Application of Nanoparticle Technology for the Prevention of Restenosis After Balloon Injury in Rats

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Abstract—Restenosis after percutaneous coronary intervention continues to be a serious problem in clinical cardiology. Recent advances in nanoparticle technology have enabled us to deliver an antiproliferative drug selectively to the balloon-injured artery for a longer time. NK911, which is a core-shell nanoparticle of polyethylene glycol-based block copolymer encapsulating doxorubicin, accumulates in vascular lesions with increased permeability. We first confirmed that balloon injury caused a marked and sustained increase in vascular permeability (as evaluated by Evans blue staining) for a week in the rat carotid artery. We then observed that intravenous administration of just 3 times of NK911, but not doxorubicin alone, significantly inhibited the neointimal formation of the rat carotid artery at 4 weeks after the injury in both a single- and double-injury model. Immunostaining demonstrated that the effect of NK911 was due to inhibition of vascular smooth muscle proliferation but not to enhancement of apoptosis or inhibition of inflammatory cell recruitment. Measurement of vascular concentrations of doxorubicin confirmed the effective delivery of the agent to the balloon-injured artery by NK911 in both a single- and double-injury model. RNA protection assay demonstrated that NK911 inhibited expression of several cytokines but not that of apoptosis-related molecules. NK911 was well tolerated without any adverse systemic effects. These results suggest that nanoparticle technology to target vascular lesions with increased permeability is a promising and safe approach for the prevention of restenosis after balloon injury.

The full text of this article is available at http://www.circresaha.org.

Key Words: nanoparticle ▪ restenosis ▪ angioplasty ▪ drug delivery

Percutaneous transluminal coronary intervention (PCI) is now widely used for the treatment of coronary artery disease; however, restenosis after the procedure continues to be a serious complication.1,2 Restenosis can be prevented by a local delivery of an antiproliferative agent to the dilated segment of the coronary artery. This strategy has been utilized for the treatment of cancers, and indeed many drug delivery systems (DDS) have been developed and tested for selective and efficient delivery of antiproliferative agents to tumor tissues.3-13 Tumor tissues are characterized by enhanced permeability and retention (EPR) effects, which include hypervasularity, enhanced permeability, and low wash-out of a drug delivered to the tissue.14 Recent advances in nanoparticle technology have enabled us to develop a nanoparticle carrier conjugated with an antiproliferative agent for the treatment of tumors with EPR effects. This includes NK911, which is a core-shell nanoparticle formed through a self-assembly of block copolymer conjugated with doxorubicin.13 NK911 consists of shell-forming hydrophilic segment (polyethylene glycol) and doxorubicin-conjugated hydrophobic segment of polyspartic acid. When NK911 is dissolved in the aqueous phase, it forms stable core-shell nanoparticles (polymeric micelles) with an average diameter of 40 nm and physically entraps free-doxorubicin to inner core (active component of the antiproliferative effect of NK911).13 NK911 can selectively penetrate through a tumor-vessel wall with EPR effects.13 Based on the previous reports concerning prolonged endothelial dysfunction after balloon injury,15,16 we hypothesized that balloon-injured coronary arteries also have EPR effects, and thus could be a good target for NK911. This prompted us to examine whether NK911 is effective for the prevention of restenosis after balloon injury in the rat carotid artery.

Materials and Methods

This experiment was approved by the Institutional Animal Care and Use Committee and was conducted in conformity with institutional guidelines. NK911 was provided by Nippon Kayaku Pharmaceutical Co (Tokyo, Japan). The pharmacologically effective dose of doxorubicin released from NK911 is 16% of the micelle.17
Time Course of the Increase in Vascular Permeability After Balloon Injury
A single balloon injury was created with a Fogarty catheter in the normal left rat carotid artery as previously described. Time course of the increase in vascular permeability was examined before, immediately after, and 1, 3, 5, and 7 days after the balloon injury (3 animals for each time point), when we administered Evans-Blue dye (35 mg/kg) intravenously and euthanized the animals 45 minutes after the administration. The balloon-injured carotid artery was carefully isolated, opened longitudinally, and analyzed at a magnification of 20×.

