Simvastatin Preserves Coronary Endothelial Function in Hypercholesterolemia in the Absence of Lipid Lowering

Stephanie H. Wilson, Robert D. Simari, Patricia J.M. Best, Tim E. Peterson, Lilach O. Lerman, Michael Aviram, Karl A. Nath, David R. Holmes, Jr, Amir Lerman

Abstract—Recent evidence suggests that some benefit from the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors may occur independent of lipid lowering. We aimed to determine the effect of simvastatin on coronary endothelial function, endothelial NO synthase (eNOS) expression, and oxidative stress in experimental hypercholesterolemia (HC) in the absence of cholesterol lowering. Pigs were randomized to 3 experimental groups: normal diet (N group), high cholesterol diet (HC group), and HC diet with simvastatin (HC+S group) for 12 weeks. Low density lipoprotein cholesterol was similarly increased in the HC and HC+S groups compared with the N group. In vitro analysis of coronary large- and small-vessel endothelium-dependent vasorelaxation was performed. The mean vasorelaxation of epicardial vessels to bradykinin was significantly attenuated in the HC group compared with the N group (32.3±1.2% versus 42.9±1.6%, respectively; P<0.0001). This attenuation was significantly reversed in the HC+S group (38.7±1.5%, P<0.005 versus HC group). The maximal vasorelaxation to substance P was significantly attenuated in the HC group compared with the N group (50.5±11.9% versus 79.3±5.3%, respectively; P<0.05). This attenuated response was normalized in the HC+S group (74.9±4.1%, P<0.05 versus HC group). The maximal arteriolar vasorelaxation to bradykinin was also significantly attenuated in the HC group compared with the N group (71.9±4.9% versus 96.8±1.34%, respectively; P<0.005). This was reversed in the HC+S group (98.4±0.6%, P<0.0001 versus HC group). Western blotting of coronary tissue homogenates for eNOS demonstrated a decrease in protein levels in the HC group compared with the N group, with normalization in the HC+S group. Elevation of plasma F2-isoprostanes and thiobarbituric acid–reactive substances, markers of oxidative stress, occurred in the HC compared with the N group. These changes were reversed in the HC+S group. In summary, simvastatin preserves endothelial function in coronary epicardial vessels and arterioles in experimental HC (in the absence of cholesterol lowering) in association with an increase in coronary eNOS levels and a decrease in oxidative stress. These alterations may play a role in the reduction in cardiac events after treatment with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors.

Keywords: hypercholesterolemia ■ nitric oxide ■ vasorelaxation ■ endothelial NO synthase

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reatment of hyperlipidemia with the 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors (HRIs) has led to a significant reduction in major coronary events in humans. One of the earliest beneficial sequece after initiation of lipid-lowering therapy with these agents is an improvement in endothelium-dependent vasorelaxation, suggesting an increase in the bioavailability of the vasorelaxant NO. NO is a powerful local vasodilator, but it is also involved in the regulation of smooth muscle proliferation and endothelial cell–leukocyte interactions and may play an important part in the atherogenic process. Thus, agents that alter NO bioavailability may have a beneficial effect on atherogenesis.

Recent evidence has suggested that the HRIs may have important therapeutic effects in hypercholesterolemia (HC), in the absence of cholesterol lowering. First, in vitro studies have shown that the HRIs directly upregulate endothelial NO synthase (eNOS), the constitutive enzyme catalyzing the synthesis of NO in the vasculature. Furthermore, a lipid-independent decrease in superoxide formation by macrophages has been demonstrated, consistent with an antioxidant effect. However, there is a paucity of data regarding the effects of the HRIs in hypercholesterolemia, in the absence of lipid lowering, in vivo.

Experimental porcine HC is characterized by a decrease in NO bioavailability, with downregulation of eNOS in association with increased production of oxygen-derived free radicals that may inactivate NO. Thus, a decrease in NO production and an increase in its breakdown may contribute to abnormal endothelium-dependent vasorelaxation. We hy-
pothesized that the HRIs may have direct beneficial effects on NO bioavailability and oxidative stress in vivo. Thus, we designed the present study to determine the effects of simvastatin, an HRI, on large- and small-vessel coronary endothelial function, coronary eNOS, cGMP production, and oxidative stress in the setting of experimental HC in the absence of cholesterol lowering.

