Homocysteine Impairs the Nitric Oxide Synthase Pathway
Role of Asymmetric Dimethylarginine

Markus C. Stühlinger, MD; Philip S. Tsao, PhD; Jeng-Horng Her, PhD; Masumi Kimoto, PhD; Robert F. Balint, PhD; John P. Cooke, MD, PhD

Background—Hyperhomocysteinemia is a putative risk factor for cardiovascular disease, which also impairs endothelium-dependent vasodilatation. A number of other risk factors for cardiovascular disease may exert their adverse vascular effects in part by elevating plasma levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase. Accordingly, we determined if homocysteine could increase ADMA levels.

Methods and Results—When endothelial or nonvascular cells were exposed to DL-homocysteine or to its precursor L-methionine, ADMA concentration in the cell culture medium increased in a dose- and time-dependent fashion. This effect was associated with the reduced activity of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades ADMA. Furthermore, homocysteine-induced accumulation of ADMA was associated with reduced nitric oxide synthesis by endothelial cells and segments of pig aorta. The antioxidant pyrrollidine dithiocarbamate preserved DDAH activity and reduced ADMA accumulation. Moreover, homocysteine dose-dependently reduced the activity of recombinant human DDAH in a cell free system, an effect that was due to a direct interaction between homocysteine and DDAH.

Conclusion—Homocysteine post-translationally inhibits DDAH enzyme activity, causing ADMA to accumulate and inhibit nitric oxide synthesis. This may explain the known effect of homocysteine to impair endothelium-mediated nitric oxide–dependent vasodilatation.

(Circulation. 2001;104:2569-2575.)

Key Words: endothelium ■ arginine ■ atherosclerosis ■ nitric oxide

Hyperhomocysteinemia impairs vascular function and is a putative risk factor for cardiovascular diseases.1 Endothelium-dependent, flow-mediated dilatation of the brachial artery is impaired in humans with elevated plasma homocyst(e)ine2 and with experimental hyperhomocysteinemia induced by oral L-methionine, the precursor of homocysteine.3 Flow-mediated vasodilatation of the brachial artery is largely due to the release of endothelium-derived nitric oxide (NO).4 The mechanisms for diminished NO bioavailability in hyperhomocysteinemia remain incompletely defined.

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Infusions of homocysteine cause patchy denudation of vascular endothelium,5 but these toxic effects occur at levels of homocysteine that are not relevant to human disease. A more plausible mechanism may be that homocysteine, through the formation of disulfides and the generation of hydrogen peroxide6 and superoxide anion,7 increases the oxidative degradation of NO. Other potential effects of homocysteine may be to decrease NO synthesis.8 This observation would be most consistent with a dysregulation of NO synthase (NOS), because protein expression is not affected by homocysteine.9

Endogenous competitive inhibitors of NOS, including L-N-monomethylarginine (L-NMMA) and asymmetric dimethylarginine (ADMA), may contribute to endothelial dysfunction. Plasma levels of ADMA are elevated in patients with hypercholesterolemia, hypertension, hyperglycemia, and tobacco exposure.10 Indeed, plasma ADMA levels correlate better with endothelial vasodilator dysfunction than do plasma LDL cholesterol levels in patients with hypercholesterolemia.11 Thus, ADMA may be an endogenous regulator of NO synthesis that becomes dysregulated in disease states.

The 2 major pathways for ADMA clearance are through renal excretion and through metabolism by dimethylarginine dimethylaminohydrolase (DDAH).12 The reduced clearance of ADMA in renal failure is associated with severe endothelial vasodilator dysfunction, which is reversed by intravenous administration of the NO precursor L-arginine13 or by dialysis (which removes plasma ADMA).14 In hypercholesterolemia, ADMA accumulation seems to be due to an impairment of DDAH activity.15

Received July 18, 2001; revision received August 29, 2001; accepted August 30, 2001.
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Accordingly, the following study was performed to determine if homocysteine could affect the NOS pathway by impairing DDAH.

