Different Frequencies of Inducible Nitric Oxide Synthase Genotypes in Older Hypertensives

Cheryl L. Glenn, William Y.S. Wang, Brian J. Morris

Abstract—A locus for essential hypertension has been found recently on chromosome 17 in the general vicinity of the inducible nitric oxide synthase (iNOS) gene (NOS2A at 17cen-q11.2). We therefore tested NOS2A markers for association and linkage with hypertension in affected Australian Anglo-Caucasians. Patients for the association study (n=112) were from our cohort of hypertensives (systolic/diastolic=175±25 SD/112±19 mm Hg) who were the offspring of 2 hypertensive parents; control subjects (n=164) were normotensives whose parents were both normotensive. The linkage study involved 156 hypertensive sib-pairs. Genotypes for an 8-allele pentameric repeat located 2.6 kb upstream of NOS2A and of a biallelic tetrancleotide repeat 0.7 kb upstream were determined by polymerase chain reaction and automated gene scan analysis. In the association study, the frequency of the minor allele of the biallelic marker was 0.18 in the hypertensives and 0.14 in the normotensives ($\chi^2_{1,df}=1.1$, $P=0.3$). Allele frequencies for the multiallelic marker were also similar in each group ($\chi^2_{7,df}=9.8$, $P=0.2$). Furthermore, no genotypic differences in blood pressure were apparent. In the sib-pair study, SPLINK APM, and MAPMAKERS/SIBS did not indicate excess allele sharing. We also examined genotype as a function of age. In the younger (<60 years) hypertensives as well as younger or older normotensives, genotype and allele frequency of the biallelic marker was similar (0.12 to 0.14). However, in hypertensives ≥60 years of age, frequency of the minor allele was 0.28 ($\chi^2=7.4$, $P=0.006$). Homozygotes for this allele were rare. Frequency of heterozygotes was 0.19 for normotensives but 0.39 for the older hypertensives ($\chi^2=8.0$, $P=0.018$) and was 0.40 for hypertensive sibs ≥60 years of age with a diastolic pressure ≥100 mm Hg. Furthermore, homozygotes for the major allele were 7 years younger than heterozygotes ($P=0.05$ by ANOVA). In conclusion, the present study shows (1) no evidence for a role of NOS2A in hypertension and (2) a genotypic difference in frequency of a NOS2A promoter variant in older hypertensives, seen in 2 different cohorts. A possible interpretation of the latter observation is that NOS2A genotype could affect longevity, at least in patients at high risk by having moderate to severe hypertension. (Hypertension. 1999;33:927-932.)

Key Words: whites ■ hypertension, essential ■ nitric oxide synthase ■ genetics, biochemical ■ polymerase chain reaction ■ linkage (genetics) ■ survival

