Association of eNOS Glu298Asp Polymorphism With End-Stage Renal Disease

Eisei Noiri, Hiroaki Satoh, Jun-ichi Taguchi, Sergey V. Brodsky, Akihide Nakao, Yumiko Ogawa, Satomi Nishijima, Takehiko Yokomizo, Katsushi Tokunaga, Toshiro Fujita

Abstract—Nitric oxide (NO) derived from endothelial cells is profoundly related to the maintenance of physiological vascular tone. Impairment of endothelial NO generation brought about by gene polymorphism is considered the major deterioration factor for progressive renal disease, including diabetic nephropathy. The present study aimed to elucidate the Glu298Asp polymorphism of endothelial NO synthase (eNOS) in patients with end-stage renal disease (ESRD) and its role as a predisposing factor for cardiovascular complications. Glu298Asp in exon 7 of the eNOS gene was determined by polymerase chain reaction, followed by restriction fragment length polymorphism analysis, in ESRD patients (n=185) and compared with that of unrelated healthy individuals (n=304). The occurrence of 298Asp was significantly higher in the ESRD group (P=0.0020; odds ratio [OR] 1.65; 95% confidential interval [CI]: 1.21 to 2.25). In this group, 72 patients had type 2 diabetes mellitus (DM). Although 298Asp did not reach a significant level in the non-DM ESRD subgroup, the occurrence of 298Asp was significantly higher in DM-derived ESRD patients (P=0.0010; OR 2.02; 95% CI: 1.37 to 3.07). The functional effect of the Glu298Asp was examined using Chinese hamster ovary (CHO) cells stably overexpressing either 1917G or 1917T. NO-selective electrode measurements and fluorometric nitrite assay revealed a statistically significant difference in NO production or nitrite accumulation between CHO 1917G and 1917T (P<0.01). These data indicated that Glu298Asp is the predisposing factor in ESRD, especially DM-derived ESRD. The functional difference in NO generation depending on eNOS with either glutamate or aspartate at position 298 was also confirmed in vitro. (Hypertension. 2002;40:535-540.)

Key Words: polymorphism • polymerase chain reaction • nitric oxide synthase • diabetes mellitus

The endothelial isoform of nitric oxide (NO) synthase (eNOS, NOSIII) is a constitutively expressed 135 kDa protein predominantly associated with the particulated specific structures in the plasmalemmal membrane, caveolae, of vascular endothelial cells.1,2 NO is produced from l-arginine and diffuses to vascular smooth muscle cells, where it increases the concentration of cGMP by stimulating soluble guanylate cyclase, leading to vascular relaxation. It also inhibits platelet and/or leukocyte adhesion to vascular endothelium. Therefore, the impairment of eNOS expression has been considered a primary factor for diseases such as hypertension, coronary artery disease, thromboembolic diseases, and atherosclerosis. Indeed, knocking out the gene encoding eNOS in mice resulted in significant hypertension, and aortic rings from these animals studied ex vivo displayed no relaxation in response to acetylcholine.3 Patients with end-stage renal disease (ESRD) derived from diabetes mellitus (DM) nephropathy have a higher prevalence of cardiovascular complications than those with non-DM ESRD, and this limits their 5-year survival rate to less than 50%. Therefore, polymorphism in eNOS is considered one of the major predisposing factors for endothelial dysfunction.

The gene polymorphisms of eNOS have been detected at 4b/4a variable number of tandem repeats in intron 4, Glu298Asp in exon 7, CA repeat in intron 13, A27 to C (A to C nucleotide conversion) in intron 18, and G10 to T in intron 23. A part of candidate variations (4b/4a tandem repeats in intron 4) has been investigated in glomerulonephritis and/or ESRD patients, but no conclusive results have been obtained so far. Recently, the G/T polymorphism in exon 7 coding for Glu298Asp was detected and reported to have a remarkable association with coronary spasm, acute myocardial infarction, and hypertension.4 To date, this is the only known eNOS polymorphism associated with an altered protein sequence, though recent expression studies have demonstrated no functional difference between 298Glu and 298Asp despite the accumulating clinical evidence.5,6

Received January 28, 2002; first decision February 19, 2002; Revision accepted August 2, 2002.
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Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000033974.57407.82
Therefore, the purpose of the present study was to elucidate the Glu298Asp polymorphism in ESRD patients, to determine its role as a predisposing factor in the disease process, and to elucidate any alteration of eNOS enzymatic activity caused by this polymorphism.

