Polymorphism in Endothelin-Related Genes Limits Exercise-Induced Decreases in Arterial Stiffness in Older Subjects

Motoyuki Iemitsu, Seiji Maeda, Takeshi Otsuki, Jun Sugawara, Takumi Tanabe, Subrina Jesmin, Shinya Kuno, Ryuichi Ajisaka, Takashi Miyauchi, Mitsuo Matsuda

Abstract—Increase in arterial stiffness is associated with aging, which is improved by regular exercise. Endothelin (ET) system has crucial roles in regulating vascular tone and in the progression of atherosclerosis. We hypothesized that molecular variations (ie, gene polymorphisms) in ET-related gene might affect exercise-induced improvement in arterial stiffness with age in human subjects. The present study provides a cross-sectional investigation of 191 healthy middle-aged and older (65±1 years) human subjects to clarify the relationship between the regular exercise-induced improvement of arterial stiffness and the gene polymorphisms of ET converting enzyme (ECE)-1, ECE-2, ET-A receptor (ET-A), and ET-B receptor (ET-B). The study subjects were divided into active and inactive groups based on the median value (186 kcal/d) of energy expenditure. Brachial-ankle arterial pulse wave velocity (baPWV) was used to evaluate arterial stiffness. All individuals were genotyped for 4 different polymorphisms of the ET system: 2013(−289)A/G in intron 17 of ECE-1, 669(−17)T/C in intron 5 of ECE-2, 958A/G in exon 6 of ET-A, and 831A/G in exon 4 of ET-B. The baseline baPWV was significantly lower in the active group without any change in blood pressure. Polymorphisms in ECE-1 influenced basal blood pressure. Polymorphisms in ECE-1 and ECE-2 had no effect on baPWV between active and inactive groups. However, polymorphisms in both ET-A and ET-B affected baPWV in the 2 groups. The present results suggest that differences in ET-A and ET-B polymorphisms may influence the response of the vascular wall to exercise whereas ET-1 polymorphisms may affect basal blood pressure. (Hypertension. 2006; 47:928-936.)

Key Words: aging ■ arteries ■ endothelin ■ exercise ■ polymorphism

The aorta and large arteries (ie, central arteries) are conduits delivering blood to the tissues and organs, and the central arteries act as mechanical buffers to normalize fluctuations in blood pressure created by cardiac pulsation and intermittent blood flow. Increased arterial stiffness impairs this buffering function, leading to increases in systolic blood pressure (SBP) and left ventricular afterload. Blacher et al1 and Laurent et al2 proposed that arterial stiffness may be an independent risk factor for mortality and cardiovascular disorder. Arterial stiffness increases with age3,4 and is associated with the development of several pathological conditions, including hypertension, atherosclerosis, congestive heart failure, stroke, and aortic root regurgitation.1,2,5,6 Several studies reported that arterial stiffness is less in physically active individuals compared with sedentary individuals.4,7,8 Furthermore, aerobic exercise training causes a reduction of arterial stiffness.8–10 Thus, regular exercise prevents or improves age-associated increase in arterial stiffness.

Endothelin (ET)-1, a peptide produced by vascular endothelial cells, is a potent vasoconstrictor that acts as a modulator of vasomotor tone, cell proliferation, and vascular remodeling.11–13 The biological actions of ET-1 are mediated by at least 2 different receptors: ET-A receptor (ET-A) and ET-B receptor (ET-B). The interaction of ET-1 with ET-A in vascular smooth muscle cells is primarily responsible for ET-1–mediated vasoconstriction, whereas endothelial cell expressing ET-B promotes vasodilation.14–16 ET-converting enzyme (ECE)-1 and ECE-2 are responsible for generating active ET-1.13

