Exaggeration of Focal Cerebral Ischemia in Transgenic Mice Carrying Human Renin and Human Angiotensinogen Genes

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Background and Purpose—We examined the possibility that activation of the human brain renin–angiotensin system is involved in enhancement of ischemic brain damage using chimeric transgenic mice with human renin (hRN) and human angiotensinogen (hANG) genes.

Methods—Chimeric (hRN/hANG-Tg) mice were generated by mating of hRN and hANG transgenic mice. Permanent occlusion of the middle cerebral artery (MCA) by an intraluminal filament technique induced focal ischemic brain lesions.

Results—hRN/hANG-Tg mice showed higher angiotensin II levels in the plasma and brain. The ischemic brain area at 24 hours after MCA occlusion was significantly enlarged in hRN/hANG-Tg mice with an enhanced neurological deficit compared to that in wild-type, hRN-Tg and hANG-Tg mice. The reduction of cerebral blood flow in the periphery region of the MCA territory after MCA occlusion was markedly exaggerated in hRN/hANG-Tg mice. Superoxide anion production in the brain and arteries was also increased significantly in hRN/hANG-Tg mice even before MCA occlusion and was further enhanced after MCA occlusion. Treatment with an AT1 receptor blocker, valsartan (3.0 mg/kg per day), for 2 weeks significantly reduced the ischemic brain area and improved the neurological deficit after MCA occlusion in hRN/hANG-Tg mice, similar to those in wild-type, hRN-Tg, and hANG-Tg mice, with restoration of cerebral blood flow in the peripheral region and decreases in superoxide anion production and blood pressure.

Conclusions—These results indicate that activation of the human renin–angiotensin system exaggerates ischemic brain damage mainly through stimulation of the AT1 receptor and marked reduction of cerebral blood flow and enhanced oxidative stress. (Stroke. 2009;40:597-603.)

Key Words: angiotensin ■ brain infarction ■ hypertension ■ receptors

Angiotensin II (Ang II), the major component of the renin–angiotensin system (RAS), plays a key role in the regulation of blood pressure and also induces cardiovascular remodeling. Recent clinical trials such as the LIFE,1 MOSES,2 and JIKEI HEART3 studies demonstrated that administration of an Ang II type 1 (AT1) receptor blocker (ARB) prevented the onset of stroke independent of its blood pressure-lowering effect. This preventive effect of ARB on the onset of stroke could be due to improvement of vascular remodeling, amelioration of the metabolic syndrome associated with antithrombotic effects, and reduction of atrial fibrillation. It has been highlighted that the local RAS in the brain could play an important role in a variety of neuronal functions as well as systemic blood pressure control, including the regulation of cerebral blood flow, and that all components of the classical RAS exist in the brain.4,5

Mice with deletion of angiotensinogen,6 or the AT1 receptor7 show a reduction of the ischemic area after middle cerebral artery (MCA) occlusion. Moreover, it has been reported that administration of an ARB decreases ischemic brain damage.8–11 Sustained blockade of AT1 receptors with an ARB could reverse the pathological cerebrovascular change, oxidative stress, and inflammation, thereby increasing cerebrovascular compliance and decreasing ischemic brain damage.12–14 ARB also could exert neuroprotective effects in ischemic neuronal tissue and improve the neurological outcome of focal brain ischemia.15,16 However, questions still remain as to whether activation of RAS could be really involved in the exaggeration of ischemic brain damage and whether treatment with an ARB could prevent enhanced ischemic brain damage induced by activation of the brain RAS. If an ARB could exert an anti-ischemic brain damage effect, the mechanisms need to be further elucidated.

We used transgenic mice carrying both the human renin and angiotensinogen genes (hRN/hANG-Tg), which have been developed as a mouse model of human hypertension...
induced by activation of the human RAS.\textsuperscript{17–19} Oxidative stress plays an important role in the actions of Ang II and consequent vascular inflammation and vascular remodeling.\textsuperscript{20,21} Ando et al reported that blockade of the AT\(_1\) receptor reverses inflammation in brain microvessels.\textsuperscript{22} Moreover, it has been reported that oxidative stress induces neural cell damage and activation of the central sympathetic nervous system.\textsuperscript{23,24} In the present study using these mice, we examined the roles of human RAS activation in ischemic brain damage focusing on cerebral blood flow and oxidative stress.