Methods

Animals
The study procedures and handling of animals were reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee. Experiments were conducted on female juvenile domestic crossbred pigs weighing 25 to 37 kg each. Twenty-nine animals were placed on a diet of 2% cholesterol and 15% lard by weight (TD 93296, Harlan Teklad) for a total of 12 weeks, as previously described. The hypercholesterolemic control group (HC group, n = 15) was not placed on any additional drug. The other 14 pigs were placed on simvastatin (HC+5 group), an HRI, initially at 40 mg/d and increasing to 80 mg/d after 5 weeks. The dosing was based on previous animal studies and clinical practice in humans. A group of 14 pigs fed a normal diet (N group) was used as the control. After 12 weeks, the animals were euthanized with an intravenous overdose of pentobarbital sodium (30 mg/kg IV), and the coronary arteries were harvested.

Lipid Parameters
Plasma total, LDL, and HDL cholesterol levels were measured after completion of 12 weeks of therapy. Plasma total and LDL cholesterol levels were determined by applying the technique of Allain et al., with use of a commercial reagent (Roche).

In Vitro Determination of Vascular Reactivity

Epicardial Vessels
The method has been previously described in detail. In brief, segments of the left circumflex artery 2 to 3 mm long were dissected. To determine the endothelium-dependent vasorelaxation properties of epicardial arteries, the arteries were first contracted with 10^{-5} mol/L endothelin-1 (Phoenix) and then relaxed with cumulative concentrations of bradykinin (10^{-11} to 10^{-6} mol/L, Sigma Chemical Co), substance P (10^{-11} to 10^{-8} mol/L, Sigma), and calcium ionophore (10^{-11} to 10^{-8} mol/L, Sigma). At the end of these experiments, 10^{-12} mol/L papaverine (Sigma) was added to determine the maximal vasodilatory capacity of the vessel.

Arterioles
Coronary vasomotor tone in the arterioles was determined by using a previously described method. In brief, segments of the secondary branch of the left circumflex artery (<500 μm in diameter) were dissected with use of a dissecting microscope. The vessels were precontracted with 10^{-6} mol/L endothelin-1, and then the response to 10^{-11} to 10^{-6} mol/L bradykinin was recorded.

In addition, epicardial vessels and arterioles were preincubated with Nω-monomethyl-l-arginine (L-NMMA, 10^{-4} mol/L, Sigma), the NO synthase inhibitor, for 20 minutes before the addition of endothelium-dependent agents to assess the contribution of endogenous NO release in vasorelaxation. The contraction attained with endothelin-1 for each vessel at baseline was considered as 0% relaxation. Subsequent measurements of coronary artery relaxation were expressed as a percent reduction in contraction (with the maximal relaxation attained with papaverine being 100% relaxation). At least 5 rings were used in each group of experiments, and no more than 2 rings from each animal were used per group of experiments.

Western Blotting for eNOS
Arteries from 3 pigs in each experimental group were snap-frozen in liquid nitrogen and subsequently homogenized in lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1% NP-40, and 0.5% sodium deoxycholate) by use of a tissue homogenizer. The lysate was analyzed for protein content by a Bradford assay (Bio-Rad). Equal amounts of protein were resolved under reducing conditions on an 8% SDS-polyacrylamide gel. Immunoblotting was performed with use of a monoclonal antibody to eNOS (Signal Transduction Laboratories) at a dilution of 1:1000 in a nonfat milk/Tris buffer. The membrane was subsequently probed with a secondary anti-mouse antibody conjugated to horseradish peroxidase (Amersham Life Sciences) at a dilution of 1:5000 and developed with chemiluminescence (Roche). The membrane was then exposed to x-ray film (Kodak), which was subsequently developed. Densitometry was performed by use of NIH image.

