG ATG CCA GTC AGT CC-3′ (from 348 to 368). PCR conditions were as follows: 30 cycles at 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute. As the G→C transition in this region destroys a DdeI restriction site, the PCR product was digested overnight at 37°C with 1 U of this enzyme. The G allele is characterized by 25-, 37-, 39-, 64-, and 135-bp fragments; and the C allele, by 37-, 39-, 64-, and 160-bp fragments (see Figure 2).

Mobility Shift Analysis of Transcription Factor Affinity
Band shift assays were performed using 15 μg of nuclear protein extracted from frozen cadaveric human hypophysis tissue. Nuclear protein extract was prepared following a previously described technique.9 Probes were either the short (S) or the long (L) TG dinucleotide PCR product labeled by incorporating [α-32P]dCTP into the reaction mixture. Fragments were purified by Sephadex superfine G50 chromatography and quantitated. DNA-protein complexes were competed by adding 0 (C), 5-, 10-, and 100-fold molar excess of unlabelled specific PCR product (S or L) to the incubation mixture, incubated for 10 minutes in an ice bath, and separated from free probes by electrophoresis by 6% polyacrylamide gels. The incubation mixture also included a mixture of various unrelated PCR products to avoid nonspecific binding.

Statistical Analysis
Quantitative data were analyzed with the Mann-Whitney test or ANOVA (Tukey’s post-hoc test) and expressed as mean±SD. Genotypes frequencies were analyzed by means of a χ2 test. Correlation between variables was assayed by the Spearman R correlation coefficient test. Logistic regression was used for testing of multivariate association between qualitative variables. We used the CSS/Statistica program package (StatSoft) to perform these analyses. Linkage disequilibrium analysis was performed by a free program that is distributed by the authors over the Internet.11

Results
We found that the TG dinucleotide repeat located in the 5′ region of the TRHR is polymorphic with mainly 3 alleles, which yielded PCR products of 123 bp, 129 bp, and 133 bp (Figure 1) and corresponded to 18, 21, and 23 TG dinucleotide repeats, respectively, as estimated by comparison with size markers and direct DNA sequencing. The frequencies in the normal population of these three alleles were 53%, 44%, and 3%, respectively. The distribution of the resulting genotypes was in Hardy-Weinberg equilibrium.

To further investigate the frequency of these genotypes in hypertensive versus normotensive subjects, for sake of simplicity we excluded the 133-bp allele because its frequency was very low and named the 123-bp allele as the short allele (S) and the 129-bp allele as the long (L). According to the nonparametric Spearman rank correlation coefficient and χ2 tests, there were significant differences in allele and genotype frequencies between adult hypertensive and normotensive...
Figure 1. Genotyping of normal subjects for the (TG)ₙ dinucleotide repeat located in the promoter region of the TRHR gene. ³²P-labeled PCR products were separated on a 8 mol/L urea and 6% polyacrylamide gel and autoradiographed. Lanes 1 and 2 show homozygotes for the 123-bp allele (S); lanes 3, 4, and 5, homozygotes for the 129-bp allele (L); and lane 6, a heterozygote 123 bp/129 bp. Lane 7 shows a much more rare genotype that corresponds to a subject heterozygote for the common allele 123 bp and the less frequent 133-bp allele.

groups (Table 1). In a discriminant logistic model, an almost 2-fold higher risk of hypertension was found among the carriers of the S variant per dose of the allele (odds ratio [OR], 1.71; 95% confidence interval [95% CI], 1.12 to 2.62; P<0.011). The significant association between the S allele and hypertension persists even when adjusted for age and sex (OR, 1.61; 95% CI, 1.02 to 2.58; P<0.04). To confirm this association, we studied a population-based sample of adolescents from a small inner city (Chacabuco). In this community, the prevalence of young-onset hypertension is ~5% (54 of 938 subjects interviewed). Allele and genotype frequencies for the S and L TRHR variants were similar to those of the adult population, and both groups differed (OR, 1.97; 95% CI, 1.13 to 3.45; P<0.02) (Table 2).

Moreover, looking for other variants in the promoter region, we found by single-strand conformational polymorphism and direct sequencing of the PCR product, a common SNP that is a G→C transition at nucleotide −221 in the promoter region. As far as we know, this polymorphism has not been described. In 282 chromosomes of the general population, we found using PCR/restriction fragment length polymorphism (RFLP) (Figure 2) that the frequencies of allele G and C were 36% and 64%, respectively. This SNP is in strong linkage disequilibrium with the STR as shown by disequilibrium analysis (Fisher exact test, P<0.0001). We basically found 2 haplotypes: S/−221C and L/−221G. Thus, a significant difference was also found in adults between the frequency of SNP C allele carriers in hypertensive and normotensive groups (OR, 2.59; 95% CI, 1.03 to 6; P<0.04). Similar results were obtained in adolescents using a multiple logistic regression analysis (age-/sex-adjusted OR, 1.82; 95% CI, 1.02 to 3.3; P<0.04).