Methods

Study Population
The Glu298Asp polymorphism in exon 7 of the human eNOS (heNOS) gene was determined in 185 patients with ESRD who were maintained on hemodialysis at 2 suburban dialysis centers in Tokyo, Japan, from 1996 to 1998. This polymorphism was then compared with that of 304 unrelated healthy individuals (control) who visited the urban health center in Tokyo, Japan, for routine health check-ups from 1997 to 1998. The average length of time from the onset of ESRD to the time of this study was 6.3±5.4 (mean±SD) years. All ESRD patients had creatinine clearance of <10 mL/min and were treated 3 times a week with hemodialysis for 4 to 5 hours. In the ESRD group, 72 patients had type 2 DM. The remaining patients had chronic glomerulonephritis (n=71; 3 of whom had previous renal transplantation), hypertensive nephrosclerosis (n=22), polycystic kidney disease (n=9), and miscellaneous conditions (n=11). There were statistical differences between the 2 groups with respect to age (71.7±12.3 years in the ESRD group versus 56.7±10.2 years in the control group, mean±SD, P<0.01) and gender (male-to-female ratio, 136:49 in ESRD versus 195:110 in control, P<0.01). In addition, in the ESRD group, the clinical data (eg, serum creatinine, serum urea nitrogen, serum uric acid, serum phosphate, serum potassium) reflected chronic renal failure, and SV1 + SV2 on the ECG was also significantly higher than that of the control group (P<0.0001).

At the time of recruitment for this study, informed consent was obtained from each individual according to the protocol approved by the Human Study Committee of the University of Tokyo.

Genomic DNA
Genomic DNA from each subject in the study population was purified from peripheral blood leukocytes by SepaGene (Sanko-Junyaku) following the manufacturer’s protocol.

Genotyping
The detection of Glu298Asp polymorphism of the heNOS gene was performed following the previously reported protocol with minor modifications (detailed in an online supplement available at http://www.hypertensionaha.org). The typical pattern of electrophoresis was demonstrated in Figure 1A. All fragments uncleaved were double-checked by direct sequencing to avoid mistyping (Figure 1B).

The variable number of tandem repeats (VNTRs) in intron 4 of the eNOS gene (eNOS4a/b) was determined as previously described by Wang et al.9

Direct Sequencing
Polymerase chain reaction (PCR) products were amplified, and both sense and antisense strands were directly sequenced using the same primer pairs as those for genotyping. Fluorescence-based automated cycle sequencing of PCR products was performed on ABI PRISM 310 (Perkin-Elmer) using a dye-terminator method according to the manufacturer’s instructions (dRhodamine Terminator Cycle Sequencing Kit; Perkin-Elmer).

Plasmid Constructs
Human wild-type–eNOS cDNA ligated into pcDNA3.1 expression vector (pcDNA3.1-WT-heNOS) (Invitrogen, Groningen, Netherlands) was constructed using a standard molecular protocol by Y.O. and S.N. The cDNA construct containing the 298Asp substitution was generated by site-directed mutagenesis using an LA PCR in vitro mutagenesis kit (Takara) and oligonucleotides, 5’-GCA GCC CCC AGA TGA TCC CCC AGA ACT CTT CC-3’, according to the manufacturer’s instructions (detailed in the online supplement). The presence of the substitution and the entire sequence of the inserted fragment were verified by DNA sequencing.