A locus for essential hypertension has been reported recently on chromosome 17 after sib-pair analysis with the use of microsatellite markers. The broad linkage region identified boarders, at cen-q11.2, the inducible nitric oxide (NO) synthase (iNOS) gene (NOS2A), which spans ~37 kb and contains 26 exons, exons 22 to 26 also being represented as 4 partially duplicated sequences in humans and great apes at this (NOS2B, −C) and other (−E) loci. NOS2A, along with the neuronal (n) NOS and endothelial (e) NOS isoform genes, NOS1 and NOS3 (chromosomes 12q24.2-q24.31 and 7q35-q36), respectively, are involved in the generation of NO, a potent vasodilator. NOS2A is expressed in various tissues, including some relevant to the cardiovascular system, viz, cardiac and vascular smooth muscle, renal tubules, and afferent arteriole. There is, moreover, evidence that intrarenal expression of iNOS can regulate arterial pressure. Although NO is reduced in essential hypertension, which may contribute to vascular and cardiac hypertrophy, and NO markers correlate inversely with blood pressure, there is no evidence to date that iNOS has a pathogenic role. In the spontaneously hypertensive rat (SHR), however, although iNOS expression is similar in vascular smooth muscle cells of prehypertensive rats and Wistar-Kyoto controls, sustained NO production is lower in SHR, and NOS2 transcription differs between cells of each strain, leading to a suggestion that iNOS could be involved in the early rise in blood pressure. Others have found abnormal expression later in SHR hypertension. In Sabra DOCA salt-hypertensive rats, iNOS expression is reduced compared with their salt-resistant control strain, and greater NO generation could contribute to the salt resistance of the latter. Dahl salt-sensitive hypertensive rats may have a defect in NO synthesis, seen in vivo as well as in primary cultures of aortic smooth muscle cells.
and a transversion, T2140C (Ser714Pro), has been noted in the \( \text{iNOS} \) gene of Dahl rats. Moreover, various rat strains show a hypertension linkage region in the vicinity of \( \text{Nos2} \). Two polymorphisms have been described for \( \text{NOS2A} \). Both concern variation in repeated sequences and each is located in the 5′-flanking DNA. One is in an AAAT/AAAAAT repeat at –756 to –716 relative to the major transcription start site and involves an insertion or deletion of 1 repeat unit. The other, located 2.7 to 2.5 kb upstream, consists of 8 alleles of a CCTTT\(_n\) pentanucleotide repeat with heterozygosity 0.80. Of possible relevance to a disease such as hypertension, strand slippage in such repeats provides a rapid evolutionary mechanism for response to environmental change, with 9 to 16 repeat units in humans but only 3 to 9 in chimpanzees. Similar sequences form a triplex structure in vivo, leading to S1 nuclease-sensitive sites that may affect iNOS hypersensitive sites in active chromatin. Moreover, variation in repeat number in the insulin promoter affects promoter activity and onset of insulin-dependent diabetes mellitus (IDDM).

Using these polymorphisms of \( \text{NOS2A} \) as markers, we have conducted the first disease association and linkage studies of this gene. As well as examining hypertension itself, we also checked whether there was any effect on mortality in the \( \text{iNOS} \) gene of Dahl rats. Moreover, various rat strains and a transversion, T2140C (Ser714Pro), have been noted in the \( \text{iNOS} \) gene of Dahl rats. Moreover, various rat strains show a hypertension linkage region in the vicinity of \( \text{Nos2} \). Two polymorphisms have been described for \( \text{NOS2A} \). Both concern variation in repeated sequences and each is located in the 5′-flanking DNA. One is in an AAAT/AAAAAT repeat at –756 to –716 relative to the major transcription start site and involves an insertion or deletion of 1 repeat unit. The other, located 2.7 to 2.5 kb upstream, consists of 8 alleles of a CCTTT\(_n\) pentanucleotide repeat with heterozygosity 0.80. Of possible relevance to a disease such as hypertension, strand slippage in such repeats provides a rapid evolutionary mechanism for response to environmental change, with 9 to 16 repeat units in humans but only 3 to 9 in chimpanzees. Similar sequences form a triplex structure in vivo, leading to S1 nuclease-sensitive sites that may affect iNOS hypersensitive sites in active chromatin. Moreover, variation in repeat number in the insulin promoter affects promoter activity and onset of insulin-dependent diabetes mellitus (IDDM).

Using these polymorphisms of \( \text{NOS2A} \) as markers, we have conducted the first disease association and linkage studies of this gene. As well as examining hypertension itself, we also checked whether there was any effect on mortality in the same way as tested previously for angiotensin-converting enzyme (ACE) genotypes.

### Methods

#### Subjects

**Association Study**

The association study involved hypertensives with a strong genetic background (2 hypertensive parents). These have a greater likelihood of showing an existing association than for only 1 affected first-degree relative or an unselected hypertensive group. This group has, moreover, been the subject of a number of previous molecular genetic studies of hypertension, wherein ascertainment details have been described. In all there were 112 unrelated, age- and sex-matched, nondiabetic, treated white essential hypertensive patients and a control group of 164 normotensive subjects who also had parents who both had the same blood pressure status as theirs. Hypertensives with 2 affected parents represent ~10% of all hypertensive patients. Characteristics of the groups are shown in Table 1. These studies had human ethics approval, and all subjects gave informed consent.