### Materials and Methods

#### Animals and Treatments

Adult male mice aged 10 to 12 weeks were used in this study. Transgenic mice carrying both the human renin and angiotensinogen genes (hRN/hANG-Tg) were generated by mating of human renin-transgenic (hRN-Tg; C57BL/6J background) mice and human angiotensinogen–transgenic (hANG-Tg; C57BL/6J background) mice purchased from Riken Bioresource Center (Tsukuba, Japan).\textsuperscript{17} C57BL/6J mice were used as genetic background-matched wild-type controls. The animals were housed in a room with a 12-hour light/dark cycle with temperature of 25±1°C. They were given standard laboratory chow (MF; Oriental Yeast Co Ltd, Osaka, Japan) and water ad libitum. Valsartan (3.0 mg/kg per day), a selective AT\(_1\) receptor blocker (provided by Novartis Pharma AG), and hydralazine (0.5 mg/kg per day) were administered orally from 10 weeks of age for 2 weeks before MCA occlusion. The Animal Studies Committee of Ehime University approved the following experimental protocol.

#### Middle Cerebral Artery Occlusion

Focal cerebral ischemia was induced by occlusion of the MCA by an intraluminal filament technique according to methods described previously.\textsuperscript{5,25} We performed MCA occlusion in each mouse at 12 weeks of age. Brain samples were obtained at 24 hours after MCA occlusion, and coronal sections of 1-mm thickness were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) as previously described.\textsuperscript{25} Neurological deficit was evaluated at 24 hours after MCA occlusion by means of neurological scores developed by the authors.\textsuperscript{26} Changes in cerebral blood flow after MCA occlusion were expressed as a percentage of the baseline value.

#### Measurement of Cerebral Blood Flow

Cerebral surface blood flow was determined under anesthesia in the territory of the MCA by laser-Doppler flowmetry (FLD-C1; Ome-gawave, Tokyo, Japan) as previously described.\textsuperscript{26,27} The tip of the probe was fixed to the intact skull over the territory supplied by the proximal part of the MCA (core; 2 mm caudal to bregma and 6 mm lateral to midline) and the peripheral part of the MCA (periphery; 2 mm caudal to bregma and 3 mm lateral to midline). Changes in cerebral blood flow after MCA occlusion were expressed as a percentage of the baseline value.

#### Detection of Superoxide Anion Production in Brain Sections

Histological detection of superoxide anion was performed as described previously.\textsuperscript{30} In brief, frozen, enzymatically intact, 10-μm thick sections were prepared from mouse brain before and after MCA occlusion and incubated immediately with dihydroethidium (DHE; 10 μmol/L) in phosphate-buffered saline for 30 minutes at 37°C in a humidified chamber protected from light. DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and fluoresces red. For detection of ethidium, tissue sections were examined with an Axioskop microscope (Axioskop 2 plus with AxiosCam; Carl Zeiss) equipped with a computer-based imaging system. The intensity of the fluorescence was analyzed and quantified using computer imaging software (Densitograph; ATTO Corporation).

### Results

#### Detection of Angiotensin II Production in the Brain of hRN/hANG-Tg Mice

Ang II content in the brain and plasma was determined at 12 weeks of age by Immunoassay Kit (EK-002–12; Phoenix Pharmaceuticals, Inc). The brain and blood samples were obtained under anesthesia. To exclude the contamination of blood, we perfused the brain with cold saline before brain sampling. The plasma was separated and the tissue was homogenized with ice-cold homogenizing buffer A (RK-BA; Phoenix Pharmaceuticals, Inc) and treated with Sep-Pak C18 column referring to the manufacturer’s method of the kit before immunoassay.

Some brain samples were separated into 7 parts such as cortex, striatum, midbrain, hypothalamus, hippocampus, cerebellum, and medulla oblongata. Content of Ang II was detected by Western blot using antiangiotensin antibodies (Santa Cruz Biotechnology, Inc).

#### Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from parietal part of the cortex. Expression of mRNA was determined by quantitative real-time reverse-transcription polymerase chain reaction. The level of target gene expression was normalized against the glyceraldehyde-3-phosphate dehydrogenase expression in each sample. Polymerase chain reaction primers for human renin were 5′-TCCGGACAGCACACACCAC-3′ (forward) and 5′-CCATGTCCACACTCGTCCGCTC-3′ (reverse); for human angiotensinogen, they were 5′-ACTCCCTCAAATGGAATTCAC-3′ (forward) and 5′-CCCTGATGCCGTCATTTGCTC-3′ (reverse); for the AT\(_1\) receptor, they were 5′-ATGTCGCCACATGGCCTGCTGCT-3′ (forward) and 5′-ACTGGTCCCTTTGTCGGTGAAG-3′ (reverse); for the AT\(_2\) receptor, they were 5′-CCTGCTATGTTTGGCTGATGG-3′ (forward) and 5′-CCGCCAGGCCGGTCCAC-3′ (reverse); and for glyceraldehyde-3-phosphate dehydrogenase, they were 5′-TGCGAC-TTCAACGCAACTC-3′ (forward) and 5′-ATGTAGCCATTGAGTTCCAC-3′ (reverse).

#### Statistical Analysis

Values are expressed as mean±SEM in the text and figures. The data were analyzed by one-way analysis of variance. If a statistically significant effect was found, post hoc analysis was performed to detect the difference between the groups. A value of \(p<0.05\) was considered to indicate statistical significance.

#### Focal Ischemic Injury of Brain After Middle Cerebral Artery Occlusion in hRN/hANG-Tg Mice

Similar to the results in the previous report,\textsuperscript{17} systolic blood pressure of hRN/hANG-Tg mice at 12 weeks was higher than that of wild-type (WT), hRN-Tg, and hANG-Tg mice (Table 1). In hRN/hANG-Tg mice at 12 weeks of age, the ischemic brain area after MCA occlusion was approximately 2-fold larger than that in WT mice (Figure 1). The neurological deficit at 24 hours after MCA occlusion was also exaggerated in hRN/hANG-Tg mice with a score of 3.8 versus 1.9 in WT mice (Figure 2). In contrast, the ischemic area and neurological score in hRN-Tg and hANG-Tg mice were not significantly different from those in WT mice.

#### Changes in Cerebral Blood Flow After MCA Occlusion in hRN/hANG-Tg Mice

Cerebral surface blood flow decreased in the core and peripheral regions of the MCA territory (Figure 3). Just after MCA occlusion, cerebral surface blood flow de-
increased to 15% of the basal level in the core region and to 55% in the peripheral region in WT mice (Figure 3A). This reduction of cerebral blood flow did not significantly change during 24 hours after MCA occlusion in WT mice. In hRN/hANG-Tg mice, the decrease in cerebral blood flow in the core region was not significantly different from that in WT, hRN-Tg, and hANG-Tg mice (Figure 3A). However, cerebral blood flow in the peripheral region further decreased in hRN/hANG-Tg mice during 24 hours after MCA occlusion (Figure 3A).

**In Situ Detection of Superoxide Anion Production in Brain of hRN/hANG-Tg Mice**

We have previously reported that an increase in oxidative stress is involved in the exaggeration of ischemic brain damage after MCA occlusion. Superoxide anion production was increased in the brain of hRN/hANG-Tg mice at 12 weeks of age, even in the basal condition without MCA occlusion (Figure 4). In the MCA, superoxide anion production was also increased in the arterial wall in these mice (Figure 4). Superoxide anion production was further increased 24 hours after MCA occlusion in the core and peripheral (penumbra) regions in hRN/hANG-Tg mice compared with that in WT, hRN-Tg, and hANG-Tg mice (Figure 5).

**Expression of Human Renin, Human Angiotensinogen, and Angiotensin I and Angiotensin II Receptors**

Expression of mRNA for both human renin and angiotensinogen was detected in the brain of hRN/hANG-Tg mice (0.00037 ± 0.00014 and 0.00087 ± 0.00025 relative to glyceraldehyde-3-phosphate dehydrogenase for renin and angiotensinogen, respectively). We could not detect human renin and angiotensinogen mRNAs in the brain of WT mice. In addition, expression of AT1 and AT2 receptor in the brain of hRN/hANG-Tg mice (0.00184 ± 0.00018 and 0.00152 ± 0.00011 rel-
ative to glyceraldehyde-3-phosphate dehydrogenase for AT₁ and AT₂ receptor, respectively) was not significantly different from that in WT mice.