The ET-1 system has been implicated in the regulation and development of arterial stiffness.17,18 Our previous study demonstrated a reduction of aging-induced increase in plasma ET-1 concentration by aerobic exercise training in older humans.19 Thus, the exercise-mediated alteration in ET-1 may have a role in the physiological mechanism responsible for exercise-induced improvements in arterial stiffness. Although regular exercise is beneficial for the
cardiovascular system, not all individuals may benefit equally from exercise. Recently, it has been reported that variations in the nucleotide sequence of both the coding and noncoding regions of genes in the different components of ET-1 system are associated with differential cardiovascular function (e.g., blood pressure).20–23 However, the relationship between molecular variations in the nucleotide sequence of genes for ET-1 system and the regular exercise-induced arterial adaptation remains unclear. Because ET-1 system is implicated in the regulation and adaptation of arterial stiffness, we hypothesized that molecular variation in the nucleotide sequence of genes for ET-1 system might affect the exercise-induced reduction in arterial stiffness associated with age in human subjects. Thus, we examined whether polymorphisms in the ECE, ET-A, and ET-B genes, important genes for ET-1 system, affected the degree of change in arterial stiffness following exercise in middle-aged and older human subjects.

In the present study, we tested our hypothesis by assessing the daily physical activity, arterial stiffness, and genotypes of single-nucleotide polymorphisms (SNPs) of 2013(+/−289)A/G in intron 17 of ECE-1 on chromosome 1, 669(+/−17)T/C in intron 5 of ECE-2 on chromosome 3, 958A/G in exon 6 of ET-A on chromosome 4, and 831A/G in exon 4 of ET-B on chromosome 13 in healthy middle-aged and older Japanese subjects. With an aim to construct an infrastructure for genome-wide association studies of common diseases or drug sensitivities in Japanese individuals, recently a vast number of genetic variations in polymorphism have been reported in the Japanese Single Nucleotide Polymorphisms (JSNP) database,24,25 which constitutes a potent infrastructure for the next step toward personalized medicine (i.e., whole-genome association studies of common diseases or drug sensitivities). The SNPs used in the present study were picked up from the JSNP public database. In addition, the polymorphisms of ET-A and ET-B studied in the present study have already been investigated in relation to blood pressure and myocardial infarction.23 We measured brachial-ankle arterial pulse wave velocity (baPWV) as index of arterial stiffness.26 Finally, the plasma ET-1 level was determined in different study groups.

Methods

An extended methods section can be found in an online supplement available at http://www.hypertensionaha.org.

Subjects

One hundred and ninety-one middle-aged and older Japanese subjects (70 males and 121 females) aged 51–78 years participated in this cross-sectional study (65±1 years). None of the participants had a history of smoking, and none were currently taking medications. All subjects were free from the signs and symptoms of any overt chronic diseases. Daily physical activity, baPWV, SBP, diastolic

TABLE 1. Characteristics of Subjects in the Active and Inactive Groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Active</th>
<th>Inactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>104</td>
<td>87</td>
</tr>
<tr>
<td>Age, years</td>
<td>63±1*</td>
<td>66±1</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>58±1*</td>
<td>55±1</td>
</tr>
<tr>
<td>Height, cm</td>
<td>157±1*</td>
<td>154±1</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.1±0.3</td>
<td>23.9±0.3</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>60±1*</td>
<td>64±1</td>
</tr>
<tr>
<td>Daily physical activity, kcal/day</td>
<td>352±15*</td>
<td>141±4</td>
</tr>
</tbody>
</table>

Values are means±SE.
*P<0.05 vs inactive.
blood pressure (DBP), heart rate (HR), polymorphisms in ET-related genes, and plasma ET-1 level were determined in all subjects. Serum levels of cholesterol, triglycerides, and insulin and plasma glucose level were also measured.

The study was approved by the Ethical Committees of the Institute of Health and Sport Sciences and the Institute of Clinical Medicine of the University of Tsukuba. The study conformed to the principles outlined in the Helsinki Declaration, and written informed consent was obtained from all subjects before inclusion in the study.