**Sib-Pair Study**

Two hundred thirty-four individuals from 98 hypertensive sibships (>2 affected sibs) were contacted by community advertising. All were Anglo-Caucasians, mainly from eastern Australia, principally Sydney, and had to have systolic/diastolic blood pressure of >140/90 mm Hg and not have diabetes or renal disease. After adjustment for 14 trios and 6 quartets, the weighted sib-pair number was 156. Table 1 shows their characteristics.

#### Genotyping

DNA was isolated from whole blood by a modified salting-out method. Genotypes for the biallelic polymorphism were determined by polymerase chain reaction (PCR) with the following primers: sense –5′-TGG TGC ATG CCT GTA GTC C-3′, antisense –5′-GAC GCC TCT GAG ATG TTG GTC-3′. The former was labeled with FAM during synthesis by Bresatec (Adelaide, South Australia). The PCR mix (25 µL) consisted of 50 ng DNA, 20 nmol each primer, 0.25 mmol/L each dNTP, 1 U AmpliTaq Gold DNA polymerase (Perkin-Elmer), 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 1.7 mmol/L MgCl\(_2\), and 4 µg BSA. A “hot-start” protocol was used in which after initial denaturation at 94°C for 5 minutes, there were 10 cycles of 94°C, 65°C, and 72°C for 1 minute each, followed by 15 cycles of 94°C, 60°C, and 72°C for 1 minute each, and finally 20 cycles of 94°C, 58°C, and 72°C for 1 minute each, finishing with a step at 72°C for 30 minutes.

For the multiallelic marker, PCR primer sequences were: forward, 5′-ACC CCT GGA AGC CTA CAA CTG CAT-3′ (FAM-labeled); reverse, 5′-GCC ACT GCA CCC TAG CCT GTC TCA-3′. The final PCR mix (8 µL) was composed of 50 ng DNA, 5.4 mmol each primer, 0.24 mmol/L each dNTP, 0.4 U AmpliTaq Gold, 49 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, and 2 mmol/L MgCl\(_2\). After an initial denaturation step at 94°C for 12 minutes, 35 cycles of 94°C, 55°C, and 72°C for 30 seconds each were performed, finishing with a 10-minute step at 72°C.

All PCR products were electrophoresed on an ABI 377 automated sequencer (Applied Biosystems), and genotypes were assigned with ABI Genotyper software.

#### Statistical Analysis

Genotype data were used to calculate total alleles on all chromosomes, and these data were tested by \( \chi^2 \) analysis with Excel.
The comparison of different parameters across genotypes involved 1-way ANOVA. Linkage analysis was performed with the use of programs suitable for complex traits, viz, SPLINK, which uses allele shared identity by state (IBD) estimates for all possible pairs in a sibship and computes probabilities for each marker genotype when parents are not available, the Affected Pedigree Member (APM) method, and MAPMAKER/SIBS. Linkage disequilibrium between the markers was tested as described.

Results

Biallelic Marker

Association Study
Genotype and derived allele frequencies are shown in Table 2. Hardy-Weinberg equilibrium was observed. Frequency of the minor allele (+) was 0.14 in the normotensive group and 0.18 in the hypertensive group. The difference was not significant. Minor allele frequency was 0.16 in male and 0.20 in female hypertensives (P = 0.5) and 0.18 in both lean (body mass index <26 kg/m²) and obese (≥26 kg/m²) patients. Comparison by 1-way ANOVA of the various parameters in Table 2 across genotypes did not reveal any significant differences. For example, systolic blood pressure (mean ± SD, mm Hg) in the hypertensives was 174 ± 25, 179 ± 25, and 170 ± 21 for −/−, +/+ , and +/− genotypes (n = 62, 22 and 5), respectively, and for diastolic pressure was 112 ± 17, 111 ± 24, and 114 ± 21. Values for plasma lipids, plasma renin, plasma angiotensinogen, and plasma ACE were similar to those described previously and did not differ between genotypes (data not shown).

