Induction by Lysophosphatidylcholine, a Major Phospholipid Component of Atherogenic Lipoproteins, of Human Coronary Artery Smooth Muscle Cell Migration

Masakazu Kohno, MD; Koji Yokokawa, MD; Kenichi Yasunari, MD; Mieko Minami, MD; Hiroaki Kano, MD; Takao Hanehira, MD; Junichi Yoshikawa, MD

Background—The objectives of the present study were (1) to determine whether lysophosphatidylcholine (lyso-PC), a prominent component of oxidatively modified LDL, induces migration of human coronary artery smooth muscle cells (SMCs) and, if so, to clarify the mechanism, and (2) to investigate the possible interactions of lyso-PC and platelet-derived growth factor (PDGF)-BB, endothelin-1 (ET-1), adrenomedullin (AM), or vitamin E on SMC migration by the Boyden’s chamber method.

Methods and Results—Lyso-PC induced SMC migration in a concentration-dependent manner between $10^{-6}$ and $5 \times 10^{-5}$ mol/L. By contrast, phosphatidylcholine was without significant activity, and lysophosphatidylinositol and lysophosphatidylserine were much less effective than lyso-PC. Lyso-PC increased basic fibroblast growth factor (bFGF) production in a concentration-dependent manner between $10^{-6}$ and $5 \times 10^{-5}$ mol/L in these cells. Furthermore, lyso-PC–induced SMC migration was inhibited by neutralizing antibody to bFGF but not by neutralizing antibody to transforming growth factor-β1. Lyso-PC–induced migration was significantly enhanced by PDGF-BB or ET-1 but was clearly inhibited by human AM and vitamin E.

Conclusions—These results indicate that (1) lyso-PC induces human coronary artery SMC migration at least in part through release of endogenous bFGF and (2) this lyso-PC–induced migration can be further induced by PDGF-BB and ET-1 and can be inhibited by human AM and vitamin E. Lyso-PC may recruit medial SMCs during the process of coronary atherosclerosis in part by releasing bFGF in concert with PDGF-BB or ET-1 in vascular tissues. This lyso-PC–induced SMC migration may be suppressed by AM and vitamin E under certain pathological conditions.

Key Words: lysophosphatidylcholine ■ growth substances ■ adrenomedullin ■ endothelin ■ migration ■ cells

Elevated levels of LDL cholesterol are an important risk factor for coronary atherosclerosis and cardiovascular morbidity. However, oxidized LDL is shown to possess more atherogenic properties than native LDL. Actually, several lines of evidence have indicated that oxidatively modified LDL plays a key role in atherogenesis. In oxidized LDL particles, the content of lyso-PC, a polar phospholipid, is found to be dramatically increased. Actually, Parthasarathy and coworkers have demonstrated that as much as 40% of the phosphatidylycholine of LDL is converted to lyso-PC during oxidized modification. In addition, the concentration of lyso-PC is shown to be increased in atherosclerotic arterial lesions in animals fed an atherogenic diet.

On the other hand, the migration of coronary artery medial SMCs into the intima is proposed to be an initial process of intimal thickening in coronary atherosclerotic lesions. Here, we show for the first time that lyso-PC, not phosphatidylcholine, potently induces migration of human coronary artery SMCs and that this lyso-PC–induced migration can be further induced by a potent mitogen, PDGF-BB, and a vasoconstrictive and growth-promoting peptide, ET-1.

Recently, Chai and coworkers demonstrated that the mechanism of enhanced DNA synthesis by oxidized LDL and lyso-PC at least in part involves an autocrine or paracrine action of release of endogenous bFGF. Therefore, we examined whether this lyso-PC–induced SMC migration is caused by the release of endogenous bFGF.

Finally, we show the interaction of lyso-PC and AM, a novel endothelium-derived vasorelaxant peptide, or vitamin E, a potent antioxidant, on the migration of human coronary artery SMCs.

Methods

Materials SmBM and human coronary artery SMCs were purchased from Clonetics Corp. FCS, trypsin, and Versine were purchased from Gibco Laboratories. Synthetic human AM (1–52) and ET-1 were purchased from Peptide Institute. 8-Bromo-cAMP, IBMX, lyso-PC
Lysophosphatidylcholine and SMC Migration

Selected Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>AM</td>
<td>Adrenomedullin</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<td>ET-1</td>
<td>Endothelin-1</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>PDGF-BB</td>
<td>Platelet-derived growth factor–binding protein</td>
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<td>SmBM</td>
<td>Smooth muscle cell basal medium</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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Culture of Human Coronary Artery SMCs

Human coronary artery SMCs were cultured in SmBM containing human epithelial growth factor (0.5 ng/mL), human FGF (2 ng/mL), insulin (5 μg/mL), 5% FCS, 50 μg/mL gentamicin sulfate, and 50 μg/mL amphotericin-B. Cells were identified as SMCs according to their morphological and growth characteristics. Cultures were maintained at 37°C with atmospheric air and 5% CO2. Cells were subcultured after treatment with 0.25% trypsin and 0.02% EDTA. Subconfluent SMCs between the fourth and eighth passages were used for the experiments.