As we found a significant association between these markers and essential hypertension in 2 independent populations and it has been described that short tandem repeats (STRs) are potential Z-DNA–forming sequences affecting gene expression, we propose that these TRHR promoter variants represent forms with different transcriptional activity by interacting differently with nuclear protein factors. Competitive-binding studies using human pituitary nuclear extracts suggested that the affinity of transcriptional factor to the S variant is higher than that of the longer allele because the corresponding unlabelled competitor is more efficient in displacing the probe from the DNA-protein complex (Figure 3). Densitometry analysis indicated that 3 times more L allele than S allele competitor (9-versus 3-fold molar excess) was needed to displace 50% of the corresponding bound probe (data not shown).

Discussion

Extrahypothalamic TRH participates in the development and/or maintenance of rat spontaneous hypertension. It was thus tempting to speculate that the component genes of the TRH system may be associated with essential hypertension. Because Lupi et al9 reported that hypertensive patients have a blunted TSH response to TRH, indicating that there is a possible defect in the TRHR activation–mediated TSH release, we postulated that TRHR may be also involved in essential hypertension. Accordingly, we searched for molecular variants of the TRHR gene promoter. We first characterized the putative variants of the STR of a TG dinucleotide located in the promoter region and found that this microsatellite was polymorphic displaying 2 highly prevalent alleles.

| TABLE 1. Clinical Characteristics of Adult Normotensive and Hypertensive Subjects According to Genotypes Corresponding to the TG Dinucleotide Microsatellite on the TRHR Gene |
|-----------------------------------|----------------|----------------|----------------|
| | Genotype | BMI, Kg/m² | SBP, mm Hg | DBP, mm Hg |
| Subject Groups | Frequencies, % | | |
| | | Normotensives (n=63) | | |
| SS | 31.5 | 24.97±1.85 | 129.3±6.6 | 73.2±6.7 |
| SL | 42.4 | 25.04±2.71 | 128.4±6.2 | 76.4±3.4 |
| LL | 26.4 | 25.30±1.85 | 129.3±2.6 | 73.8±5.4 |
| Hypertensives (n=120) | | | |
| SS | 45.7 | 27.68±4.26 | 148.9±13.4 | 94.7±8.5 |
| SL | 41.7 | 27.22±4.56 | 146.5±14.7 | 93.1±7.4 |
| LL | 12.6 | 28.15±2.93 | 151.9±13.5 | 95.2±7.0 |

S and L stand for PCR products of 123 bp and 129 bp, respectively. TRHR genotype frequencies of normotensives and hypertensives are significantly different (P<0.013). Body mass indices (BMI) and systolic (SBP) and diastolic (DBP) blood pressures were not different across genotypes within each group, but BMI differed between groups (P<0.001).

Values are mean±SD.
rendering PCR products of 123 and 129 bp and a rare allele of 133 bp. The distribution of the resulting genotypes was in Hardy-Weinberg equilibrium, and significant differences were found between allele and genotype frequencies of hypertensive and normotensive groups because the S allele was more frequent in adult hypertensives than in normotensive subjects.

To confirm this association, a population-based study was performed on adolescents from a small inner community with a small genetic admixture, on the rationale that a genetic component of the disease should be more apparent in a group of patients with young-onset hypertension. Allele and genotype frequencies for the S and L TRHR variants significantly differed between hypertensive and normotensive groups as in adults. Moreover, we looked for other variants in the promoter region, exons and intron-exon joints of the TRHR. Besides several SNPs that are rare, we found a common SNP, a G for C conversion at nucleotide −221 in the promoter region. As this SNP is in strong linkage with the STR, it was not surprising to find basically 2 haplotypes, the short STR combined with −221C and the long STR combined with −221G. Thus, a significant difference was also found in adults between the frequency of SNP C allele carriers in hypertensive and normotensive groups. Similar results were obtained in adolescents using a multiple logistic regression analysis. Our data imply that an estimated risk of hypertension as high as ≈1.8 per TRHR −221C allele can be assigned to an individual who carries it.