Sib-Pair Study
Minor allele frequency in hypertensive sibs was 0.17. The biallelic marker was not very informative (information content = 0.17). Linkage analysis of sib-pair data by SPLINK, either weighted or unweighted, gave P = 0.50, and analysis by APM produced P = 0.66. Thus each method showed no significant excess allele sharing.

Multillexic Marker

Association Study
Frequencies of the 8 alleles in each group are shown in Table 3. Observed heterozygosity was 0.74 in the normotensive group, 0.81 in the hypertensive group, and 0.77 in the hypertensive sibs. Comparison of allele frequencies by χ² analysis showed no significant difference between the groups. Blood pressure and other parameters were also similar for each genotype.

The 2 markers were in weak linkage disequilibrium (D = 70%, P = 0.05) with alleles 193, 198, and 203 of the multiallelic marker begin associated with the + allele of the biallelic marker, and alleles 178, 183, 188, 208, and 213 being associated with the − allele.

TABLE 2. Genotype and Allele Frequencies of Deletion/Insertion (−/+ ) Polymorphism of NOS2A in the Hypertensive Group and Different Age Groups of HTs, Compared With the Normotensive Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype Frequency (Proportion)</th>
<th>Total Alleles (Proportion)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = −/−</td>
<td>−/+</td>
</tr>
<tr>
<td>All NTs</td>
<td>149</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>(0.76)</td>
<td>(0.19)</td>
</tr>
<tr>
<td>All HTs</td>
<td>109</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>(0.70)</td>
<td>(0.25)</td>
</tr>
<tr>
<td>Different age groups of HTs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;50 y</td>
<td>51</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>(0.78)</td>
<td>(0.18)</td>
</tr>
<tr>
<td>Age 50–59</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(0.80)</td>
<td>(0.15)</td>
</tr>
<tr>
<td>Age ≥60 y</td>
<td>38</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>(0.53)</td>
<td>(0.39)</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1. χ² values are for comparison of data for HT, or each age group of HTs, with data for NT group.

TABLE 3. Frequency of Alleles of 8-Allele Pentameric Repeat Upstream of NOS2A in the Hypertensive Association and Sib-Pair Groups, the Normotensive Group, and in Another White Population

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype (Size of PCR Product in bp) and Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht sibs</td>
<td>n = 178</td>
</tr>
<tr>
<td>HT</td>
<td>112</td>
</tr>
<tr>
<td>NT</td>
<td>164</td>
</tr>
<tr>
<td>Xu et al*</td>
<td>202</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1. *Data for a general population in which the polymorphism was first described.
**Sib-Pair Study**

Linkage analysis of sib-pair data by SPLINK, unweighted and weighted, gave probability values of 0.56 and 0.57, respectively, indicating no excess allele sharing. APM produced $P=0.4$. A loglike value of 0.00 was obtained by MAPMAKER/SIBS, and Lod score was $-1.6$.