Measurement of Daily Physical Activity

Daily physical activity was measured using a uniaxial accelerometer (Life-Corder, Suzuken Co., Nagoya, Japan) as described in previous studies with minor modifications. All subjects wore the uniaxial accelerometer on the waist continuously for 14 days, except for sleeping and bathing, and data from a continuous 7-day period were used to assess physical activity. The total energy expenditure (kcal) was calculated as the sum of the energy measured by both the uniaxial accelerometer and the questionnaire of activities with the Compendium of Physical Activity. Subjects were divided into inactive lifestyle and active lifestyle groups, with the dividing line set at the median value (186 kcal/d) of mean energy expenditure per day.

Measurement of Arterial Pulse Wave Velocity

baPWV (using formPWV/ABI; Colin Medical Technology, Komaki, Japan) was measured as previously described with minor modifications. The value of baPWV mainly reflects stiffness in the central arteries, because baPWV correlates well with the aortic pulse wave velocity (PWV) using a catheter tip with pressure manometer.

SNP Genotyping

Genomic DNA was extracted from plasma buffy coats and buccal cells using the QIAamp DNA Blood Maxi Kit (QIAGEN, Tokyo, Japan). ECE-1, ECE-2, ET-A, and ET-B SNP genotypes were determined using real-time polymerase chain reaction (PCR) with TaqMan probes using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster, CA) as previously described with minor modification. The gene-specific primers and TaqMan probes for each SNP were synthesized using Primer Express version 1.5 software (Perkin-Elmer) according to the published DNA sequences for each SNPs as follows: 2013(A/G) in intron 17 of ECE-1 (NCBI accession #rs212528), 669(T/C) in intron 5 of ECE-2 (NCBI accession #rs2272471), 958A/G in exon 6 of ET-A (NCBI accession #rs5333), and 831A/G in exon 4 of ET-B (NCBI accession #rs5351). The sequences of the oligonucleotides were as follows:

- ECE-1 forward: 5'-GGCTGACTTGGTGTTAGCTTGTT-3'
- ECE-1 reverse: 5'-GGGCTTTCATTCCGCTTGA-3'
- ECE-1/A probe: 5'-TCCTGAGAGATGAGAAGGAA-3'
- ECE-1/G probe: 5'-TCCTGAGAGATGAGAAGGAA-3'
- ECE-2 forward: 5'-ACTGAGAGATCATTCTTTTGGTTGAGAAGGTA-3'
- ECE-2 reverse: 5'-GGGCCATTCTCCTCTCCTTGAAG-3'
- ECE-2/T probe: 5'-ACTGAGAGATCATTCTTTTGGTTGAGAAGGTA-3'
- ECE-2/C probe: 5'-ACTGAGAGATCATTCTTTTGGTTGAGAAGGTA-3'
- ET-A forward: 5'-ACTGAGAGATCATTCTTTTGGTTGAGAAGGTA-3'
- ET-A reverse: 5'-CATTGAGCCGCGTGGTTGAGAAGGTA-3'
- ET-A/A probe: 5'-ACTGAGAGATCATTCTTTTGGTTGAGAAGGTA-3'
- ET-A/C probe: 5'-ACTGAGAGATCATTCTTTTGGTTGAGAAGGTA-3'

Figure 2. Fasting serum concentrations of cholesterol (total, A; HDL, C; and LDL, D), triglycerides (B), and insulin (E), and plasma concentrations of glucose (F) in active subjects and inactive subjects. Data are expressed as mean±SE.
ET-A/G probe: 5'-TACGGCTTAAGTGAAAGA-3'
ET-B forward: 5'-CTCATCCCTATAGTTTTACAAGACAGC-3'
ET-B reverse: 5'-GCCAATGGCAAGCAGAAATGAA-3'
ET-B/A probe: 5'-TCTGGTGCTATTCGTT-3'
ET-B/G probe: 5'-ATTGGTGCTGGTACAGT-3'

PCR 96-well plates were read on an ABI-7700 with end point analysis mode of the SDS v1.7a software package (Perkin-Elmer Applied Biosystems). Genotypes were determined automatically by the single-processing algorithms in the software.