Immunohistochemistry for eNOS
One section taken from the proximal segment of the circumflex artery was analyzed from 8 animals in each group, as previously described. The sections were mounted, dried, and fixed. Endogenous peroxidase activity was blocked by incubating slides in 1.5% hydrogen peroxide/50% absolute methanol for 10 minutes. The slides were then placed in 0.25% SDS for 10 minutes at room temperature. Nonspecific protein binding sites were blocked with 5% goat serum diluted in PBS/0.05% Tween 20 at room temperature for 10 minutes. A monoclonal antibody to eNOS was then used (Signal Transduction Laboratories). This antibody was diluted to 1:500 in 1% goat serum and PBS/0.05% Tween 20 and applied to the slides overnight at 4°C. A biotinylated secondary antibody was then applied (mouse IgG) at a 1:400 dilution for 30 minutes with 1% normal goat serum and 2% normal swine serum. The slides were subsequently incubated for 30 minutes with streptavidin–horseradish peroxidase (Dako, 1:500 dilution). After a further washing, color development was performed with the use of 3-aminophthalene dihydrochloride substrate solution for 15 minutes at room temperature. A counterstain was then performed with the use of hematoxylin for 30 seconds. The slides were then rinsed for 5 minutes in running tap water and mounted in aqueous glycerol gelatin media.

cGMP Production
Vascular rings were collected from 10 animals in each experimental group, and the endothelium was manually removed by rolling the vessel with wire. The rings were then placed in an organ chamber filled with Krebs’ solution. After 1 hour of incubation, 146 μL of 3-isobutyl-1-methylxanthine (10^{-4} mol/L) and 100 μL of indomethacin (10^{-4} mol/L) were added to the solution for 30 minutes. Samples were then randomized to either control or the NO donor, diethylamine NONOate (DEA) (10^{-6} mol/L), for 1 minute and then shock-frozen.

Homogenization was carried out on dry ice with the addition of 2 mL of absolute alcohol. After being allowed to sit for 5 minutes, the samples were centrifuged for 10 minutes at 10°C. Samples were dried, and 500 μL of Tris buffer was added, after which they were centrifuged as before and placed on ice. After the preparation of standards, 50 μL of radioactive dye and 50 μL of antiserum were subsequently added to both samples and standards. After vortex mixing, tubes were placed on ice and refrigerated for 90 minutes, ammonium sulfate was added, and samples were allowed to sit for 5 minutes. The samples were subsequently centrifuged for 10 minutes at 4000 rpm at 10°C. Supernatant was discarded, 1.1 mL of water added, and the samples were vortexed again. Finally, samples were placed in a scintillation counter.

Plasma Lipid Peroxidation

Plasma F_{2}-Isoprostanes
Blood samples were taken from each group after 12 weeks of the experiment. Samples were collected in EDTA tubes, and the plasma was stored at -80°C until the time of the assay. The total levels of 8-isoprostaglandin F_{2α} were measured with an enzyme immunoassay kit (EIA, Cayman). Before the enzyme immunoassay, an alkaline hydrolysis was used. Plasma samples were purified by Sep-Pak C18 columns (Milford) before analysis. The samples, tracer, and antiserum were added to wells precoated with mouse monoclonal antibody. The plates were washed to remove all unbound reagents. Ellman’s reagent (containing the substrate to acetylcholinesterase) was added and the absorbance was measured at 412 nm. The rate of reaction was used to calculate the amount of F_{2α} in the plasma.
Potassium phosphate (pH 7.0), 1 mmol/L EDTA, 1 mmol/L NaN₃, 1 mL) was added to 0.8 mL of mixture consisting of 50 mmol/L sucrose by using a Polytron homogenizer (Brinkmann) for 15 seconds. The homogenates were centrifuged for 1 hour at 105 000 g.

In Vitro Determination of Vascular Reactivity

Epicardial Vessels

The mean vasorelaxation to bradykinin was significantly attenuated in the HC group compared with the N group (32.3±1.2% versus 42.9±1.6%, P<0.0001; Figure 1). There was increased vasorelaxation to bradykinin in the HC+S group compared with the HC group (38.7±1.5%, P<0.005 versus HC group; Figure 1). There was no difference in the precontraction to endothelin-1 between the groups. The vasorelaxation response to bradykinin in all groups was significantly attenuated by preincubation of the vessels with the NO synthase inhibitor L-NMMA (P<0.0001 in all cases).