**Analysis in Different Age Groups**

Genotype frequency is fixed from conception and should remain constant throughout life. Any deviation with age could indicate an effect on longevity. To test this, data in Table 2 were reanalyzed after subdivision into age groups of $<50$ years, 50 to 59, and 60 to 69, as done previously to reveal a deleterious effect of the deletion allele of an ACE gene variant in the same group. As can be seen in Table 2, although frequencies were virtually identical with control in the younger age groups, the older hypertensives had double the frequency of the “+” allele (0.28), with correspondingly lower “−” allele frequency ($\chi^2 = 7.4$, $P = 0.006$). In contrast, the normotensives showed no difference with age (data not shown). Comparison of $−/−$ with $+/+$ and $+++-$ frequencies combined produced $\chi^2 = 7.9$, $P = 0.005$. The hypertensive group but not the normotensives displayed, moreover, a significant interaction of age with genotype: $−/−$ = 50.7 $\pm$ 11.9 SD $\gamma$ (n = 76), $+/+$ = 57.3 $\pm$ 11.8 SD (n = 27), and $+/++$ = 53.8 $\pm$ 13.3 SD (n = 6) ($P = 0.05$ by ANOVA). Pretreatment blood pressures were similar for each genotype, in each age group; for example, in the older subgroup, systolic = 183 $\pm$ 24 SD, 182 $\pm$ 20, 180 $\pm$ 14 for $−/−$, $+/+$, and $+/+$ (n = 16, 20, and 2, respectively); diastolic = 112 $\pm$ 15, 112 $\pm$ 23, 113 $\pm$ 4. All parameters were similar for older versus younger patients, except that pretreatment systolic pressure was higher in the older subgroup (182 $\pm$ 21 vs 171 $\pm$ 26 SD mm Hg; $P = 0.03$).

Similar analyses in the hypertensive sibss, whose blood pressure was lower overall (mean diastolic = 103 $\pm$ 112 mm Hg; Table 1), showed no difference across age groups (“−” frequency: 0.15, 0.20, 0.16 for age <50, 50 to 59, and ≥60 years, respectively). However, restriction to sibs with more severe hypertension (diastolic $\geq$ 100 mm Hg; mean $= 107 \pm 10$ SD) revealed a genotypic difference in the 20 sibs ≥60 years: viz, 0.60, 0.40, and 0.0 for $−/−$, $+/+$, and $+/+$, respectively ($P = 0.03$ vs normotensives). For the 38 sibs <60 years of age (with diastolic $\geq$ 100 mm Hg), genotype values (0.76, 0.21, and 0.03) for $−/−$, $+/+$, and $+/+$ were similar to the normotensives. Age for each genotype of the more severely hypertensive sibss was 57 $\pm$ 10 (SD) years, 59 $\pm$ 10, and 57, respectively (n = 48, 20, and 1).

For the multiallelic marker, although numbers for each allele were smaller, no age-related differences could be seen (data not shown).

**Discussion**

The present study found no evidence for association or linkage of markers at the NOS2A locus with essential hypertension. We tested both biallelic and multiallelic polymorphisms. The former are preferred in association studies, whereas the latter can provide the necessary informativeness for linkage analyses. Association studies test whether a disease and an allele show correlated occurrence in a population, whereas linkage studies test whether they show a correlated transmission within a pedigree.37

Biallelic allele frequency in our normotensives and younger hypertensives was similar to what others have observed in unselected European whites (minor allele frequency = 0.15, n = 35). Since, in the absence of exhaustive studies, it is generally believed that results of association analyses only apply to the polymorphism tested, the present findings do not rule out hypertension association for (another) variant(s) in or near NOS2A that is not in linkage disequilibrium with the markers tested.

The negative sib-pair linkage result suggests either that the NOS2A locus is not linked to essential hypertension or the size of the study group was insufficient to provide sufficient power to reveal a small genetic contribution of NOS2A or a linked gene to hypertension. For strong family history and disease onset before age 55 years, relative risk to a sibling ($\lambda_s$) is 3.8,38 meaning that in a complex disease ≈100 sib-pairs would be sufficient to provide 90% power to show significant linkage at the Lod $\geq 3$ ($P \leq 0.001$) level.40 For a disease with multiple weak contributing loci (eg, $\lambda_s$ values of ≈2), 156 sibs should have 90% power to provide evidence at the Lod $\geq 2$ ($P \leq 0.001$) level, as indeed we have found with our number of sibs for markers on chromosome 17, and as others have reported for the angiotensinogen locus.41

Thus a negative result from both association and linkage analysis helps provide some assurance about the validity of the conclusion reached but does not completely rule out a contribution to the disease tested.