Statistical Analysis
The allelic frequencies of ECE-1, ECE-2, ET-A, and ET-B were calculated using a gene-counting method, and Hardy-Weinberg equilibrium was confirmed using a χ² test. To evaluate differences between active and inactive groups or the different genotype groups, a Student t test for unpaired values was used. Furthermore, the comparisons of PWV between the genotype groups in each active and inactive group were assessed by covariance analysis (ANCOVA) model that included age, SBP, and sex as covariates. Values are expressed as mean±SE. P<0.05 was accepted as significant.

Results
Comparison of Characteristics in Low and High Physical Activity Groups
The total energy expenditure of the active group was significantly greater than that of the inactive group (Table 1). Additionally, the baPWV of the active group was significantly lower than that of the inactive group (Figure 1), indicating that arterial stiffness in the active group was lower. There were no significant differences in body mass index (BMI), SBP, DBP, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), glucose, and insulin between the active and inactive groups (Table 1 and Figures 1 and 2). Triglycerides and HR were significantly lower in the active group than in the inactive group (Table 1 and Figures 1 and 2). There were no significant differences in daily physical activity, height, body weight, BMI, baPWV, total cholesterol, HDL, LDL, triglycerides, and glucose between these groups (Figure 3 and Table 3). In the genotypes of 669(+17)T/C in intron 5 of ECE-2, there were no significant differences in any parameters seen between the TT and TC+CC groups (Figure 3 and Table 3). This was also the case with the 958A/G polymorphism in exon of ET-A and the 831A/G polymorphism in exon of ET-B (Figure 3 and Table 3).

Comparison of PWV Between Genotypes and Activity Groups
No significant differences were seen in baPWV between the active or inactive groups with either the ECE-1 2013(+289)A/G polymorphism or the ECE-2 669(+17)T/C of ECE-2 polymorphism (Figure 4A and 4B). However, the baPWV of subjects with the AA genotype of 958A/G of ET-A was significantly lower in the active group compared with the inactive group, whereas no significant differences were seen in baPWV of subjects with the AG+GG genotype (Figure 4C). Additionally, the baPWV of the individuals with the AG+GG genotype of 831A/G of ET-B was significantly lower in the active group compared with the inactive group, whereas there was no significant difference in the baPWV for individuals with the AA genotype regardless of activity (Figure 4D).
Plasma ET-1 Level
Finally, to explore if there was any relation between the arterial stiffness observed in the present study and the plasma ET-1 level, we performed regression analyses between the baPWV and the plasma ET-1 level of subjects with different gene polymorphisms. There were positive and significant correlations between baPWV and plasma ET-1 level in all the study subjects (See Figure I, available online). As shown in Figure 5, there were positive and significant correlations between baPWV and plasma ET-1 level of the individuals with the ECE-1 2013(+289)A/G polymorphism, ECE-2 669(+17)T/C of ECE-2 polymorphism, or the ET-B 831A/G polymorphism (Figure 5A-D, 5G, and 5H). In the genotypes of 958A/G of ET-A, there was positive and significant correlation between baPWV and plasma ET-1 level of the individuals with the AA genotype (Figure 5E), whereas no significant correlations were observed with the AG/GG genotype (Figure 5F).

Discussion
The present study investigated the relationship between arterial stiffness, physical activity, and polymorphisms in genes related to ET system in middle-aged and older human subjects. Interestingly, using baPWV as a measure of arterial stiffness, increased physical activity by individuals with the AA genotype at 958A/G of ET-A and the AG+GG genotype at 831A/G of ET-B led to decreased arterial stiffness, but in individuals with the opposite genotype (eg, AG+GG or AA, respectively), physical activity did not affect arterial stiffness. Additionally, the polymorphisms examined in ECE-1 and ECE-2 were not related to the changes in arterial stiffness with physical activity. Thus, genetic polymorphisms in components of the ET-1 system may cause differential response of age-related arterial stiffness to physical activity.