**Methods**

**Cell Culture**
Primary bovine aortic endothelial cells (BAEC; single-donor; passage 2 to 6; Clonetics) were cultured to confluence in microvascular endothelial growth medium (Clonetics) containing 5% FCS. ECV304 cells (American Type Culture Collection) were cultured to confluence in Medium 199 (Gibco-BRL) containing 10% FCS.

Cells were washed with Hank's balanced salt solution (HBSS; Irvine Scientific) and cultured in minimal essential medium (MEM Select-Amine Kit; GibcoBRL) containing 0.5% fetal bovine serum in the absence or presence of L-methionine (30 μmol/L to 1 mmol/L; GibcoBRL), DL-homocysteine (10 μmol/L to 1 mmol/L), and/or pyrrolidine dithiocarbamate (PDTC; 100 μmol/L). Cells and media were harvested to measure dimethylarginine concentrations, DDAH protein expression, and enzyme activity. To assess stimulated NO synthesis, BAEC (passage 2 to 4) were exposed to calcium ionophore (A23187; 1 μmol/L) for 2 hours before collecting the medium for chemiluminescence analysis.

All measurements were performed in triplicate. Cell viability was tested by measuring lactate dehydrogenase activity in the medium by a spectrophotometric assay. Cell number was assessed using a hemocytometer counting.

**Ex Vivo Exposure of Porcine Aortic Segments**
Harvested aortas from 4 Yorkshire-cross pigs were placed in cold physiological solution. The vessels were opened longitudinally and incubated in 3 mL of Hanks' buffered saline solution (HBSS) (Gibco BRL) in the absence or presence of L-homocysteine (30 μmol/L or 300 μmol/L). After 2 hours, aortic segments were stimulated with calcium ionophore (1 μmol/L) in the absence or the presence of L-arginine or D-arginine (500 μmol/L), and nitrogen-oxide (NOx) elaboration was measured by chemiluminescence.

**Determination of Dimethylarginines and Nitrogen Oxides**
Concentrations of ADMA and symmetric dimethylarginine (SDMA) in the conditioned media of BAEC and in aortic segments incubated with HBSS were determined with a chemiluminescence apparatus (Diasorin 2108) after reduction of the samples in boiling acidic vanadium (III) chloride at 98°C.7,16 Standard curves for NOx were linear over a range between 100 nmol/L and 100 μmol/L (r²=0.99). The detection limit of this assay was 10 nmol/L. Intra-assay and interassay variability was <6%. Western Blotting

**Cloning and Expression of Recombinant Human DDAH-1**
Full-length human DDAH-1 was cloned into a pGEX-5X-1 vector (Amersham) with a glutathione-S-transferase (GST) sequence at the N-terminus of the DDAH sequence and transformed into BL21-DE3 Escherichia coli. GST-DDAH was expressed in bacteria according to Kaelin et al19; this was followed by separation of recombinant protein from other bacterial proteins with a GST Micro Spin Purification Module (Amersham). The eluate was dialyzed 4 times with PBS, dissolved in 50% glycerol, and stored at -20°C until used for enzyme activity measurements or binding assay.

The final concentration of recombinant GST-DDAH was calculated by determining the total protein concentration by Bradford assay (Bio-Rad) after SDS-PAGE of the soluble protein fraction.

**Results**

**Western Blotting**
Cells were lysed in 0.1 mol/L sodium phosphate buffer (pH=7) containing 1% IGEPAL CA-630, trypsin inhibitor (10 mg/L), leupeptin (10 mg/L), pepstatin (10 mg/L), and antipain (10 mg/L). Cell lysates were centrifuged at 2000g for 45 minutes, and supernatants were collected for Western assay, Western blotting, and DDAH enzyme activity assay. Hybod enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham) with proteins transferred from SDS-PAGE gels were incubated with a mouse monoclonal antibody raised against purified human DDAH-120 (1:500) in Tris-buffered saline containing 4% nonfat dry milk and probed with an anti-mouse IgG horseradish peroxidase-linked antibody (Amersham), with detection by enhanced chemiluminescent Western blotting reagents (Amersham).