Our 2 groups of hypertensives had similar inclusion criteria and geographic location. However, the stronger family history of hypertenives with 2 hypertensive parents may explain why their age of onset of hypertension (32 $\pm$ 10 [SD] years) was earlier than the hypertensive sibs (43 $\pm$ 13 years), so accounting for their lower age (53 $\pm$ 12 [SD] vs 61 $\pm$ 10 years) and more severe hypertension (Table 1). Moreover, the fact that age of onset for the older hypertensives (33 $\pm$ 7 years) was similar to the younger hypertensives rules out a different pathogenesis involving late-onset disease.

There has been no previous molecular genetic study of NOS2A in hypertension, so our results add to findings for NOS1, in which an 8-allele microsatellite (heterozygosity 0.52) in exon 29 failed to show association with hypertension in Japanese patients, and NOS3, in which a 24-allele dinucleotide repeat (polymorphism information content 0.92) in intron 13 showed no linkage with hypertension in 269 white sib-pairs nor association in 88 hypertensives with 2 hypertensive parents. In Japanese patients, although no difference was observed for the whole group, hypertensives without left ventricular hypertrophy showed a weak ($P = 0.02$) association with hypertension. 55 Two other single-base substitution polymorphisms, in introns 18 and 23, have also proved negative for hypertension in whites, but a T/G variant in exon 7 that causes an amino acid substitution (Asp298Glu) was 16% more frequent ($P = 0.004$). It displayed, however, no association with blood pressure. Thus results to date for NOS1 and NOS3 also provide little support for each in the pathogenesis of hypertension.
Although we found no association in the group as a whole or in younger hypertensives, in the case of the biallelic variant, older more severely affected hypertensives of either group displayed a 2-fold elevation in $+/-$ genotype frequency and a one-third reduction in $+/+$ genotype frequency. $+/+$ hypertensives were also 7 years older than $+/+$ hypertensives. No conclusion could be made about $+/+$ homozygotes because patient number and population frequency for this genotype was low.

A possible cause of altered genotype frequency with age may involve effects on survival or mortality. Our results would be consistent with a deleterious effect in high-risk patients with the $+/-$ genotype or a survival advantage in those with the $+/+$ genotype. What this would mean is that at the level of the gene, the promoter variant tested could affect NOS2A expression or be in linkage disequilibrium with (an)other variant(s) that confers altered promoter activity, mRNA stability, or is a sequence variant of iNOS having different enzymatic activity. Thus alteration in iNOS-mediated NO formation could affect death rate in at-risk individuals.

Depending on the tissue, cytotoxic effects of elevated iNOS activity might be either beneficial, for example, in reducing tumor growth, or harmful, for example, in $\beta$-cell destruction and onset of non–insulin-dependent diabetes mellitus (NIDDM), atherosclerosis, and coronary disease. Infections result in an iNOS response, which may be beneficial or deleterious, depending on the pathogen. Survival effects could involve its immune or proinflammatory functions, and enhanced vasodilatory actions could be cardioprotective. The effects of iNOS therefore appear dichotomous. However, the greatest cause of death in patients >60 years of age with moderate to severe hypertension is heart attack and stroke. Atherosclerosis is an exacerbatory factor, for which iNOS has both pathogenic and protective functions. Both iNOS and ACE are elevated markedly in human coronary plaque macrophages. Higher iNOS levels in $+/-$ patients could thus have a survival disadvantage.

If our results do indeed reflect an effect on longevity, the rarity of the $+/+$ allele makes it difficult to say whether the $+/+$ allele might be genetically dominant, that is, whether it has a similar effect in $+/+$ and $+/+$ patients or whether heterozygosity provides a greater relative survival advantage than either allele alone.

In conclusion, the present work provides no support for involvement of the iNOS gene (NOS2A) in the genetics of essential hypertension but suggests that genetic variation involving a tetranucleotide repeat in the promoter is associated with mortality.

Acknowledgments
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References


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