Arterial infusion of exogenous ET-1 increased PWV, and infusion of BQ-123, a selective ET-A antagonist, decreased PWV in vivo. Furthermore, Vuurmans et al reported that infusion of VML 588, an ET receptor antagonist with higher affinity for ET-A than ET-B, decreased both PWV and blood pressure in humans. Taken together, these data suggest that events downstream of ET-A are associated with increased arterial stiffness. In the present study, regular exercise im-
proved the age-related increase in arterial stiffness of the individuals with the AA genotype of 958A/G of ET-A. The mechanism by which the polymorphism in ET-A identified in this study affects the ability of exercise to decrease arterial stiffness remains unclear, but it further supports the importance of ET-A in modulating endothelial function. On the contrary, in the present study, arterial distensibility following exercise has been shown to be increased in subjects with the AG+GG genotype in ET-B, but not in the AA genotype. Thus, ET-B might have a role in the reduction of age-related arterial stiffness in humans with active exercise.

In the present study, the polymorphism of 2013(+289)A/G of ECE-1 did not affect the individual difference of arterial stiffness although it had an association with the basal SBP, DBP, and HR. The observed association of the polymorphism of ECE-1 with the blood pressure was supported by the other reports showing a relationship between blood pressure or hypertension and Lys198Asn of ET-1, −338C/A of ECE1B, or −231A/G of ET-A. Collectively, the finding in the present study together with the other observations suggest that the polymorphism of the ECE-1 may be one of the molecular variations of ET-1 system, which affects the basal hemodynamic parameters. Thus, while the polymorphism of ECE-1 seems to be important in regulating cardiovascular parameters, the involvement of the polymorphism of ECE-2 remains to be investigated. Accordingly, the present study

### Table 3. Genotypes of ECE-1, ECE-2, ET-A, and ET-B Genes and Characteristics of the Subjects

<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
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<td>No. of subjects</td>
<td>AA 113</td>
<td>78</td>
<td>97</td>
<td>68</td>
</tr>
<tr>
<td>Age, years</td>
<td>Age 64±1</td>
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<td>65±1</td>
<td>64±1</td>
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<tr>
<td>Body weight, kg</td>
<td>56±1</td>
<td>56±1</td>
<td>56±1</td>
<td>56±1</td>
</tr>
<tr>
<td>Height, cm</td>
<td>156±1</td>
<td>156±1</td>
<td>156±1</td>
<td>156±1</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.0±0.3</td>
<td>24.1±0.3</td>
<td>24.0±0.2</td>
<td>24.0±0.3</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>60±1</td>
<td>64±1</td>
<td>61±1</td>
<td>61±1</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>200±4</td>
<td>193±7</td>
<td>190±7</td>
<td>198±6</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>59±1</td>
<td>62±2</td>
<td>61±1</td>
<td>61±1</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>129±3</td>
<td>130±4</td>
<td>126±3</td>
<td>125±4</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>96±4</td>
<td>108±8</td>
<td>98±6</td>
<td>101±8</td>
</tr>
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<td>Insulin, μU/mL</td>
<td>5.4±0.2</td>
<td>6.9±0.6*</td>
<td>6.4±0.5</td>
<td>5.9±0.5</td>
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<tr>
<td>Glucose, mg/dL</td>
<td>99±2</td>
<td>100±1</td>
<td>101±2</td>
<td>99±2</td>
</tr>
<tr>
<td>Daily physical activity, kcal/day</td>
<td>272±17</td>
<td>226±15</td>
<td>261±15</td>
<td>260±21</td>
</tr>
</tbody>
</table>

Values are means±SE.

*P<0.05 vs AA of ECE-1 [2013(+289)A/G]
attempted to clarify the association of the polymorphism of ECE-2 [such as 669(+17)T/C of ECE-2] with arterial stiffness and hemodynamic parameters, and no significant association was observed between the polymorphism of ECE-2 and hemodynamic parameters as well as the arterial stiffness.