**DDAH Enzyme Activity Assay**
DDAH enzyme activity was assayed by determining L-citrulline formation, as previously described.21 DDAH activity of recombinant GST-DDAH was assessed by incubating the purified enzyme with exogenous ADMA and 0.1 mol/L sodium phosphate buffer (pH=7) in the absence and presence of DL-homocysteine (1 μmol/L to 1 mmol/L), L-methionine (1 mmol/L), L-cysteine (1 mmol/L), and PDTC (100 μmol/L to 1 mmol/L). After 2 hours, the reaction was stopped, and activity was calculated after measuring the concentration of L-citrulline with a chromogenic reaction that specifically determines ureido groups.22 One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol/L L-citrulline per min at 37°C.

**DDAH Binding Assay**
To assess the binding of DL-homocysteine to recombinant DDAH-1, homocysteine was biotinylated with an EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce). Biotinylated homocysteine (10 μmol/L and 100 μmol/L) was incubated with recombinant GST-DDAH (1 μg and 0.5 μg) for exactly 60 minutes, and the mix was subsequently placed on a Reacti-Bind streptavidin-coated Polystyrene Strip Plate (Pierce) and washed. Human DDAH was then detected with a primary antibody raised against purified human DDAH-1 and a secondary anti-mouse horseradish peroxidase–linked antibody, as described above under Western blotting. Immunoblot x-ray pictures obtained from homocysteine-DDAH binding experiments were digitalized, saved as bitmap files, and quantified (Scion Image for Windows, version β4.0.2; Scion Corp). To assess the specificity of the homocysteine reaction with DDAH, biotinylated L-methionine (100 μmol/L) and L-cysteine (100 μmol/L) were used in place of biotinylated homocysteine.

**Calculations and Statistical Analyses**
Data are reported as mean±SEM. Group differences were examined by Statview software (SAS Institute Inc) using ANOVA, and post hoc analysis was performed using Fisher’s protected least-significant difference test. Statistical significance was set at P<0.05.
### Effect of Homocysteine and/or PDTC on ADMA Accumulation and DDAH Activity in BAEC

<table>
<thead>
<tr>
<th>Homocysteine, μmol/L</th>
<th>ADMA</th>
<th>SDMA</th>
<th>DDAH Activity</th>
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<tr>
<td>0</td>
<td>0.33±0.02</td>
<td>0.31±0.02</td>
<td>0.14±0.02</td>
</tr>
<tr>
<td>10</td>
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<td>0.45±0.07</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>30</td>
<td>0.82±0.11*</td>
<td>0.57±0.05†</td>
<td>0.11±0.03</td>
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<tr>
<td>100</td>
<td>0.99±0.12*</td>
<td>0.65±0.04†</td>
<td>0.13±0.04</td>
</tr>
<tr>
<td>300</td>
<td>1.32±0.18*</td>
<td>0.78±0.05†</td>
<td>0.14±0.02</td>
</tr>
</tbody>
</table>

Values are expressed as μmol/L ADMA or SDMA per 10^6 cells and DDAH activity in percent of control. Data are mean±SEM of n=3 different experiments performed in triplicate.

*P<0.05 vs no homocysteine; †P<0.05 vs without PDTC.

2). Adding the intracellular antioxidant PDTC (100 μmol/L) significantly blocked the effect of homocysteine on ADMA accumulation in both BAEC and ECV304 cells (Table and Figure 2). SDMA concentrations per 10^6 cells were not affected by culturing either cell line in the presence of homocysteine, nor were they affected by PDTC or homocysteine plus PDTC (Table).

### Elaboration of NO Metabolites by Endothelial Cells and Pig Aortas

As shown before by Upchurch et al in the same cell line, homocysteine dose-dependently reduced NO elaboration in both BAEC and ECV304 cells (Figure 3). This effect was reversed by increasing L-arginine concentration in the medium (Figure 3). Furthermore NO elaboration by porcine aortic segments was reduced when strips were preincubated with homocysteine (Figure 4). This effect was reversed by adding 500 μmol/L L-arginine, but not D-arginine (Figure 4).

### DDAH Protein Expression and Enzyme Activity

Because ADMA (but not SDMA) levels were increased by supplemental methionine or homocysteine, we hypothesized that this elevation is due to reduced degradation of ADMA by DDAH. This enzyme metabolizes ADMA but not SDMA. Accordingly, we determined if the effect of L-methionine or DL-homocysteine on increasing ADMA accumulation was due to a change in the expression or activity of DDAH.