In addition, the enhanced vasorelaxation response to bradykinin in the N and HC+S groups compared with the HC group was abolished by preincubation of the vessels with L-NMMA.

Statistical Analysis

Data are expressed as mean±SEM. In vitro vascular reactivity was analyzed by a 2-way ANOVA, followed by a Bonferroni t test. TBARS, F₂-isoprostanes, catalase, glutathione peroxidase, cGMP, and eNOS protein levels were analyzed by a 1-way ANOVA, followed by a Bonferroni t test. Statistical significance was inferred at a value of P<0.05.

Results

Plasma Lipid Parameters

There was a significant increase in the plasma total, LDL, and HDL cholesterol concentrations in all the cholesterol-fed pigs (HC and HC+S groups) compared with the pigs fed a normal diet (N group, P<0.005 in all cases; see Table). The lipid levels of the HC group and the HC+S group were not significantly different from each other.

Plasma HMGCoA Reductase Activity

HMGCoA reductase activity was measured in 6 pigs in the HC and HC+S groups. There was no detectable activity in any of the HC group, but there was detectable activity in all of the 6 HC+S pigs measured (median 0.7, interquartile range 0.5 to 1.0; P<0.005 between groups).

Figure 1. Concentration-response curves to bradykinin in epicardial arteries in N, HC, and HC+S groups. *P<0.05 for HC group compared with HC+S group.

Figure 2. Maximal vasorelaxation to substance P in epicardial vessels in N, HC, and HC+S groups. *P<0.05 for HC group compared with HC+S and N groups.
The maximal vasorelaxation to substance P was significantly attenuated in the HC group compared with the N group (50.5 ± 11.9% versus 79.3 ± 5.3%, respectively; \( P < 0.05 \)). This attenuated response was normalized in the HC + S group (74.9 ± 4.0%, \( P < 0.05 \) versus HC group; Figure 2). In addition, although there was no significant attenuation of vasorelaxation to another endothelium-dependent agent, calcium ionophore, in the HC group compared with the N group, there was enhanced vasorelaxation in the HC + S group (40.0 ± 1.3%) compared with the HC group (31.8 ± 1.3%, \( P < 0.05 \)). There was no attenuation of the vasorelaxation to sodium nitroprusside, an endothelium-independent agent, in the HC compared with the N group.

**Arterioles**

The maximal arteriolar vasorelaxation to bradykinin was also significantly attenuated in the HC pigs compared with the N pigs (71.9 ± 4.9% versus 96.8 ± 1.34%, respectively; \( P < 0.005 \)). This was completely reversed in the HC + S group (98.4 ± 0.6%, \( P < 0.0001 \) versus HC group; Figure 3). The difference between the HC + S and HC groups was abolished by preincubation of the HC + S vessels with L-NMMA.

**Immunohistochemistry for eNOS**

Immunoreactivity for eNOS was present in an intact endothelial cell layer in the N, HC, and HC + S groups, by use of labeling with the monoclonal antibody to eNOS (Figure 4). Western blotting was used to quantify the amount of eNOS present in the 3 groups.

**Western Blotting**

Western blotting is shown in Figure 4. eNOS protein was present in the vessel wall in all 3 homogenates from the N group and was significantly decreased in the HC group (\( P < 0.05 \)). eNOS protein was also present in all 3 of the coronary tissue homogenates from the HC + S group and was significantly increased compared with that in the HC group (\( P < 0.05 \)). Ponceau staining confirmed equal protein loading between lanes.

**cGMP Levels**

Baseline generation of cGMP was the same in all 3 groups (\( P = NS \)). However, there was significantly greater generation of cGMP in response to DEA in the HC group compared with the N group (99.2 ± 10.9 versus 67.1 ± 3.7 pmol/mg protein, \( P < 0.001 \)).
P < 0.05). This was completely reversed after treatment with simvastatin (60.2 ± 4.5 pmol/mg protein, P < 0.05).

**Plasma Lipid Peroxidation**

**Plasma F₂-Isoprostanes**
There was a significant increase in the plasma F₂-isoprostane levels in the HC group (n = 14) compared with the N group (n = 10; 203.4 ± 12.1 versus 124.3 ± 16.0 pg/mL, respectively; P < 0.005). There was a significant decrease in the levels in the HC + S group (n = 9) compared with the HC group (150.7 ± 12.1 pg/mL, P < 0.05; Table). There was no significant difference in levels between the HC + S and N groups.