Cell Culture and Stable Transfection
Chinese hamster ovary (CHO) cells were obtained from Riken Cell Bank (Wako) and grown in Ham F12 (Sigma) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco).

CHO-, wild-type– and mutant-type–heNOS cell clones were established by stable transfection with zeocin selectable pcDNA3.1 plasmid (Invitrogen). Transfection was performed using lipofectamine-plus (Gibco) according to the manufacturer’s instructions. Three days after transfection, cells were placed into the selection medium containing 1 μg/mL zeocin (Invitrogen). On day 14 after selection, individual zeocin-resistant colonies were subcloned.

Western and Southern Analysis
CHO cells (1×10^7) were dissolved in the ice-cold extraction buffer and standard Western analysis was performed using monoclonal antibodies to heNOS (Transduction Laboratory) (detailed in the online supplement).

Genomic DNA of CHO cells was isolated by the Wizard genomic DNA purification kit (Promega) following the manufacturer’s protocol. Seven micrograms of genomic DNA was digested with one Hind III; Takara) or no (ClaI, Takara) recognition site within the zeocin-selectable pcDNA3.1-heNOS plasmid. After electrophoresis on 1% agarose gel, transfer to nylon membrane (Hybond) was performed using SouthernMax reagents (Ambion) following the...
Fluorometric Nitrite Analysis

Nitrite production by CHO cells was measured by the fluorometric nitrite assay,9 2,3-diaminonaphtalene kit (Dojindo), which forms a fluorescent product of 1H-naphthotriazole reacting with nitrite. Fluorometric assay is about 50 to 100 times more sensitive than the Griess reaction, as shown in Figure 2A. Cells were seeded at 2 × 10^4 per well and grown in 12-well plates (Corning) in DMEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, 1× penicillin/streptomycin, and 1× nonessential amino acids (Invitrogen). One day after cell passage, A23187 at the concentration of 5 μM was added and incubated for 15 minutes in a regular CO2 incubator, and 150 μL of the incubation medium was withdrawn for determination of nitrite concentration. Simultaneously, the nitrite concentration was determined using a calibration curve with NaNO2 as the standard. The aliquot was centrifuged once at 9000g for 1 minute at 4°C, and 2,3-diaminonaphthalene was added to both the supernatant (100 μL) and the standard. After the alkalization by NaOH and a 15-minute incubation at room temperature, all the aliquot was transferred to black 96-well glass bottom plates (Iwaki). The fluorescence was measured at the excitation of 365 nm and the emission of 450 nm using a fluorescent plate reader (f-max; Molecular Devices). Results were presented as relative fluorescence units (RFUs) and converted to micromoles using a standard curve.

NO Monitoring

NO release was monitored by the NO selective electrochemical sensor following our previous reports.10,11 The change in NO level was monitored before and after the addition of A23187 at the concentration of 5 μg/mL.

Statistical Analysis

The statistical difference in genotype distribution between the groups was examined by the Mantel-Haenszel test, considering confounding factors (eg, age and gender) between groups, the stratification of which is shown in Tables 1 to 3. Where appropriate, the common odds ratios (ORs) were calculated. Standard error by Robins-Greenland-Breslow, 95% confidential interval (CI), and probability of a and indicate each allele of the polymorphism; SE, standard error; CI, confidence interval.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n=304)</th>
<th>ESRD (n=185)</th>
<th>Control (n=304)</th>
<th>ESRD (n=185)</th>
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<tbody>
<tr>
<td>y&lt;50</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>23</td>
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<tr>
<td>OR^4</td>
<td>1.72</td>
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</table>

Results

Association Between ESRD and Polymorphism of the eNOS Gene

The frequency of Glu298Asp (G1917T) in 185 ESRD patients and in 304 healthy individuals (controls) was compared (Table 1). The distribution of genotypes in the control group did not differ significantly from that expected under Hardy-Weinberg equilibrium. The positivity of 298Asp was significantly higher in the ESRD patients (P=0.0020). The OR for the ESRD group was 1.65 (95% CI: 1.21 to 2.25). On the other hand, the positivity of eNOS4a allele in the ESRD was not statistically different from the control group (P=0.187).