Western analysis revealed that the expression of DDAH was not altered by the addition of DL-homocysteine or PDTC to the media (data not shown). However, the studies of enzyme kinetics revealed that DDAH activity was reduced by homocysteine. Treatment of BAEC or ECV304 cells with homocysteine for 24 hours reduced DDAH enzyme activity in cell lysates (Table and Figure 5) in a dose-dependent fashion. Coincubation with the intracellular antioxidant PDTC (100 μmol/L) restored DDAH activity (Figure 5). Supplemental L-methionine also reduced DDAH enzyme activity in ECV304 cells (87% with 100 μmol/L; 76% with 300 μmol/L; 58% with 1 mmol/L; all P<0.05).

### Enzyme Activity of Recombinant GST-DDAH

Homocysteine, but not L-methionine or L-cysteine, reduced the activity of recombinant DDAH in a dose-dependent fashion, as shown in Figure 6. Adding homocysteine did not change medium pH and did not interfere with the L-citrulline detection method (data not shown).

The thiol antioxidant PDTC (100 μmol/L) enhanced DDAH activity and prevented the impairment of DDAH activity by homocysteine (Figure 6). Similarly, the reducing
agent dithiothreitol (DTT) enhanced DDAH activity and prevented the impairment of DDAH activity by homocysteine (Figure 6).

**DDAH Enzyme Kinetics**

Michaelis-Menten and Lineweaver-Burk analysis of enzyme kinetics indicate that homocysteine acts as a competitive antagonist of recombinant DDAH. The $K_m$ was 214 μmol/L ADMA in control buffer and increased to 462 μmol/L ADMA at 300 μmol/L homocysteine and to 857 μmol/L ADMA at 1 mmol/L homocysteine, without significantly changing maximum velocity ($V_{\text{max}}$) (769±55 U/mg DDAH). The calculated $K_i$ for homocysteine in vitro was 333 μmol/L homocysteine.

**Binding of Homocysteine and Recombinant DDAH**

Because DDAH contains a sulfhydryl group that is critical for its activity,15,21 we hypothesized that homocysteine may directly reduce DDAH activity by forming a disulfide bridge with the critical sulfhydryl of DDAH. Accordingly, to test this hypothesis, biotinylated DL-homocysteine, L-methionine, or L-cysteine were incubated for 60 minutes with recombinant GST-DDAH. The mixture was placed on a streptavidin-coated plate, and DDAH protein was detected by immunoblotting.

Homocysteine bound significantly to recombinant GST-DDAH in a dose-dependent fashion, whereas methionine and cysteine did not (Figure 7a). To determine if this interaction was due to the formation of a disulfide bridge, we repeated the experiment in the presence of the thiol antioxidant PDTC (which reduces the disulfide bonds). We observed that the binding of homocysteine to DDAH could be significantly attenuated by addition of 100 μmol/L PDTC (Figure 7b).

**Discussion**

The salient findings of this study are as follows. (1) Homocysteine and its precursor L-methionine increase ADMA elaboration by endothelial cells in a dose-dependent fashion at pathophysiologically relevant concentrations. (2) This effect is associated with a dose-dependent impairment of the activity of endothelial DDAH, the enzyme that degrades ADMA. (3) In a cell-free system, homocysteine directly inhibits the activity of DDAH. (4) This effect of homocysteine seems to be due to its attack on a critical sulfhydryl group of DDAH. These data provide the first evidence for a novel mechanism of homocysteine-induced impairment of the NOS pathway.