Migration Assay

Migration of SMCs was assayed by a modification of the Boyden’s chamber method using microchemotaxis chambers (Nucleopore Corp) and polycarbonate filters (Nucleopore Corp), as previously reported. After preincubation, the cell monolayers were washed twice with Dulbecco’s PBS containing 5×10−5 mol/L EDTA, pH 8.0, 2×10−3 mol/L N-ethylmaleimide, and 10−3 mol/L PMSF, as previously reported. After cells were disrupted by three cycles of freezing and thawing followed by sonication for 1 minute, the homogenate was centrifuged at 25 000g for 30 minutes. The supernatant was stored at −80°C until bFGF quantification was performed with a solid-phase ELISA (Quantikine FGF Basic Immunoassay Kit; R&D Systems, Inc). The average recovery rate of bFGF in cell culture medium was 89%. The minimal detectable dose of bFGF was 1 pg/mL. The interassay variation was 8.7%, and the intra-assay variation was 4.6%.

cAMP Measurement

After preincubation, the cell monolayers were washed twice with PBS and then stimulated for 30 minutes with different concentrations of AM or vitamin E on SmBM-containing SMCs. The intracellular cAMP levels were determined by radioimmunoassay done with the cAMP assay kit, as previously described.

Calculations and Analysis

The statistical significance of differences in the results was evaluated with an unpaired ANOVA, and P values were calculated by Scheffe’s method. All values were expressed as mean±SD.

Results

Effect of Lyso-PC on SMC Migration

Figure 1 shows concentration-dependent effects of lyso-PC (10−6, 5×10−7, 10−7, 5×10−8, 10−8, and 5×10−9 mol/L) on SMC migration for 6 hours of incubation. Lyso-PC significantly induced migration in a concentration-dependent manner between 10−6 and 5×10−5 mol/L. By contrast, the migration-stimulatory effects of the higher range of concentrations (10−4 and 5×10−4 mol/L) of lyso-PC were weak or none (Figure 1).

Micrographs of human coronary artery SMCs taken after a 6-hour migration period clearly show that lyso-PC, not...
phosphatidylcholine, induced migration in a concentration-dependent manner and without causing apparent morphological injury (Figure 2).

Table 1 shows the effects of other lysophospholipids structurally similar to lyso-PC on SMC migration. When these phospholipids were tested at two comparable concentrations, phosphatidylcholine was without significant activity, and lysophosphatidylglycerol and lysophosphatidylserine appear to be much less effective than lyso-PC.

**Effects of Neutralizing Antibody to bFGF on Lyso-PC–Induced SMC Migration**

To clarify the role of endogenous bFGF in lyso-PC–induced SMC migration, cells were treated with antibody that neutralize bFGF. The results of these experiments are shown in Figure 3. bFGF slightly but significantly stimulates SMC migration in a concentration-dependent manner (Figure 3A).

The addition of neutralizing antibody to bFGF to the upper chamber clearly abolished bFGF-induced SMC migration (Figure 3A). The addition of 1 μg/mL of neutralizing antibody to bFGF to the upper chamber significantly inhibited lyso-PC–induced migration. When 10 μg/mL of antibody was added, the effect was even greater (Figure 3B). By contrast, neither 1 μg/mL nor 10 μg/mL of neutralizing antibody to TGF-β, to the upper chamber affected lyso-PC–induced migration (Figure 3B).

Furthermore, we examined the effect of lyso-PC on bFGF production in cultured human coronary artery SMCs. Lyso-PC significantly increased bFGF production in a

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**Table 1. Effects of Other Lysophospholipids Structurally Similar to Lyso-PC on Human Coronary Artery SMC Migration**

<table>
<thead>
<tr>
<th>Migration Activity, Cells/4HPF/6 h</th>
<th>5×10⁻⁴ mol/L</th>
<th>5×10⁻⁵ mol/L</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>9.5±1.3</td>
<td>11.3±2.5</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>8.8±2.6</td>
<td>10.8±2.1</td>
</tr>
<tr>
<td>Lysophosphatidylinositol</td>
<td>13.0±1.8</td>
<td>16.5±2.6†‡§</td>
</tr>
<tr>
<td>Lysophosphatidylserine</td>
<td>13.8±2.4</td>
<td>15.5±1.3†‡§</td>
</tr>
<tr>
<td>Lyso-PC</td>
<td>23.5±3.3*†‡§</td>
<td>36.3±2.6†‡§</td>
</tr>
</tbody>
</table>

Values are mean±SD of four measurements. Migration activities are expressed as number of cells per four high-power fields (4HPF).