Association Between ESRD From Type 2 DM and Polymorphism of the eNOS Gene

In the ESRD group, 72 patients had ESRD resulting from type 2 DM nephropathy. The frequency of Glu298Asp in this subgroup was compared with that of controls (Table 2). The
positivity of 298Asp was significantly higher in the patients with DM-derived ESRD (P = 0.0010), and its OR was 2.02 (95% CI: 1.34 to 3.07). In contrast, there was no significant effect of this genotype on the incidence of ESRD without DM (Table 3).

Expression of heNOS in Transfected CHO Cells
To examine whether heNOS with glutamate or aspartate at position 298 influenced the level of NO production, CHO cells were stably transfected with 2 eukaryotic expression vector constructs, heNOS 1917G (W1, W2, W3, W6 in Figure 3) and 1917T (M2, M3, M6, M9), resulting in heNOS 298 glutamic acid and aspartic acid, respectively. Western blot analyses of heNOS in CHO 1917G and CHO 1917T are shown in Figure 3. No difference in heNOS protein expression was found among cells. Copy numbers of each construct were further examined by Southern analysis (data not shown), which confirmed Western analysis results.

Functional Analysis of heNOS With or Without heNOS Polymorphism
Calcium ionophore, A23187, initiated NO production by heNOS. This NO production was measured by both NO-selective electrode and fluorescent analysis. As shown in Figure 4, NO production from CHO 1917G detected by the NO-selective electrode was 188 ± 18.8 nmol/L (n = 10; mean ± SE) compared with CHO 1917T of 56.1 ± 9.4 nmol/L (n = 9; P < 0.01). Fluorescent nitrite detections from CHO 1917G or 1917T of 2 × 10^5 cell population are shown as RFUs in Figure 2B, which was calculated as 4.33 ± 0.030 μmol/L and 3.89 ± 0.20 μmol/L (mean ± SE; P < 0.01), respectively, using a standard curve (Figure 2A).

Discussion
Endothelial dysfunction and premature atherosclerosis may have potential genetic factors that could accelerate to ESRD in chronic glomerulonephritis and DM nephropathy. The Glu298Asp polymorphism of heNOS needed to be evaluated to determine whether it is a major candidate gene for progressive renal disease, because significant accumulation of the Glu298Asp mutation in coronary artery disease,12 acute

TABLE 2. Distribution of Genotype in Patients With ESRD Resulting From Type 2 DM and in Healthy Controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n = 304)</th>
<th>ESRD From Type 2 DM (n = 72)</th>
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<td>Glu/Glu, n</td>
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<td>4</td>
</tr>
<tr>
<td>Glu/Asp + Asp/Asp, n</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>50 ≤ y &lt; 60</td>
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<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>92</td>
<td>13</td>
</tr>
<tr>
<td>Glu/Asp + Asp/Asp, n</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>60 ≤ y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>95</td>
<td>32</td>
</tr>
<tr>
<td>Glu/Asp + Asp/Asp, n</td>
<td>30</td>
<td>18</td>
</tr>
</tbody>
</table>

Female
| Genotype       |                  |                             |
| Glu/Glu, n     | 92               | 3                           |
| Glu/Asp + Asp/Asp, n | 18 | 8                           |

OR* 13.6

Mantel-Haenszel OR 2.02

SE by Robins-Greenland-Breslow

95% CI

Lower limit 1.34

Upper limit 3.07

Significance, P 0.001

*OR that met only the criteria satisfying both P < 0.05 and CI lower limit of >1.0 is shown here.