ADMA is derived from the catabolism of ubiquitous proteins containing methylated arginine residues. The methylated proteins that have been characterized are largely found in the nucleus, and they seem to be involved in RNA processing and transcriptional control.23

When these proteins undergo hydrolysis, their methylated arginine residues are released. Methylated arginines are excreted in the urine.24 Thus, patients with renal insufficiency have expected elevations in plasma ADMA levels. Methylated arginines may also be metabolized. The major metabolic pathway for ADMA is the enzyme DDAH.12 Two isoforms of DDAH are known, DDAH-1 and DDAH-2, and either or both
have been found in every cell type examined. DDAH-1 is typically found in tissues expressing neuronal NOS, whereas DDAH-2 predominates in tissues containing the endothelial isoform of NOS. ADMA undergoes extensive metabolism by DDAH (to citrulline and dimethylamine), such that only 5% of parenterally administered ADMA is recovered in the urine. Pharmacological inhibition of DDAH activity in isolated vascular rings induces a gradual vasoconstriction, which is reversed by adding L-arginine to the medium. 27 Pharmacological inhibition of DDAH activity can be prevented by its metabolism by DDAH. This interaction may block ADMA binding to the active center of the enzyme or may change the conformation of DDAH. This interaction may block ADMA binding to the active center of the enzyme or may change the conformation of DDAH.

In the present investigation, we show that the homocysteine-induced increase in ADMA is associated with a temporally related decline in DDAH activity and reduced NO elaboration by endothelial cells and aortic vessel segments. We have previously shown that a similar mechanism is operative in hypercholesterolemia. When cultured endothelial cells are exposed to oxidized LDL cholesterol, ADMA accumulation in the medium is associated with a temporally related decline in DDAH activity. 15 We now find that homocysteine increases ADMA accumulation in endothelial and nonvascular cells (the ECV304 cell line is a derivative of the human bladder line T24). It seems likely that ADMA levels in both vascular and nonvascular cells are regulated by DDAH activity and that impairment of the NOS pathway by DDAH inhibition could have biological implications beyond the vasculature.

Both isoforms of DDAH are expressed in endothelial cells, although DDAH-1 seems to be the predominant isoform in ECV304 cells. Homocysteine did not change the expression of DDAH-1. There are currently no available monoclonal antibodies specifically directed against DDAH-2, so we cannot comment on the effect of homocysteine on the expression of this isoform. Therefore, it is possible that homocysteine induced a change in the expression of DDAH-2 that was not detected in this study. However, this deficiency does not detract from the observation in our cell-free system that homocysteine directly impairs DDAH activity, apparently by a direct oxidative attack on critical sulfhydryl groups.

Our study indicates that homocysteine can directly bind to and functionally interfere with DDAH-1. DDAH-1 contains 4 cysteine groups on its amino acid sequence. The sulfhydryl groups on these cysteines seem to be very important for its enzyme function, because sulfhydryl-blocking agents such as p-chloromercuribenzoate and HgCl₂ act as potent inhibitors, the effect of which can be reversed by the reducing agent DTT, which restores sulfhydryl groups. Homocysteine contains a very reactive sulfhydryl group and avidly forms the disulfide dimer homocystine, mixed disulfides with cysteine, and amino-thiols with proteins. In fact, ~98% of total plasma homocysteine is covalently bound to protein. Therefore, it is possible that homocysteine forms a mixed disulfide with DDAH. This interaction may block ADMA binding to the active center of the enzyme or may change the conformation of DDAH and, therefore, inhibit its action. This hypothesis is supported by our observation that the homocysteine-induced impairment of endothelial DDAH activity can be prevented by PDTC, a thiol antioxidant that protects sulfhydryl groups, and by the reducing agent DTT. Surprisingly, analysis of
enzyme kinetics revealed competitive inhibition of DDAH-1 by homocysteine, which is structurally unrelated to the substrate ADMA. Therefore, the active site nucleophile for the hydrolysis of ADMA is likely to be a thiol amino acid (ie, cysteine).

Furthermore, in vitro, PDTC reduced the ability of homocysteine to bind to DDAH-1. Our observations are consistent with the hypothesis that homocysteine impairs the NOS pathway by directly inhibiting DDAH activity, which leads to accumulation of ADMA.