*P<0.05 vs baseline level.
†P<0.05 vs level of phosphatidylcholine.
‡P<0.05 vs level of lysophosphatidylinositol.
§P<0.05 vs level of lysophosphatidylserine.

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**Figure 2.** Micrographs of human coronary artery SMCs after a 6-hour migration period (magnification ×400). A 200-μL volume of SMC suspension was placed in upper chamber, and 40-μL volumes of medium containing various concentrations (b–e) of lyso-PC or 5×10⁻⁵ mol/L of phosphatidylcholine (f) were placed in lower chamber. a, Control; b, 10⁻⁶ mol/L lyso-PC; c, 5×10⁻⁶ mol/L lyso-PC; d, 10⁻⁵ mol/L lyso-PC; e, 5×10⁻⁵ mol/L lyso-PC; and f, 5×10⁻⁵ mol/L phosphatidylcholine.

**Figure 3.** A, Effect of bFGF on SMC migration and effect of neutralizing antibody to bFGF (Ab-FGF) on bFGF-induced SMC migration. Various concentrations (5, 10, and 20 ng/mL) of bFGF were added to lower chamber. Ab-FGF (10 μg/mL) was added to upper chamber 30 minutes before addition of 20 ng/mL bFGF to lower chamber. bFGF significantly stimulated SMC migration, and Ab-FGF clearly abolished bFGF-induced SMC migration. *P<0.05 vs control; B, Effect of neutralizing antibody to bFGF or TGF-β2 on lyso-PC–induced SMC migration. Two concentrations (1 and 10 μg/mL) of neutralizing antibody to bFGF or TGF-β2 were added to upper chamber 30 minutes before addition of 5×10⁻⁵ mol/L lyso-PC to lower chamber. IgG represents an irrelevant neutralizing antibody, Ab-FGF represents a neutralizing monoclonal antibody against bFGF, and Ab-TGF represents a neutralizing polyclonal antibody against TGF-β2. Ab-FGF significantly inhibited SMC migration stimulated with lyso-PC. Irrelevant antibodies (IgG and Ab-TGF) did not significantly alter migration activities. *P<0.05 vs control; †P<0.05 vs 5×10⁻⁵ mol/L lyso-PC alone; ‡P<0.05 vs lyso-PC plus Ab-FGF (1 μg/mL).
concentration-dependent manner between $10^{-6}$ and $5 \times 10^{-5}$ mol/L (Figure 4). bFGF concentration caused by $5 \times 10^{-5}$ mol/L were 9-fold greater than nonstimulated controls. These results suggest that lyso-PC induces SMC migration at least in part through release of endogenous bFGF.

To further clarify the mechanism by which lyso-PC induces human coronary artery SMC migration, we examined the ability of vitamin E, a potent antioxidant, to inhibit lyso-PC–induced chemotaxis. Vitamin E ($5 \times 10^{-5}$ mol/L) significantly inhibited SMC migration induced by $5 \times 10^{-5}$ mol/L lyso-PC, whereas vitamin E could not inhibit the nonstimulated and bFGF-induced SMC migration (Figure 5). This suggested that the vitamin E–inhibitable step in the lyso-PC–induced migration was before stimulation by bFGF.

**Effects of PDGF-BB and ET-1 on Lyso-PC–Induced SMC Migration**

Figure 6 shows the effects of PDGF-BB and ET-1 on lyso-PC–induced SMC migration. Lyso-PC–induced migration was significantly enhanced by PDGF-BB. Lyso-PC–induced migration was also enhanced by ET-1. It is of note that ET-1 alone cannot induce SMC migration at the concentrations used in this study (Figure 6B).