Single-Injury Model
Male Wistar-Kyoto rats (240 to 260 g) were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), and then a balloon injury of the left carotid artery was made as previously described. Six sham-operated rats also underwent the same surgical procedure except that the balloon was not inserted. NK911 (0.1, 1, and 10 mg/kg), doxorubicin alone (0.016, 0.16, and 1.6 mg/kg; adjusted for the corresponding content of doxorubicin in NK911) or saline vehicle was administered intravenously 3 times, immediately after, and 3 and 6 days after the balloon injury. For each dose, 6 animals were assigned in a random and blind manner. At 4 weeks after the balloon injury, the animals were killed with an overdose of pentobarbital, and the carotid artery was perfusion-fixed at 100 mm Hg with 10% formaldehyde, excised, and embedded in paraffin. The carotid segment (10 mm in length) was isolated from the middle of the balloon-injured artery, cut into 3 sections, and stained with hematoxylin-eosin in each rat (n=6 each for 3 doses of NK911 or doxorubicin alone). The medial and intimal areas, luminal area, and the length of the internal (IEL) and the external elastic lamina (EEL) were measured with a computerized digital image analysis system and averaged for 3 (distal, middle, and proximal) sections.

Double-Injury Model
In order to induce preceding vascular lesions, we made an initial balloon injury with a Fogarty catheter 2 weeks before creating a second balloon injury in the rat carotid artery. For the initial injury, we inserted the balloon catheter through the right iliac artery into the left carotid artery and performed balloon-injury of the artery as in the single-injury model. For the second injury, we inserted the catheter into the previously balloon-injured carotid artery, confirmed the position of the catheter under direct view, and performed balloon injury at the same site as in the first injury. The manner of drug administration (1 and 10 mg/kg for NK911 and 0.16 and 1.6 mg/kg for doxorubicin alone) and that of tissue analysis were the same as in the single-injury model. For each dose, 6 animals were assigned in a blind manner.

Cell Proliferation, Apoptosis, and Infiltration of Inflammatory Cells in the Injured Artery
In each serial section, proliferating cells were evaluated by PCNA staining and apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method using an in vivo apoptosis detection kit (WAKO). Inflammatory cells were evaluated by ED-1 immunostaining. Those analyses were performed 7 days after the balloon injury based on the previous studies with the same rat model of balloon injury. Six animals were assigned for 3 different doses of either NK911 or doxorubicin alone. In each experiment, rat small intestine was used as a positive control. A negative control was made without the PCNA, TdT, or ED-1 antibody. The number of cells positive for PCNA, TUNEL, or ED-1 staining was counted at a magnification of 400× in a blind manner. The quantitative analysis was performed in 3 sections (distal, middle, and proximal) from each carotid artery and averaged in each animal. The number of PCNA-positive cells in the 3 vascular layers (the intima, media, and adventitia) was counted for each section. The number of TUNEL-positive cells was expressed as a TUNEL index (TUNEL-positive cells/total nucleated cells). The number of ED-1-positive cells was counted in a whole section. All specimens were prepared at 1 week after the balloon injury.

RNA Protection Assay for Cytokines and Apoptosis-Related Molecules
We also performed RNA protection assay for control, NK911 (10 mg/kg), and doxorubicin (1.6 mg/kg) groups (n=12 each) at 1 week after the balloon injury in the injured carotid artery that was flash-frozen in liquid nitrogen. The stored artery was homogenized using the Isogen kit (WAKO), and mRNA was isolated. The mRNA expression was examined by RNA protection assay (RPA) using RNA template kit, RPA kit, and transcription kit (Farmingen, San Diego, Calif). The actual mRNA expression was corrected by GAPDH signal in each column.

Tissue Concentration of Doxorubicin
For measurement of tissue concentration of doxorubicin, the carotid arteries from the 3 groups were carefully excised and flasked by saline and weighed. The measurement was made at 8 time points, including before and 3 hours after the first drug administration on day 1, before and 3 hours after drug administration on day 3, on day 4, before and 3 hours after drug administration on day 6, and on day 7 (4 animals for each time point). In a single injury model, we also measured the concentration of doxorubicin in the contralateral artery. The measurement of doxorubicin was performed by the HPLC method.

Possible Side Effects of NK911
We measured body weight on a weekly basis, whereas we measured hemodynamic variables (by the tail-cuff method in conscious conditions) and liver/renal functions at 4 weeks after balloon injury with NK911 administration. We also checked hematology at 1 and 4 weeks after the NK911 treatment.

Statistical Analysis
Statistical analysis was performed by unpaired Student’s t test or ANOVA followed by Scheffé’s post hoc test. A value of P<0.05 was considered to be statistically significant.