**TBARS Assay**
With use of the TBARS assay, the HC group had a significantly higher level of aldehydes compared with the N group (6.2 ± 0.2 versus 4.5 ± 0.3 nmol MDA equivalent per milliliter, P < 0.005; both n = 6). This high plasma lipid peroxidation in the HC group was significantly decreased by treatment with simvastatin (3.9 ± 0.1 nmol MDA equivalent per milliliter, P < 0.0001; n = 6; Table). There was no significant difference in levels between the HC + S group and the N group.

**Catalase and Glutathione Peroxidase Assay**
There was a decrease in the catalase levels in the HC group compared with the N group (6.0 ± 0.4 versus 7.3 ± 0.8 μmol H₂O₂ consumption per minute per milliliter), which was reversed in the HC + S group (7.5 ± 0.5 μmol H₂O₂ consumption per minute per milliliter). However, these changes did not reach statistical significance (P = 0.14). There was no significant difference between the glutathione peroxidase levels in the 3 groups (see Table).

**Discussion**

The present study demonstrates that simvastatin, an HRI, preserves endothelium-dependent vasorelaxation in large and small coronary vessels in porcine experimental HC, despite no change in plasma lipids. This effect was associated with normalization of eNOS protein levels and cGMP activity. Moreover, in vivo plasma markers of oxidative stress were attenuated by treatment with simvastatin. These studies suggest a role for the HRIs in reducing cardiac morbidity and mortality, beyond their effect on cholesterol levels.¹ ²

One of the beneficial effects of the HRIs is a restoration of endothelial function.²⁰ ²¹ Multiple studies in the setting of HC have demonstrated an improvement in endothelium-dependent vasodilation after treatment,³ ⁴ ²² ²³ suggesting an increase in NO bioavailability. This improvement has been attributed primarily to the ability of these agents to inhibit cholesterol biosynthesis by blocking the conversion of HMG-CoA to mevalonate, with a subsequent increment in LDL receptor activity, resulting in a reduction in plasma cholesterol levels. Increasing evidence is emerging that the HRIs may have benefits independent of their lipid-lowering effects. In support of this, some HC studies have shown no correlation between the extent of lipid lowering by the HRIs and improvement in endothelial function.²² In addition, a recent study in primates showed that pravastatin, in combination with a lipid-lowering diet, improved acetylcarnine-mediated vasodilatation in vivo more than a lipid-lowering diet alone, despite no incremental decrease in plasma lipids.²³ We have previously demonstrated that porcine experimental HC is characterized by a decrease in coronary eNOS immunoreactivity and decreased endogenous NO activity.⁵ In the present study, we have provided multiple lines of evidence that suggest a functional increase in NO via a direct simvastatin effect. Bradykinin, substance P, and calcium ionophore lead to vasorelaxation via different endothelial receptors, but the release of NO is involved in the action of each.²⁴ ²⁵ Furthermore, when vessels were preincubated with L-NMMA, an NO synthase inhibitor, the enhanced vasorelaxation response to bradykinin in the simvastatin-treated group was abolished. In contrast to previous animal and human data, a recent study suggested that patients with only modestly elevated cholesterol levels did not have improvement in endothelial function after treatment with a statin.²⁶ It may be that significantly elevated cholesterol levels need to be present at baseline to benefit from either the lipid-lowering or direct effect of the statins.

The abnormal endothelial function seen with HC also occurs in the microcirculation.²⁷ ²⁸ Impaired vasodilation of the microcirculation may lead to myocardial perfusion abnormalities and ischemia, even without obstructive coronary disease.²⁹ In addition, an improvement in myocardial ischemia has been demonstrated after treatment with HRIs,³⁰ ³¹ out of proportion with alterations in epicardial artery narrowing. However, these improvements have thus far been demonstrated only in the presence of significant plasma cholesterol lowering. We have shown a significant improvement in microvascular endothelial function after treatment with simvastatin, again despite no changes in plasma cholesterol levels. The enhanced response to bradykinin was more prominent in the microcirculation than the epicardial vessels, although this enhanced response was blocked by preincubation with L-NMMA, again suggesting a role for NO. This may have important implications for myocardial perfusion, because treatment with HRIs, even in the absence of cholesterol lowering, may lead to a decrease in the potential for myocardial ischemia.