**Table 2. Effect of ET\_A Receptor Antagonist BQ-123 on Enhancement by ET-1 of Lyso-PC–Induced Human Coronary Artery SMC Migration**

<table>
<thead>
<tr>
<th>Migration Activity, Cells/4HPF/6 h</th>
<th>Baseline</th>
<th>ET-1</th>
<th>Lyso-PC</th>
<th>Lyso-PC + BQ-123 $10^{-6}$ mol/L</th>
<th>ET-1 + lyso-PC</th>
<th>ET-1 + lyso-PC + BQ-123 $10^{-6}$ mol/L</th>
<th>ET-1 + lyso-PC + BQ-123 $10^{-7}$ mol/L</th>
<th>ET-1 + lyso-PC + BQ-123 $10^{-8}$ mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.7±2.2</td>
<td>11.1±2.5</td>
<td>24.1±3.7</td>
<td>23.4±4.2†</td>
<td>39.7±4.2‡</td>
<td>34.5±4.7†‡</td>
<td>29.3±4.1§</td>
<td>25.7±3.0†§</td>
</tr>
</tbody>
</table>

Values are mean±SD of four measurements. Migration activities are expressed as number of cells per four high-power fields (4HPF). ET-1 $10^{-7}$ mol/L and/or lyso-PC $5 \times 10^{-5}$ mol/L were added to the lower chamber with or without values concentrations ($10^{-5}$, $10^{-7}$, and $10^{-8}$ mol/L) of BQ-123.

*P<0.05 vs baseline.
†P<0.05 vs ET-1 alone.
‡P<0.05 vs lyso-PC alone.
§P<0.05 vs ET-1 plus lyso-PC.
SMC migration, cellular cAMP increased after treatment with AM also inhibited in a concentration-dependent manner between 10^{-5} and 10^{-6} mol/L adrenomedullin. In parallel with the inhibition by AM of lyso-PC–induced SMC migration, cellular cAMP, we examined the effect of 8-bromo-cAMP, a cAMP analogue, on lyso-PC (5 mol/L)–induced SMC migration. Human AM significantly inhibited 5 mol/L lyso-PC–induced SMC migration at concentrations of 10^{-5} and 10^{-6} mol/L. Human AM also inhibited 5 × 10^{-5} mol/L lyso-PC–induced migration in a concentration-dependent manner between 10^{-5} and 10^{-6} mol/L.

The effects of AM on cellular cAMP levels in cells treated with 5 × 10^{-6} or 5 × 10^{-5} mol/L lyso-PC are shown in Figure 7B. In parallel with the inhibition by AM of lyso-PC–induced SMC migration, cellular cAMP increased after treatment with AM (Figure 7A and 7B).

**Effect of AM on Lyso-PC–Induced SMC Migration**

Figure 7A shows effects of various concentrations (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} mol/L) of human AM on 5 × 10^{-5} mol/L or 5 × 10^{-5} mol/L lyso-PC–induced SMC migration. Human AM significantly inhibited 5 × 10^{-5} mol/L lyso-PC–induced migration at concentrations of 10^{-5} and 10^{-6} mol/L. Human AM also inhibited 5 × 10^{-5} mol/L lyso-PC–induced migration in a concentration-dependent manner between 10^{-5} and 10^{-6} mol/L.

The effects of AM on cellular cAMP levels in cells treated with 5 × 10^{-6} or 5 × 10^{-5} mol/L lyso-PC are shown in Figure 7B. In parallel with the inhibition by AM of lyso-PC–induced SMC migration, cellular cAMP increased after treatment with AM (Figure 7A and 7B).

**Effects of 8-Bromo-cAMP and Forskolin on Lyso-PC–Induced SMC Migration**

To elucidate whether the inhibitory effect of AM on lyso-PC–induced SMC migration is causally linked to the increase in cellular cAMP, we examined the effect of 8-bromo-cAMP, a cAMP analogue, on lyso-PC (5 × 10^{-5} mol/L)–induced SMC migration. Inhibition of lyso-PC–induced SMC migration by AM could be reproduced by this analogue at concentrations of 10^{-6} and 10^{-5} mol/L (Figure 8A).

Furthermore, the effect of forskolin, an activator of adenylate cyclase, on lyso-PC (5 × 10^{-5} mol/L)–induced SMC migration was examined. The addition of forskolin also reduced lyso-PC–induced SMC migration at concentrations of 10^{-5} and 10^{-4} mol/L (Figure 8B).

**Discussion**

In the present study, we show that lyso-PC, a prominent component of oxidized LDL,1,5 induces migration of human coronary artery SMCs. To the best of our knowledge, this is the first demonstration concerning the relationship between lyso-PC and SMC migration. This may support the finding shown by Auto and coworkers12 and subsequently by our group12 that oxidized LDL stimulates the migration of cultured rat or human SMCs, because phosphatidylcholine of LDL is found to be converted to lyso-PC during oxidized modification.5 Furthermore, both lyso-PC and oxidized LDL are shown to stimulate SMC proliferation.9,18 Several lines of evidence suggest that oxidized LDL19 and its major phospholipid component, lyso-PC, are present in the intima. Therefore, these observations raise the hypothesis that the conversion of phosphatidylcholine into lyso-PC in the intima may render it chemotactic for medial SMCs, thus contributing to plaque formation and atherogenesis in the coronary artery.