Results
Animal Assignment
We used a total of 398 rats. In the protocol on the time-course of vascular permeability, we used 18 rats (3 each for 6 time
points after balloon injury). In the histology protocol, we used 48 in the single-injury model (3 different doses of NK911 and doxorubicin alone) and 36 in the double-injury model (2 different doses of NK911 and doxorubicin alone). For immunostaining protocol at 1 week after the injury, we used 48 for 3 different doses of NK911 and doxorubicin alone, while we used another 48 for cell count protocol in the same manner. Finally, we used 128 for measurement of tissue concentration of doxorubicin (4 each at 8 time points after either a single or double balloon injury for NK911 and doxorubicin alone), while we used the remaining 72 for RPA analysis.

**Sustained Increase in Vascular Permeability After Balloon Injury**

We first examined the time-course of the increase in vascular permeability at 6 time points after balloon injury using Evans-Blue dye staining (n = 3 each). The balloon-injured area was blue-stained, and the staining was noted at least for 7 days after the injury, confirming the presence of the EPR effects in the balloon-injured artery (Figure 1).

**Inhibitory Effect of NK911 in a Single-Injury Model**

We then examined whether NK911 inhibits vascular lesion formation 4 weeks after a single balloon injury in the rat carotid artery. When compared with the control group (balloon injury with no treatment), NK911 significantly and dose-dependently inhibited neointimal formation as evaluated by intima/media ratio (Figures 2A and 2B) and therefore maintained the lumen area at a maximum dose, whereas doxorubicin alone showed no inhibitory effects and failed to prevent the reduction in lumen area (Figure 2C). The inhibitory effect of NK911 was noted at a dose of 1.0 mg/kg (0.16 mg/kg of doxorubicin), which is approximately one fourth of its effective concentration for the treatment of cancers. In contrast, neither NK911 nor doxorubicin alone affected vascular remodeling (reduction in total cross-sectional area) as evaluated by the length ratio of IEL and EEL (data not shown).

Measurement of doxorubicin concentration in the balloon-injured artery showed that NK911 delivered the antiproliferative agent more effectively than intravenous administration of the drug alone to the balloon-injured arteries, whereas the concentration was low in the contralateral arteries at all time points (Figure 2D). Especially at 3 hours after the balloon injury, the doxorubicin concentration in the balloon-injured artery showed that NK911 delivered the antiproliferative agent more effectively than intravenous administration of the drug alone to the balloon-injured arteries, whereas the concentration was low in the contralateral arteries at all time points (Figure 2D). 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concentrations were 4-fold higher in the NK911 group than in the doxorubicin-alone group (Figure 2D).

Inhibitory Effect of NK911 in a Double-Injury Model
We further examined whether NK911 also inhibits vascular lesion formation in a double-injury model. The carotid artery lesion was induced by a balloon injury 2 weeks before a second balloon injury. At 4 weeks after the second injury, NK911 again inhibited the neointimal formation compared with the control group (Figures 3A and 3B) and maintained the lumen area at its maximum dose (Figure 3C). Again, neither NK911 nor doxorubicin affected the vascular remodeling (data not shown). In this double-injury model, NK911 again delivered doxorubicin to the balloon-injured artery more effectively than intravenous administration of the drug alone (Figure 3D). Thus, the efficacy of NK911 for the prevention of restenosis after balloon injury has been confirmed in those two models.

Mechanisms for the Inhibitory Effects of NK911
We then attempted to elucidate the mechanisms for the inhibitory effect of NK911 on neointimal formation after balloon injury. For this purpose, we performed immunostainings for PCNA, TUNEL, and ED-1, and RNA protection assay (RPA) for the expressions of cytokines and apoptosis-related molecules. The number of total cells in the injured carotid arteries was significantly decreased in the NK911 group as compared with the control or doxorubicin alone group in all 3 layers (Figure 4A). Furthermore, the inhibitory effect of NK911 was due in part to the inhibition of VSMC proliferation (Figure 4B) rather than enhancement of VSMC apoptosis (Figure 4C) or inhibition of macrophage recruitment (Figure 4D). The RPA analysis further demonstrated that NK911 significantly suppressed the expressions of several cytokines (e.g., IL-1α, IL-6, IL-10, and TNF-β) (Figure 5A) but did not affect the expressions of apoptosis-related molecules (Figure 5B). These results are consistent with the findings with the PCNA and TUNEL stainings.

Side Effects of NK911
NK911 was well tolerated, and no side effects of NK911 were noted in terms of the time course of body weight (Table 1), hemodynamic variables, or liver/renal functions at 4 weeks after the NK911 treatment (Table 2). No abnormality was also found in hematology at 1 (data not shown) or 4 weeks (Table 2) after the treatment.