Increase in Angiotensin II Production in the Brain of hRN/hANG-Tg Mice Before Middle Cerebral Artery Occlusion

Production of Ang II was increased in hRN/hANG-Tg mice. As shown in Table 2, the Ang II content in the brain was significantly increased as well as that in plasma in hRN/hANG-Tg mice. In Western blot analysis, the increase in Ang II content was observed in most parts of the brain in these mice (see supplemental Figure I, available online at http://stroke.ahajournals.org).

Effect of AT₁ Receptor Blockade on Focal Brain Ischemia After Middle Cerebral Artery Occlusion in hRN/hANG-Tg Mice

Treatment of hRN/hANG-Tg mice with an ARB, valsartan, decreased blood pressure to a level similar to that in WT mice (Table 1). The same dose of valsartan did not significantly affect blood pressure in WT mice. Valsartan significantly inhibited the exaggeration of ischemic brain area and neurological deficit after MCA occlusion in hRN/hANG-Tg mice (Figures 1 and 2). Moreover, valsartan restored the reduction of cerebral blood flow after MCA occlusion in the peripheral region in hRN/hANG-Tg mice (Figure 3B). Valsartan also inhibited the increase in superoxide anion production in the infarct core and peripheral (penumbra) regions as well as in

Figure 3. Change in cerebral blood flow after MCA occlusion in hRN/hANG-Tg mice. Surface cerebral blood flow was determined before MCA occlusion and immediately (time 0), 1 hour, and 24 hours after MCA occlusion by laser-Doppler flowmetry as described in “Materials and Methods.” Blood flow change was expressed as a percentage of basal flow. n=5 to 6 for each group. Values are mean±SEM. Val, valsartan; Hyd, hydralazine. *P<0.05 versus WT, hRN-Tg, or hANG-Tg. †P<0.05 versus hRN/hANG-Tg without valsartan or hydralazine.

Figure 4. Detection of superoxide anion production in brain and MCA in hRN/hANG-Tg mice without MCA occlusion. A, Representative staining of brain cortex and middle cerebral artery with DHE in different experiments. B, Intensity of fluorescence after DHE staining in the brain cortex (left) and middle cerebral artery (right). Val, valsartan; Hyd, hydralazine. n=6 to 7 for each group. Values are mean±SEM. *P<0.05 versus WT, hRN-Tg, or hANG-Tg. **P<0.01 versus WT, hRN-Tg, or hANG-Tg. †P<0.01 versus hRN/hANG-Tg without valsartan.
versus hRN/hANG-Tg without valsartan.

Table 2. Ang II Levels in the Plasma and Brain

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>hRN-Tg</th>
<th>hANG-Tg</th>
<th>hRN/hANG-Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Ang II, pg/mL</td>
<td>266.0 ± 15.0</td>
<td>268.5 ± 53.2</td>
<td>283.3 ± 94.3</td>
<td>555.3 ± 162.7*</td>
</tr>
<tr>
<td>Brain Ang II, pg/g</td>
<td>99.5 ± 9.5</td>
<td>100.2 ± 5.9</td>
<td>114.4 ± 11.9</td>
<td>204.4 ± 28.8*</td>
</tr>
</tbody>
</table>

Plasma and brain Ang II levels were measured by using Immunoassay Kit as described in “Materials and Methods.” n = 5 to 6 for each group.

Figure 5. Detection of superoxide anion production in infarcted side of brain at 24 hours after MCA occlusion. MCA occlusion was performed, and brain samples were prepared and stained with DHE. A, Representative staining of brain sections with DHE. B, Intensity of superoxide anion production in brain area. Val, valsartan; Hyd, hydralazine; Core, core region on infarcted side; Penumbra, penumbral region on infarcted side. n = 6 to 7 for each group. Values are mean ± SEM *P < 0.05 versus WT, hRN-Tg, or hANG-Tg. †P < 0.05 versus hRN/hANG-Tg without valsartan.