Aerobic exercise–induced reduction of the age-related increase in arterial stiffness is associated with the improvements in endothelial function.8–10,19,32 In light of this fact, we tried to measure the plasma ET-1 level in the present study subjects. Interestingly, the plasma ET-1 level has shown a positive and significant correlation with the arterial stiffness, and this correlation is independent of the polymorphism sites in case of ECE-1, ECE-2, and ET-B. However, the plasma ET-1 level of the individuals with the AA genotype of 958A/G of ET-A corresponded to the arterial stiffness, but not to the AG+GG genotype. Thus, the effects of polymorphism in the different components of ET-1 system might cause differential changes in the various functional aspects of ET-1 system like the modulation of plasma ET-1 level, differences in binding affinity between ligands and the receptors, internalization and desensitization of the receptors, G protein coupling (downstream of ET-1 signaling), and so on.

The present results provided only an association between arterial stiffness, physical activity, and polymorphism in ET system-related genes, but did not confer cause and effect. Although we measured the plasma ET-1 levels, it is unclear whether the different genotypes result in functional difference of ET system. It would be important to test our hypothesis by inducing different polymorphism in various components of ET system in an animal model mimicking the human older subjects with age-related arterial stiffness and to clarify the functional modulations in the different components of ET system, such as ET activity or function, ET receptor affinities, tissue levels of ET-1, and vascular responses to exogenous ET-1. Thus, the present study cannot shed light on the effects of ET polymorphism in regards to the functional aspects of ET system. Although several studies showed an association between the polymorphisms in preproET-1 gene and the cardiovascular function,20,21 the present study did not address the changes in the polymorphisms of ET-1 gene in relation to aging-induced arterial stiffness. Thus, more genotypes of different components of ET system should be evaluated in the current experimental setting in human
subjects. In addition, the present findings using Japanese subjects cannot at this point exclude the possible differences in polymorphisms of ET receptors and ECE between Japanese versus individuals of European descent subjects. To bring our hypothesis at the stage of clinical implication, a vast number of Japanese subjects as well as the individuals of other races and geographic continents should be investigated.

In conclusion, we investigated the relationship of polymorphisms in ECE-1, ECE-2, ET-A, and ET-B with arterial stiffness and daily physical activity. We identified 958A/G of ET-A and 831A/G of ET-B as gene polymorphisms affecting the improvement of arterial stiffness following daily physical activity. Furthermore, the polymorphisms in 2013(+289)A/G in intron 17 of ECE-1 affected basal SBP, DBP, and HR. However, this polymorphism as well as the 669(+1)T/C polymorphism of ECE-2 did not affect exercise-associated changes in arterial stiffness. We conclude that molecular variations in genes of the ET-1 system affect the differences in regular exercise-induced improvement in arterial stiffness in middle-aged and older subjects.

Perspectives
We examined the relationship between gene polymorphisms in ECE-1, ECE-2, ET-A, and ET-B and the exercise-induced improvement of arterial stiffness with age. From the present findings, increased physical activity in individuals with the AA genotype at 958A/G of ET-A and the AG+GG genotype at 831A/G of ET-B led to decreased arterial stiffness, but in individuals with the opposite genotype, physical activity did not affect arterial stiffness. From the therapeutic and clinical point of views, based on these findings one can suggest that with the advancement of age, physical exercise alone may not be sufficient to improve the arterial stiffness in individuals with the AG+GG genotype of ET-A or the AA genotype of ET-B, and these genotype groups might need additional other therapeutic approaches such as diet, salt restriction, hormone replacement, or drugs. Further studies to estimate precise phenotype (ET function) in the response to exercise by these polymorphisms and the effect of diverse polymorphisms in genes related to ET system are essential. Moreover, to strengthen our hypothesis, future studies should use an intervention protocol, which would add exercise to the sedentary subjects from responding and nonresponding genotypes of ET-A and ET-B. The present results may be only an entry point to explore an important area in the health promotion, development of cardiovascular diseases, and associated mortality with the therapeutic and preventive measures, focusing on the polymorphisms in genes in the different components of ET system because ET acts as a major player in cardiovascular morbidity and mortality.

Acknowledgments
This work was supported by a Grant-in-Aid for Scientific Research (14380008, 16500391, 17700484) and Special Coordination Founds from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Hypertension. 2006;47:928-936; originally published online March 27, 2006; doi: 10.1161/01.HYP.0000217520.44176.73

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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