The specific mechanisms of action of lyso-PC as a chemotactic factor for human coronary artery SMCs remains to be established, but the present findings at least allow us to say something about the possibilities. bFGF was a weak chemotactant for human coronary artery SMCs and neutralizing antibody to bFGF–inhibited lyso-PC–induced migration of human coronary artery SMCs. Actually, lyso-PC increased bFGF production in these cells. These results raise the possibility that lyso-PC stimulates human coronary artery SMC migration at least in part through release of bFGF, which is similar to the mechanism involved in stimulation of SMC proliferation by lyso-PC.5 Furthermore, we showed that vitamin E significantly inhibited lyso-PC–induced SMC migration but could not inhibit the bFGF–induced SMC migration. It is therefore likely that the vitamin E–inhibitable step in the lyso-PC–induced SMC migration is before stimulation by bFGF.

Next, we showed that lyso-PC–induced migration could be enhanced by a potent mitogen, PDGF-BB, and the endothelium–derived vasoconstrictive and growth-promoting peptide ET-1. It is of note that ET-1 significantly enhanced lyso-PC–induced migration even though ET-1 alone had no effects on...
SMC migration, which means the synergism of ET-1 with lyso-PC. Actually, a low concentration of exogenous ET-1 is found to potentiate the vasoconstrictive or mitogenic action of norepinephrine, serotonin, or PDGF. In addition, ET-induced enhancement of SMC migration was clearly inhibited by the ET<sub>A</sub> receptor antagonist BQ-123. It is therefore likely that this naturally occurring phospholipid may function in combination with PDGF or ET-1 (possibly mediated by ET<sub>A</sub> receptors) and may contribute to the intimal thickening in coronary atherosclerotic lesions. Conversely, phosphatidylcholine was without significant activity, and lysophosphatidylinositol and lysophosphatidylserine were much less effective than lyso-PC. However, the precise structural specificity of lyso-PC that has such an effect on SMC migration remains to be clarified at this time.

Then, we showed that human AM strongly inhibits lyso-PC–induced migration of human coronary artery SMCs in a concentration-dependent manner. Actually, 5×10<sup>−7</sup> mol/L lyso-PC–induced SMC migration was significantly inhibited by human AM at concentrations of 10<sup>−8</sup> to 10<sup>−6</sup> mol/L. Although human AM (1–52) is the major circulating form of AM, the normal plasma concentrations (≈10<sup>−11</sup> to 10<sup>−10</sup> mol/L) are much lower than those of synthetic human AM that inhibited SMC migration in our study. However, local levels of AM in coronary artery tissues may be much higher than plasma concentrations of AM, because it has been demonstrated that a considerable amount of AM is synthesized in and secreted from vascular endothelial cells. Taking the matter into account, our results suggest that AM, by acting locally as a paracrine agent, inhibits the migration of human coronary artery SMCs. Recently, we showed that AM had a modest antiproliferative effect in cultured rat aortic SMCs. Consequently, these findings may raise the possibility that human AM in local vascular tissues antagonizes the development of intimal thickening during the process of coronary atherosclerosis. However, this experiment was done on cultured SMCs. Therefore, any extrapolation from the present experiment on cultured SMCs to in vivo conditions should be carefully performed.

Finally, we have shown that the inhibitory effect of AM on lyso-PC–induced SMC migration is causally linked to the increase in the cellular cAMP. Actually, human AM increased cAMP levels, and this effect paralleled the mediation of the inhibition effect of AM. The cAMP-elevating agents 8-bromo-cAMP and forskolin significantly inhibited lyso-PC–induced migration. These results suggest that AM inhibits lyso-PC–induced migration, at least in part, through a cAMP-dependent process.

In conclusion, our results indicate that AM, a novel endothelium-derived relaxing factor, and vitamin E, an antioxidant, inhibit lyso-PC–induced human coronary artery SMC migration. However, further studies will be necessary to clarify the exact cellular mechanisms responsible for cellular actions of lyso-PC and the interaction of this phospholipid and other vasoactive substances.

Acknowledgments

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture, Japan. The authors gratefully acknowledge the technical assistance of Atsumi Ohnishi, Yuka Inoshita, and Miyuki Kitakaze of the Division of Hypertension and Atherosclerosis, The First Department of Internal Medicine, Osaka City University Medical School.

References


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Circulation. 1998;98:353-359
doi: 10.1161/01.CIR.98.4.353

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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