At any rate, in 2 independent populations from different geographic regions of our country, we found a significant association between these markers and essential hypertension. Because STR have been described as potential Z-DNA–forming sequences in several promoter genes and may affect gene expression because dinucleotide repeats exerts negative effects on gene transcriptions,12 we propose that these TRHR promoter variants represent forms with different transcriptional activity. This hypothesis is supported by the fact that the nuclear protein factors extracted from human pituitary glands showed a higher affinity to the S allele fragment than to the L allele. It is worthy of mention that the promoter region of the TRHR containing the STR and the SNP

<table>
<thead>
<tr>
<th>Subject Groups</th>
<th>Genotype Frequencies, %</th>
<th>BMI, Kg/m²</th>
<th>SBP, z Score</th>
<th>DBP, z Score</th>
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<td>Normotensives (n=119)</td>
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<tr>
<td>SS</td>
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<td>2.83±1.16</td>
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<tr>
<td>SL</td>
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<td>2.59±1.07</td>
<td>0.83±0.71</td>
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<tr>
<td>LL</td>
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<td>25.38±3.76</td>
<td>2.56±0.85</td>
<td>0.94±0.78</td>
</tr>
</tbody>
</table>

Values are mean±SD.

S and L stand for PCR products of 123 bp and 129 bp, respectively. TRHR genotype frequencies of normotensives and hypertensives are significantly different (P<0.019). Body mass indices (BMI) and the Z scores for systolic (SBP) and diastolic (DBP) blood pressures were not different across genotypes within each group, but BMI differed between groups (P<0.001).

Besides several SNPs that are rare, we found a common SNP, a G for C conversion at nucleotide −221 in the promoter region. As this SNP is in strong linkage with the STR, it was not surprising to find basically 2 haplotypes, the short STR combined with −221C and the long STR combined with −221G. Thus, a significant difference was also found in adults between the frequency of SNP C allele carriers in hypertensive and normotensive groups. Similar results were obtained in adolescents using a multiple logistic regression analysis. Our data imply that an estimated risk of hypertension as high as ≈1.8 per TRHR −221C allele can be assigned to an individual who carries it.

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**Figure 2.** Inverted image of the normal subject genotyping for the G-221C SNP located in the promoter region of the TRHR gene. PCR products were digested by a overnight incubation with 1 U of Ddel at 37°C, and fragments were detected by electrophoresis in 3.0% metaphor agarose gel stained with ethidium bromide. The homozygous GG shows 37-, 39-, 64-, and 135-bp bands and the CC with 37-, 39-, 64-, and 160-bp bands. Heterozygous GC has the 37-, 39-, 64-, 135-, and 160-bp bands.

**Figure 3.** Mobility shift analysis of nuclear factor affinity. Band shift assays were performed using 15 μg of nuclear protein extracted from frozen cadaveric human hypophysis tissue. Probes were the Sephadex G-50-purified PCR products corresponding to S or L TRHR alleles labeled with [α-32P]dCTP (NEN-Life Science). The complexes were competed by adding 0 (C)-, 5-, 10-, and 100-fold molar excess of unlabelled specific PCR product (S or L) to the preincubation mixture and separated by electrophoresis using 6% polyacrilamide gels in 0.5×tris-boric-EDTA (TBE) buffer at 4°C during 8 hours at 250 V.
described is highly homologous to the same region of the bovine TRHR gene (GenBank accession No. AB001751), indicating that it is probable that this sequence was conserved during the evolution and is a consensus for transcription factor(s). In addition, this region is also rich in the GC dinucleotide, which can be methylated as a mechanism of transcriptional activity regulation. Clearly, further studies are necessary to clarify these issues, particularly as to whether the nuclear factor(s) that bound to this TRHR region are truly enhancer(s) of the transcriptional gene activity.

At any rate, the frequency of the short allele was significantly higher in hypertensive than in normotensive subjects. No conclusions can be drawn for any cause-effect relationships from case-control studies and gene polymorphisms. Moreover, the TRHR gene polymorphism may be a simple molecular marker linked to the true genetic factor(s), which may confer susceptibility for the disease. However, provide the above-mentioned evidence for the hypertensive effects of the TRH system hyperactivity, these results pointed out, for the first time, that the S allele of the STR and/or the −221C variant in the promoter region of the TRHR gene may be a susceptibility factor for the development of essential hypertension. Given the high frequency of the S allele, even a 1.5-fold increased risk of hypertension among the carriers of this allele might account for a significant number of cases of hypertension in our population.

Therefore, the TRHR gene variants could participate in the etiopathogenesis of essential hypertension at least in populations of European origin.

From a more general point of view, the possible contribution of common variants of promoter regions of candidate genes to essential hypertension suggest that common variation in regulatory noncoding gene regions may be important contributors to complex diseases.

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