Simvastatin Has Anti-Inflammatory and Antiatherosclerotic Activities Independent of Plasma Cholesterol Lowering

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Abstract—Inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, such as simvastatin, lower circulating cholesterol levels and prevent myocardial infarction. Several studies have shown an unexpected effect of HMG-CoA reductase inhibitors on inflammation. Here, we confirm that simvastatin is anti-inflammatory by using a classic model of inflammation: carrageenan-induced foot pad edema. Simvastatin administered orally to mice 1 hour before carrageenan injection significantly reduced the extent of edema. Simvastatin was comparable to indomethacin in this model. To determine whether the anti-inflammatory activity of simvastatin might affect atherogenesis, simvastatin was tested in mice deficient in apoE. Mice were dosed daily for 6 weeks with simvastatin (100 mg/kg body wt). Simvastatin did not alter plasma lipids. Atherosclerosis was quantified through the measurement of aortic cholesterol content. Aortas from control mice (n=20) contained 56±4 nmol total cholesterol/mg wet wt tissue, 38±2 nmol free cholesterol/mg, and 17±2 nmol cholesteryl ester/mg. Simvastatin (n=22) significantly (P<0.02) decreased these 3 parameters by 23%, 19%, and 34%, respectively. Histology of the atherosclerotic lesions showed that simvastatin did not dramatically alter lesion morphology. These data support the hypothesis that simvastatin has antiatherosclerotic activity beyond its plasma cholesterol–lowering activity. (Arterioscler Thromb Vasc Biol. 2001;21:115-121.)

Key Words: atherosclerosis □ apoE □ mice □ HMG-CoA reductase □ inflammation

Elevated plasma cholesterol level is a major risk factor for atherosclerosis and myocardial infarction. The statin class of drugs inhibits the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the first committed step of sterol synthesis, and lowers plasma cholesterol levels. In large clinical trials, the use of statins reduces coronary events (see review in Bucher et al1), and 1 drug in this class, simvastatin, has been shown to reduce total mortality rates in patients with coronary heart disease.2

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The effectiveness and rapidity with which statins decrease coronary events have led to the speculation that statins may favorably alter vascular biology via mechanisms other than lowered plasma cholesterol. Inhibitors of HMG-CoA reductase might directly alter cellular events other than cholesterol synthesis, because the product of the enzyme, mevalonate, is an important precursor for many isoprenoids. The isoprenoids farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate play important roles in signal transduction via their attachment to critical signaling proteins, such as Ras and Rho.3

In vitro studies have documented cellular effects of statins that may be beneficial in atherosclerosis; these include the inhibition of leukocyte adhesion4,5 and decreased production of cytokines.6 Atherosclerosis is clearly an inflammatory disease,7 and the in vitro observations may be regarded as anti-inflammatory. However, it is not clear that these outcomes would occur in vivo at the circulating concentrations of statins achieved after conventional oral doses. Moreover, it is difficult to determine whether statins have direct anti-inflammatory activity on human atheroma in the face of strong cholesterol lowering.

Here, we use murine models to test the potential anti-inflammatory and antiatherosclerotic effects of simvastatin. The critical feature of these models is that simvastatin does not affect plasma lipid levels, and therefore the results may be interpreted without this confounding variable. A well-characterized foot pad swelling model demonstrated a dose-dependent anti-inflammatory action of simvastatin, with efficacy observed at doses comparable to those of indomethacin, a well-known anti-inflammatory drug. In additional studies, we address the potential anti-inflammatory activity of simvastatin on atherosclerosis using the apoE knockout mouse model. Simvastatin substantially reduced aortic cholesterol accumulation in this model, suggesting an important and direct antiatheroma effect of simvastatin.

Methods

Animals
Normal C57BL/6NTac female mice were obtained from Taconic. Mice homozygous for disruption of the apoE gene (apoE−/−)8,9 were

Received September 5, 2000; revision accepted September 27, 2000.
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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

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bred at the Merck Research Laboratories after being originally obtained from Jackson Laboratories. All mice were barrier housed, specific pathogen free, and maintained in static microisolator cages. Autoclaved food and water were provided ad libitum. The Institutional Animal Care and Use Committee of Merck Research Laboratories approved the animal use for experimentation, and all animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (revised 1996, National Academy Press, Washington, DC).

Carrageenan Footpad Edema Assay of Anti-Inflammatory Activity
Normal C57BL/6NTac female mice between 8 and 12 weeks of age were used in all experiments to induce footpad swelling. Mice received a single subplantar injection of 0.05 mL of a sterile 1% solution of carrageenan in water. Four hours later, footpad volume was measured with a mercury plethysmograph and compared with the preinjection volume of the same paw. Swelling (in microliters) was then calculated, and in drug-treated animals, percent inhibition was derived through comparison with the vehicle (methylcellulose) control group. Both simvastatin and indomethacin were administered orally in aqueous methylcellulose.

Atherosclerosis Studies in apoE−/− Mice
Male apoE−/− mice were weaned at 4 weeks of age onto a high-fat, Western-type diet that contained 21.22% (g/100 g) fat, 17.01% protein, 48.48% carbohydrate, and 0.15% cholesterol (TD88137; Harlan Teklad). Three separate studies were performed. In the first study, animals were dosed daily via oral gavage with 10 or 100 mg/kg simvastatin in 0.5% methyl-cellulose or administered methylcellulose alone (vehicle control) starting at 16 weeks of age. After 6 weeks of dosing, mice were killed, and tissues were processed as described later. The second study was identical to the first, except that the dosing began at 20 weeks of age. The results from these 2 studies were pooled (see Statistical Analysis). The final study included 3 groups of animals: 1 group was analyzed at 20 weeks of age to determine the baseline extent of atherosclerosis. The other 2 groups were analyzed at 26 weeks of age after 6 weeks of daily dosing with simvastatin or vehicle control.