Discussion

The present study indicated that the focal ischemic area after MCA occlusion was exaggerated with enhanced neurological deficit in hRN/hANG-Tg mice, in which brain RAS was activated. The reduction of cerebral blood flow in periphery (penumbra) region and the superoxide production after MCA occlusion were exaggerated in hRN/hANG-Tg mice. These changes observed in hRN/hANG-Tg mice were significantly inhibited by an ARB, valsartan. These results suggest that brain RAS contributes to the exaggeration of ischemic brain damage through the change in blood flow and oxidative stress.

It has been reported that circulating and locally formed Ang II controls cerebral blood flow through AT1 receptor mediated by stimulation in cerebral vessels and sympathetic nerves.19,31–33 Overproduction of Ang II may contribute to aggravation of cerebral vascular remodeling through sustained AT1 receptor stimulation. It is expected that an ARB could reverse pathological vascular remodeling in the brain. Moreover, an excess of AT1 receptor-mediated oxidative stress and inflammation contributes to worsening of ischemic brain damage through cerebral vascular remodeling, effects on the blood–brain barrier, and direct effects on neural cells.13 These results have been obtained mainly using genetic hypertensive models such as SHR-SP.34 However, the question still remains as to whether activation of the brain RAS could actually play an important role in enhancement of ischemic brain damage. To explore this possibility, we used hRN/hANG-Tg mice, which produce brain Ang II through human renin and angiotensinogen and subjected them to MCA occlusion. We observed a significant increase in the ischemic area and superoxide anion production with a decrease in cerebral blood flow in the periphery (penumbra) of the ischemic area in hRN/hANG-Tg mice compared with those in WT, hRN-Tg, and hANG-Tg mice (Figures 1, 3, and 4). We also observed that administration of an ARB, valsartan, improved this ischemic brain damage. These results suggest that increased production of Ang II in the brain is involved in acceleration of ischemic brain damage. It has been reported that elevated angiotensin II is associated with development of ischemic brain damage using angiotensinogen-overexpressing mice.7 Angiotensinogen-overexpressing mice were generated by injecting the entire rat angiotensinogen gene. On the other hand, hRN/hANG-Tg mice used in this present study were generated by mating human renin and human angiotensinogen–transgenic mice. Therefore, RAS in hRN/hANG-Tg mice were activated mainly by both human renin and angiotensinogen by cross-mating. These mice could be particularly suitable for the experimental model of human hypertension induced by RAS. Moreover, in case of transgenic mice of rat angiotensinogen, it might be possible that there would be feedback regulation or downregulation of mouse RAS components, including renin and angiotensin II production. However, in our study, the expression of mouse renin, angiotensinogen (data not shown) as well as AT1 and AT2 receptor was not changed in hRN/hANG-Tg mice, supporting that hypertension in this mouse model was actually induced by human renin and angiotensinogen. In addition, we directly demonstrated that overproduction of brain angiotensin II and circulating angiotensin II were observed in hRN/hANG-Tg mice. In the present study, we observed that hRN-Tg and hANG-Tg mice did not have a significant change in terms of blood pressure, production of angiotensin II, and ischemic brain injury.
compared with those in WT mice. Neither human renin nor human angiotensinogen enzymatically interacts with the endogenous mouse renin and angiotensinogen. Therefore, hRN/hANG-Tg mice, but not hRN-Tg or hANG-Tg mice, could be used as a human hypertension model.