TABLE 3. Distribution of Genotype in Patients with Non-DM ESRD and in Healthy Controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n = 304)</th>
<th>Non-DM ESRD (n = 113)</th>
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<tr>
<td>Glu/Glu, n</td>
<td>64</td>
<td>20</td>
</tr>
<tr>
<td>Glu/Asp + Asp/Asp, n</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>OR*</td>
<td>5.33</td>
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<tr>
<td>50 ≤ y &lt; 60</td>
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<tr>
<td>Glu/Glu, n</td>
<td>92</td>
<td>21</td>
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<tr>
<td>Glu/Asp + Asp/Asp, n</td>
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<tr>
<td>Glu/Glu, n</td>
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<td>46</td>
</tr>
<tr>
<td>Glu/Asp + Asp/Asp, n</td>
<td>30</td>
<td>11</td>
</tr>
</tbody>
</table>

Female
| Genotype       |                  |                       |
| Glu/Glu, n     | 92               | 29                    |
| Glu/Asp + Asp/Asp, n | 18 | 9                  |

Male
| Genotype       |                  |                       |
| Glu/Glu, n     | 159              | 58                    |
| Glu/Asp + Asp/Asp, n | 35 | 17                  |

Mantel-Haenszel OR 1.39

SE by Robins-Greenland-Breslow

95% CI

Lower limit 0.95

Upper limit 2.01

Significance, P 0.1035

*OR that met only the criteria satisfying both P < 0.05 and CI lower limit of >1.0 is shown here.
myocardial infarction,4 essential hypertension,5 carotid atherosclerosis,13 and preeclampsia14 have recently been reported. Because Glu298Asp missense was found in exon 7, the functional relevance in altering endothelial vasodilatation was expected and indirectly examined. Philip et al15 injected the alpha-adrenergic agonist, phenylephrine, into patients undergoing cardiac surgery and found the enhanced responsiveness to alpha-adrenergic stimulation in patients with the 894T allele. Therefore, we examined the involvement of Glu298Asp missense in susceptibility to ESRD and constructed CHO cells stably overexpressing either 1917G or 1917T to elucidate functional consequences directly.

The relevance of polymorphism in heNOS to type 2 DM is expected after the recent analysis of linkage of type 2 DM nephropathy with the heNOS region on chromosome 7q in Pima Indians.16 The deletion of 1 of 5 nucleotide repeats in intron 4 of heNOS may be the best-studied candidate gene for diabetic nephropathy in both type 1 and type 2 DM. The level of mRNA and protein of heNOS between normal and variant alleles is equivocal because the intron may be excised during RNA processing. Therefore, it is expected that the mutation within introns could affect rates of heNOS transcription and/or processing rate, which may affect heNOS enzymatic activity. The significant association of this variation in intron 4 with diabetic nephropathy has recently been observed in both type 1 and type 2 diabetic nephropathy.17,18 though Glu298Asp did not reach a statistically significant level in type 1 diabetic nephropathy.17 The deletion of 1 of 5 nucleotide repeats in intron 4 was also not statistically significant in type 2 diabetic nephropathy with proliferative diabetic retinopathy.18,19 It was also insignificant in hypertension5,18 and ischemic heart disease in the Japanese population.18 In addition, Miyamoto et al15 addressed the significant linkage disequilibrium between eNOS4a/b and G to T conversion in intron 23, which is the polymorphism not related to essential hypertension as reported by Bonnardreux et al.20 No significant linkage disequilibrium between eNOS4a/b and Glu298Asp (P=0.773, χ²=0.083), as well as no significant association of eNOS4a allele with ESRD, was observed in the present study.