The underlying mechanisms leading to an increase in NO bioavailability by simvastatin may be multifactorial. In vitro data have strongly suggested that the HRIs have a direct endothelial effect, preventing downregulation of eNOS protein and mRNA by oxidized LDL.³² ³³ The present study extends these observations and demonstrates in vivo upregulation of eNOS protein levels after simvastatin treatment in experimental HC, independent of lipid lowering. The HRIs, in addition to playing a pivotal role in alteration in cholesterol synthesis, are known to have an impact on other metabolites of the mevalonate pathway. In vitro studies have suggested a role for these metabolites in the regulation of eNOS expression,³³ although the in vivo mechanism remains to be elucidated. Clearly, the in vivo mechanism remains to be elucidated. The present study could have significant impact on endothelial NO production.

Previous studies have demonstrated that removal of the endothelial NO synthesis pathway by denuding the endothelium results in an augmented response and increased sensitivity of the smooth muscle to NO donors.³⁴ We have previously reported an increase in production in cGMP in the vascular smooth muscle in response to the NO donor DEA in
Simvastatin Preserves Endothelial Function

HC in the renal vasculature. In the present study, we have confirmed these findings in the coronary vasculature. Furthermore, this augmented production of cGMP found in the HC group was completely reversed after treatment with simvastatin. This is consistent with simvastatin treatment leading to a chronic increase in endothelial NO bioavailability, resulting in downregulation of the smooth muscle cell responsiveness to this increased production.

Although a direct increase in eNOS protein levels and increased production of NO may contribute to the improvement in endothelial function seen with the HRIs, other mechanisms may be involved. As an alternative or perhaps additional mechanism, simvastatin may attenuate the degradation of NO by reducing oxidative stress. The overproduction of reactive oxygen species has previously been demonstrated in experimental HC with subsequent enhanced LDL oxidation. The HRIs have been shown to attenuate oxidative stress in the context of lipid lowering, with decreased levels of lipid peroxides and attenuation of the response of LDL to oxidation in vitro. Recent in vitro studies have demonstrated a lipid-independent decrease in superoxide formation by macrophages after HRI treatment, consistent with an antioxidant effect. We have now reported, for the first time in vivo, a decrease in 2 independent parameters of oxidative stress (isoprostanes and aldehydes) after treatment with simvastatin, despite no reduction in total plasma or LDL cholesterol levels. One of these parameters, 8-epi-prostaglandin F₂, is one of a novel family of prostaglandin isomers, the F₂-isoprostanes, which have recently been described as a reliable measure of in vivo oxidative stress. The decrease in oxidative stress was not due to an increase in the antioxidant defense enzymes, glutathione peroxidase or catalase. However, we cannot rule out the possibility that other mechanisms may be involved, including alterations in the enzyme, superoxide dismutase. Although the decrease in oxidative stress after simvastatin treatment may lead to a diminution of NO inactivation by free radicals with enhanced NO bioavailability and improved endothelium-dependent vasodilation, it is also possible that normalized production of NO by simvastatin may itself lead to inactivation of oxygen-derived free radicals and reduced oxidative stress.

In summary, the present study demonstrates that simvastatin, an HRI, preserves endothelial function in large and small coronary vessels, despite no change in plasma lipid concentrations. This increase in NO activity, including an upregulation of eNOS and a reduction in oxidative stress, may mediate altered platelet aggregation, smooth muscle proliferation, and endothelium-leukocyte interactions, potentially regulating the atherogenic process. These alterations may play a role in the reduction of mortality and major morbidity that occurs with the HRIs beyond their effect on lipid lowering.

Acknowledgments

This work was supported by the Miami Heart Research Institute, Mayo Foundation, Bruce and Ruth Rappaport Program in Vascular Biology and the Rappaport Family Institute for Research in the Medical Sciences, and an unrestricted medical school grant from Merck.

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doi: 10.1161/01.ATV.21.1.122
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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