SHR are widely used as an experimental model of acceleration of the brain RAS.14,21 However, the genetic basis governing the development of hypertension in SHR remains unclear. Moreover, in SHR the changes in NAD(P)H oxidase subunits are not the same as those observed after continuous infusion of Ang II.13,35 In contrast, it has been demonstrated that the interaction of human renin with human angiotensinogen in hRN/hANG-Tg mice induces blood pressure elevation through activation of RAS.17 In addition, hRN/hANG-Tg mice are moderately hypertensive compared with SHR and SHR-SP. In the present study, treatment of hRN/hANG-Tg mice with valsartan (3.0 mg/kg per day) decreased blood pressure, whereas this dose of valsartan did not significantly affect the blood pressure of WT mice (Table 1). We also previously reported that administration of this dose of valsartan in WT mice reduced the ischemic brain area and oxidative stress with an increase in cerebral blood flow in the penumbra.9 Moreover, treatment of hRN/hANG-Tg mice with hydralazine (0.5 mg/kg per day) did not significantly reduce the ischemic brain area (Figure 1), although this dose of hydralazine decreased blood pressure to the same level as that with valsartan. Previous papers indicated that the blood pressure-lowering reduces the incidence of stroke.56 Because valsartan lowered the blood pressure of hRN/hANG-Tg mice to around the control level, we tried to evaluate whether the effects of valsartan were mainly due to the direct inhibition of human RAS with elevated angiotensin II or due to indirect action through blood pressure-lowering. These results indicate that the acceleration of ischemic brain damage in this model is mainly caused by overstimulation of the AT1 receptor and is partly due to an increase in blood pressure.

Oxidative stress is recognized as one of the important mechanisms involved in neuronal damage induced by brain ischemia.13 It has been reported that chronic Ang II infusion impairs cerebral blood flow regulation through activating AT1 receptors and inducing oxidative stress.57,58 Moreover, Krizbai et al reported that oxidative stress causes breakdown of the blood–brain barrier and plays an important role in brain ischemia.59 The blood–brain barrier constitutes an important line of defense for neurons and glial cells against ischemic brain damage. In the present study, we observed that superoxide anion production was increased in the brain and cerebral artery even in the basal condition without MCA occlusion in hRN/hANG-Tg mice, and valsartan treatment attenuated superoxide anion production in the brain (Figure 4). In Figure 4, we examined the cortex in the parietal region. In our study, the fluorescence seemed almost homogenous in the cortex. This result suggests that the angiotensin II production in the brain of hRN/hANG-Tg mice is observed in the whole area of the cortex. Superoxide anion is one of the most important reactive oxygen species involved in pathogenesis of ischemic brain damage.13,40 These results suggest that the enhancement of ischemic brain damage in hRN/hANG-Tg mice after MCA occlusion might be partly due to brain dysfunctions not associated with MCA occlusion such as impairment of compliance of cerebral arteries and blood–brain barrier permeability induced by oxidative stress. The mechanism of the increase in superoxide anion production in the each brain area was not revealed in detail. However, previous reports indicated that the AT1 receptor stimulation activates NADPH oxidase, a critical enzyme of superoxide anion production. It is also reported that NADPH oxidase is expressed in neural and glial cells.41 In our study, angiotensin II production was increased in the brain of hRN/hANG-Tg mice and expression of NADPH oxidase subunits such as p47phox was also elevated in the brain of hRN/hANG-Tg mice (data not shown). These results suggest that an increase in superoxide anion production seems to be caused by NADPH oxidase activation in neural and glial cells through AT1 receptor stimulation. Pretreatment with valsartan could improve such an unfavorable status in the brain by decreasing oxidative stress. As previously reported, Ang II promoted NAD(P)H oxidase-derived reactive oxygen species formation through AT1 receptors, leading to vascular inflammation, apoptosis, remodeling, and endothelial dysfunction.22 AT1 receptors are known to exist in the brain and cerebral arteries as well as in neurons. Therefore, these data suggest that sustained stimulation of the AT1 receptor by circulating and locally formed Ang II increases superoxide anion production in the brain and cerebral arteries, and inhibition of the circulating and brain Ang II systems with an ARB, valsartan, may result in significant protection during brain ischemia.

Our present findings suggest the possibility that in an experimental model with activated human brain RAS, blockade of the AT1 receptor with valsartan inhibits ischemic brain damage induced by cerebral infarction. The preventive effects of valsartan on ischemic brain damage are at least partly dependent on a decrease in oxidative stress and an increase in cerebral blood flow in the penumbra. Therefore, sustained reduction of activation of AT1 receptors by an ARB could be a therapeutic approach to prevent brain ischemia in addition to its hypotensive effect and preventive effect on the onset of stroke.

Sources of Funding
This work was supported in part by grants from the Ministry of Education, Science, Sports, and Culture of Japan.

Disclosures
None.

References
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*Stroke*. 2009;40:597-603; originally published online November 20, 2008; doi: 10.1161/STROKEAHA.108.519801
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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