The present study, in which Glu298Asp polymorphism in ESRD and type 2 diabetic nephropathy associated with it was found for the first time, demonstrated a significant increase of 298Asp in ESRD patients. It also revealed significant accumulation of this mutation predominantly in patients with ESRD resulting from type 2 DM, compared with patients with non-DM ESRD. These observations point to 298Asp not only as the candidate gene of progressive renal disease, but also as a possible prognostic factor in patients with DM-derived ESRD, because the 5-year survival rate of these patients is significantly shorter than that of patients with non-DM ESRD.21 One of the major reasons for this poor prognosis is that the prevalence of atherosclerotic complicating diseases, such as cerebrovascular accidents, coronary artery disease, and peripheral vascular disease, is much higher in the DM-ESRD population.22,23 Lesser NO production in 298Asp individuals may increase the proinflammatory leukocyte adhesion to endothelial cells and may decrease the vasodilatory capability, which could further exacerbate atherosclerotic lesions in these DM-ESRD patients with 298Asp.

The 3D representation of heNOS dimer demonstrates position 298 of amino acid at the exterior of the dimer surface, which apparently is not close to the catalytic site or the cofactor binding domains of heNOS.24 One possible explanation for its location may relate to the modification of protein-protein interaction, especially with caveolae, though the specific binding interaction of heNOS with endothelial membrane structures is not well defined. Another possible mechanism of functional alteration may be the stability of the heNOS enzyme and/or interactions with other parts of heNOS activity. Tesauro et al6 and Fairchild et al7 recently investigated the difference in NO generation between heNOS with glutamate and with aspartate at position 298, using a transient expression system of COS7 cells, and found no difference. However, it was reported that 298Asp was more vulnerable to enzymatic cleavage in cell lysates compared with Glu298 protein.6 The discrepancy in findings between those data and ours could be attributed to the differences in copy numbers expressed in cells and the proper expression of proteins at high levels, which are difficult to adjust in a transient transfection system,25 as well as to the sensitivity of methods used to measure NO generation. Because in our study the CHO cells with mutation showed a lower level of NO generation, it would be reasonable to compare NO production after performing both the adjustment of copy numbers and the confirmation of heNOS protein levels.

In the present study, CHO cells with 298Asp showed a measurable amount of NO, which suggests that this polymorphism does not impair NO production in an all-or-nothing manner. However, the graded differences in NO generation could play a much more critical role in the pathophysiological condition at the microvascular level. Conservation of the vasodilatory potential in eNOS-deficiency has been attributed to the upregulation of other NOS isoforms and the induction of vasodilatory prostaglandins or endothelium-derived hyperpolarizing factor.26,27 However, 298Asp mutation does not produce a phenotype of null mutation and, therefore, should have a lesser effect on other vasodilatory mediators.

**Perspectives**

The observed accumulation of 298Asp, especially in patients with DM as a cause of ESRD, suggests that this polymorphism is the candidate factor for the accelerated nephrop-
athy in type 2 DM. In addition, the accumulation of 298Asp may contribute to the poor prognosis for the 5-year survival rate in DM-ESRD patients compared with that of other ESRD patients. This inference is logical, given that, in Japan, the principal life-threatening complication of type 2 DM in ESRD is ischemic heart disease, which has been remarkably associated with 298Asp. This mutation is becoming more important because DM nephropathy has become the principal cause of the initiation of elective hemodialysis in Japan. To elucidate this possibility, a prospective study based on hNOS polymorphism is needed. In future studies, it is also certainly necessary to investigate the association of the current polymorphism with other variants and/or a specific haplotype, which should provide a more comprehensive analysis of the eNOS gene. [Author: McMillan et al was not cited in the article.]

Acknowledgments

The authors are grateful to Dr Michael S. Goligorsky (M.S.G.) for his critical reading of the manuscript. These studies were supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (E.N., K.T.), Jinsei-Hinketsu Kenkyukai Foundation (Japan: E.N., A.N.), and Takeda Science Foundation (Japan: E.N.), as well as by grant support from M.S.G. (S.V.B. is a fellow of the American Heart Association.)

References


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Hypertension. 2002;40:535-540; originally published online September 3, 2002;
doi: 10.1161/01.HYP.0000033974.57407.82
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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