There are other potential mechanisms by which homocysteine may impair the NOS pathway. Homocysteine may generate hydrogen peroxide and superoxide anion via disulfide formation and thereby increase oxidative degradation of NO. In a murine model of mild hyperhomocysteinemia, endothelial vasodilator function is impaired, an effect that is associated with peroxynitrite formation and that can be reversed by antioxidants. These observations have lead to the proposal that homocysteine impairs endothelial function by the oxidative degradation of NO. These observations are not exclusive to our group. We previously showed that oxidative stress (induced by oxidized LDL cholesterol) impairs DDAH activity and increases ADMA elaboration by endothelial cells. The vulnerability of DDAH to oxidative attack is likely conferred by the sulfhydryl groups that are critical for its activity.

Parenthetically, it is notable that NO may detoxify homocysteine. When homocysteine is briefly exposed to stimulated endothelial cells, it becomes nitrosylated to form S-NO-homocysteine, a potent agent that has anti-platelet and vasodilatory properties. Nitrosylated homocysteine does not support H2O2 generation and does not undergo conversion to homocysteine thiolactone, which are reaction products believed to contribute to endothelial toxicity.

Boeger and colleagues also observed that homocysteine increases the elaboration of ADMA (but not SDMA) in the media of cultured vascular cells exposed to higher homocysteine concentrations. They speculated that homocysteine is converted to methionine and that the increase in methionine availability drives the formation of ADMA. This mechanism seems less plausible. Cellular methylation reactions are governed by the ratio of S-adenosyl-homocysteine/S-adenosyl-methionine. Elevated levels of homocysteine are associated with an increase of this ratio and a consequent decrease in methylation reactions. Moreover, the methylation of arginine residues on proteins is a specific and highly regulated process that is necessary for key functional attributes of these proteins (eg, RNA processing and transcriptional control). It seems unlikely that methionine availability would be rate-limiting in the formation of these proteins. This view is supported by the observation that the proportions of methylated and nonmethylated arginine residues on methyl proteins are fairly constant for a given species. Furthermore, dimethylarginine concentrations in urine remain unchanged during a protein-free diet (which would reduce methionine availability). Finally, proteolysis is necessary to release free methylarginine residues. Indeed, measurements of methylarginines are used as an index of protein degradation. Therefore, it is highly unlikely that this process is regulated by methionine availability.

Furthermore, we now present compelling evidence that ADMA levels are regulated by alterations in DDAH activity. This hypothesis is more consistent with our observations (and those of Boeger et al) that the accumulation of ADMA (but not SDMA) is increased by homocysteine. ADMA (but not SDMA) is a substrate for DDAH. In contrast, if the Boeger mechanism were operative, increased methylation of proteins would be expected to result in increased elaboration of both forms of dimethylarginine. Accordingly, we propose that the regulation of ADMA occurs at the point of its degradation. We conclude that homocysteine impairs the NO pathway by inhibiting DDAH activity, increasing ADMA accumulation and, subsequently, reducing NO elaboration by endothelial cells and aortic segments. These observations may explain the observation that endothelial vasodilator function is impaired in individuals with hyperhomocysteinemia. Furthermore, these observations may explain, in part, the acceleration of vascular disease by hyperhomocysteinemia. Chronic suppression of endothelial NOS activity accelerates atherosclerosis, whereas chronic enhancement of the NOS pathway attenuates and even reverses the progression of vascular disease in experimental models. There is accumulating evidence that endothelium-derived NO is an endogenous antiatherogenic molecule and that impairment of the NOS pathway is a predictor of vascular morbidity and mortality. These insights may lead to new diagnostic modalities and therapeutic interventions for cardiovascular disease.

Acknowledgments

This study was supported in part by a grant from the National Heart, Lung, and Blood Institute (R01 HL-58638) and by a grant from the Tobacco-Related Diseases Research Program of the University of California (7RT-0128). Dr Cooke is an Established Investigator of the American Heart Association. He is also a consultant to United Therapeutics, which makes the L-arginine–enriched medical food HeartBar. Dr Stühlinger is a recipient of an “Erwin Schrödinger Auslandsstipendium” provided by the Austrian “Fonds zur Förderung der wissenschaftlichen Forschung” (J1893-MED). For the work presented in this manuscript, Dr Stühlinger received the 2001 Young Investigator Award of the American College of Cardiology.

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Circulation. 2001;104:2569-2575
doi: 10.1161/hc4601.098514

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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