Discussion
The present study demonstrates that NK911, a nanoparticle carrier conjugated with doxorubicin, may be an effective and...
safe treatment for the prevention of restenosis after balloon injury.

**EPR Effect**

EPR effect was first recognized in tumor tissues. Most solid tumors have elevated levels of several factors that enhance vascular permeability, such as bradykinin, nitric oxide, peroxynitrite, prostaglandin, VEGF, and matrix metalloproteinases. Furthermore, high vascular density and impaired lymphatic drainage enhance the accumulation of delivered agents. Enhanced vascular permeability is also observed in granuloma and inflammatory and infected tissues. After balloon injury, endothelial cells are removed, and consequently, local inflammation occurs at the balloon-injured site. In the present study with balloon injury in the rat carotid

Figure 4. Mechanisms for the inhibitory effects of NK911 on neointimal formation after balloon injury. A, Total cell count per section at 1 week after balloon injury in the rat carotid arteries. B through D, Number of cells (per section) positive for PCNA (B), TUNEL (C), or ED-1 (D) immunostaining at 1 week after the injury. Results are mean ± SEM. **P < 0.01 vs control group; †P < 0.05, ‡P < 0.001 vs doxorubicin (DOX) alone group.

Figure 5. RNA protection assay for cytokines and apoptosis-related molecules. RNA protection assay for cytokines (A) and apoptosis-related molecules (B). Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01 vs control group; †P < 0.05 vs doxorubicin (DOX) alone group.
The NK911 accumulation is accelerated in the balloon-injured artery with a lower entrapment rate by the liver or the spleen when compared with the micelle with a neutral charge. In this study, NK911 significantly suppressed the vascular lesion formation after balloon injury more effectively than the drug alone (0.16 mg/kg content of doxorubicin) at lower concentrations required for the treatment of cancers (0.2 to 0.6 mg/kg, administered intravenously, 3 to 4 times a week, repeated 2 to 3 times). Furthermore, measurement of tissue concentrations of doxorubicin confirmed the effectiveness of NK911 to selectively deliver the agent to the balloon-injured artery in vivo. Because the most significant difference in the tissue doxorubicin concentrations was noted immediately (3 hours) after balloon injury, it is conceivable that a single intravenous administration of NK911 might be enough to suppress the subsequent vascular lesion formation, although this point remains to be examined in a future study.

Selective Delivery of Doxorubicin by NK911 In Vivo

NK911 is observed to accumulate in the vascular lesion where permeability is increased. The NK911 accumulation may be mediated by two mechanisms; first, the size of the micelle may be adequate for enhanced accumulation. Indeed, it was demonstrated that the size of the micelle is critical for accumulation of the nanoparticle and that oversized micelles may result in reduced tissue accumulation. Second, the surface charge of the micelle may be important. The surface of NK911 is negatively charged, whereas the luminal surface of the injured blood vessel is positively charged. Therefore, the NK911 accumulation is accelerated in the balloon-injured artery, which should facilitate the efficient delivery of doxorubicin by NK911 to the balloon-injured artery in vivo.

Mechanisms for the Inhibitory Effect of NK911 on Neointimal Formation After Balloon Injury In Vivo

It was recently suggested that one of the antiproliferative effects of doxorubicin is mediated by enhanced apoptosis through several apoptotic pathway. However, it was also reported that doxorubicin damages the cell membrane but does not induce cell apoptosis, unlike other anthracycline family members. In this study, NK911 inhibited vascular proliferation but did not enhance apoptosis. Indeed, NK911 suppressed neointimal formation and the expression of several cytokines that promote VSMC proliferation. Adriamycin downregulates the expression of the cyclin D1, the major regulator of cell cycle into the proliferative stage in several tumor cell lines. It was also reported that doxorubicin suppressed the expression of oncogene c-my c and c-jun in rat tumor cell lines.

TABLE 1. Time Course of Body Weight (kg)

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<tr>
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<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
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<tr>
<td>Control</td>
<td>296±11</td>
<td>292±8</td>
<td>311±11</td>
<td>319±10</td>
<td>324±11*</td>
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<td>Doxorubicin, mg/kg</td>
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<td>294±4</td>
<td>297±5</td>
<td>303±5</td>
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<td>NK911, mg/kg</td>
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<td>300±9</td>
<td>316±11</td>
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<td>10</td>
<td>295±9</td>
<td>298±10</td>
<td>307±8</td>
<td>320±10</td>
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Results are expressed as mean±SEM (n=6 each). Control indicates control group without any treatment; doxorubicin, doxorubicin alone group; NK911, NK911 group. *P<0.05 vs week 0.

TABLE 2. Hemodynamic Variables, Liver/Renal Functions, and Blood Analysis (4 Weeks After Balloon Injury)

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<td>0.17±0.03</td>
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<td>141±10</td>
<td>124±26</td>
<td>118±19</td>
<td>136±23</td>
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<td>131±15</td>
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<td>ALT, IU/L</td>
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<td>BUN, mg/dL</td>
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<td>WBC, per μL</td>
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<td>2267±211</td>
<td>2583±381</td>
<td>3117±457</td>
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<td>2350±145</td>
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<td>RBC, ×10⁵/μL</td>
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<td>801±24</td>
<td>852±22</td>
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<td>799±30</td>
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<td>Ht, %</td>
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<td>Plts, ×10⁴/μL</td>
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Results are expressed as mean±SEM (n=6 each). Control indicates control group without any treatment; doxorubicin, doxorubicin alone group; NK911, NK911 group; T. Bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Ht, hematocrit; and Plts, platelets.
glioblastoma cell line. Furthermore, doxorubicin directly inhibits the release of cytokine (eg, IL-6, IFN-γ) from stimulated peripheral mononuclear cell at its nontoxic concentration in vitro. These mechanisms may contribute to the inhibition of VSMC proliferation by NK911 after balloon injury. In contrast, NK911 did not affect vascular remodeling. Thus, NK911 may be more useful for the prevention of vascular lesion formation after coronary stenting rather than that after balloon angioplasty alone, because the contribution of neointimal formation to restenotic vascular lesion formation is greater after stenting than after balloon injury alone.

Safety of NK911
In this study, NK911 caused no side effects while it significantly suppressed the restenotic changes of the carotid artery after balloon injury. Indeed, it was previously confirmed that NK911 causes no damage of major organs when examined histologically. Furthermore, it was previously shown that the cardiotoxicity of doxorubicin is markedly reduced when selectively delivered in NK911. It has been recently reported that NK911 exerts anticancer effects at a dose of up to 24 mg/kg without any major side effects. In this study, we were also able to rule out the involvement of the potential cytotoxic effect of doxorubicin in the biological effect of NK911.

Limitations of the Study
Several limitations of the present study should be mentioned. First, the effect of bare micelle alone was not examined in this study. NK911 consists of PEG-conjugated doxorubicin in the micelle that is pharmacologically ineffective and free doxorubicin incorporated inside the micelle that is pharmacologically effective. Both components of doxorubicin are required for the stability of NK911. Thus, the “bare micelle” alone may not be a suitable control for NK911, and instead, we examined the effect of doxorubicin alone as a control in this study. Second, the double-injury model in this study may not represent atherosclerotic blood vessels in humans. Thus, the inhibitory effects of NK911 should be tested in atherosclerotic animal models in primateS before its application to humans. Third, the frequencies of TUNEL- or PCNA-positive cells were relatively high in this study although the results are consistent with those of the previous studies. Thus, we consider that the frequencies may not represent the actual rate of apoptosis or proliferation but may rather reflect the relative extent of those processes. Fourth, doxorubicin has a potential cytotoxicity that may affect DNA function with resultant carcinogenicity. Although intravenous administration of the agent just 3 times appears to be enough to suppress the vascular lesion formation, it may be more appropriate to deliver antiproliferative agents with less cytotoxicity by using nanoparticles. Fifth, NK911 is a first-generation nanoparticle that utilizes only the EPR effects of balloon-injured artery. More sophisticated nanoparticle systems need to be developed. Indeed, with recent advances in nanotechnology, several nanoparticles that can specifically recognize surface antigens or differences in tissue composition or temperature have already been developed.

In summary, the present study demonstrates that nanoparticle technology targeting balloon-injured arteries with increased permeability is a promising and safe approach for the prevention of restenosis after the procedure.

Acknowledgments
This study was supported in part by the grants-in-aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan (Nos. 09470169, 10177223, 10357006, 12032215, and 12470158) and from the Japanese Ministry of Health, Labor and Welfare, Tokyo, Japan. We thank Prof S. Mohri at the Center of Biomedical Research, Kyushu University Graduate School of Medical Sciences, for cooperation in this study, M. Sonoda and E. Gunshina for excellent technical assistance, and Nippon Kayaku Co, LTD, for providing NK911.

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_Circ Res._ 2003;92:e62-e69; originally published online March 27, 2003;
doi: 10.1161/01.RES.0000069021.56380.E2

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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