Analysis of Plasma Lipids
At the time the mice were killed, they were weighed. Blood was collected from the vena cava into syringes that contained EDTA as an anticoagulant. Plasma was prepared via centrifugation at 850 g for 15 minutes at 4°C and stored at −20°C for later evaluation of plasma cholesterol and triglyceride levels. Plasma cholesterol and triglyceride measurements were made with standard enzymatic kits (Sigma Chemical Co). For a subset of animals, plasma was subjected to lipoprotein analysis via FPLC size exclusion chromatography with the BioLogic Chromatography System (Bio-Rad Life Science). Some of the samples subjected to FPLC lipoprotein analysis were pools of plasma from multiple animals. In all cases, 200 μL of plasma was chromatographed onto a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated and run in PBS containing 1 mMol/L EDTA, pH 7.4. The column was run at a flow rate of 0.2 mL/min, and 0.27-mL fractions were collected. Of each fraction, 100 μL was assayed for cholesterol (Cholesterol CHI Kit; Wako Diagnostics).

Aortic Cholesterol Measurements
After collection of the blood sample, the vasculature was gently perfused through the left ventricle with cold PBS and 5 mMol/L EDTA. For collection from the aorta for biochemical analysis, all branches and any adipose tissue connected to the aorta were removed, and each aorta was carefully excised from the aortic root to the right renal artery. The aortas were stored briefly on ice in PBS, 0.02% NaN3, and then frozen in OCT (Optimal Cutting Temperature) embedding medium (Fisher Scientific) over liquid nitrogen. The fresh frozen hearts were used to examine the morphology of lesions in the aortic root area. Sequential 20-μm thick sections were cut until the aortic valve leaflets appeared. From this point on, serial 6-μm sections were collected on 10-well masked slides (Erie Scientific). Sections were stained with hematoxylin-phyloxine-saffron stain (Polyscientific) for morphology. Additional sections were stained with oil red O (Polyscientific) for lipids. For immunohistochemistry, sections were fixed in Nakane fixative, washed in PBS, and blocked with 1.5% normal goat serum, followed by avidin-biotin block (Vector). For detection of CD18, sections were reacted with monoclonal anti-CD18 antibody (Endogen). Primary antibody was followed by incubation with biotinylated goat anti-hamster IgG antibody in the presence of 200 μg/mL normal mouse IgG. Antibody reactivity was detected using HRP-conjugated biotin-streptavidin complexes and developed with diaminobenzidine tetrahydrochloride as substrate.

Statistical Analyses
ANOVA was used for the assessment of effects of simvastatin treatment on atherosclerosis. A 2-way ANOVA model with 2 main factors, treatment and study, and treatment × study interaction was applied to the data from the first 2 studies. The results indicated that there was no significant interaction between study and treatment (0.10 < P < 0.75). Therefore, data from the first 2 studies were combined to test the pooled treatment effects. The final number of animals analyzed were 20 control animals, 20 animals dosed with 10 mg/kg simvastatin, and 22 animals dosed with 100 mg/kg simvastatin. To better meet the normality assumption required by the model, natural logarithm transformation was applied to 4 parameters: aortic total cholesterol, aortic free cholesterol, aortic cholesteryl ester, and body weight. Analyses of the plasma lipid parameters were based on the original scale.

Results
Simvastatin Exhibits Anti-Inflammatory Activity In Vivo
Simvastatin was tested in a murine model of acute inflammation. Subplantar injection of carrageenan is followed by swelling of the footpad that can be reproducibly measured after 4 hours, and inhibition of footpad swelling provides a well-characterized gauge of anti-inflammatory activity.13

Histology
The aortas from 4 or 5 mice in each treatment group were harvested for histological analysis by removing the heart with ~1 mm of proximal aorta attached, and the portion distal to the tips of the auricles was excised and discarded. The top half of the heart containing the aortic root was stored briefly on ice in PBS, 0.02% NaN3, and then frozen in OCT (Optimal Cutting Temperature) embedding medium (Fisher Scientific) over liquid nitrogen-isopentane. The fresh frozen hearts were used to examine the morphology of lesions in the aortic root area. Sequential 20-μm thick sections were cut until the aortic valve leaflets appeared. From this point on, serial 6-μm thick sections were collected on 10-well masked slides (Erie Scientific). Sections were stained with hematoxylin-phyloxine-saffron stain (Polyscientific) for morphology. Additional sections were stained with oil red O (Polyscientific) for lipids. For immunohistochemistry, sections were fixed in Nakane fixative, washed in PBS, and blocked with 1.5% normal goat serum, followed by avidin-biotin block (Vector). For detection of CD18, sections were reacted with monoclonal anti-CD18 antibody (Endogen). Primary antibody was followed by incubation with biotinylated goat anti-hamster IgG antibody in the presence of 200 μg/mL normal mouse IgG. Antibody reactivity was detected using HRP-conjugated biotin-streptavidin complexes and developed with diaminobenzidine tetrahydrochloride as substrate.
Simvastatin Does Not Alter Plasma Lipids in apoE^−/− Mice

We sought evidence that simvastatin affects not only acute inflammation but also the chronic inflammation that occurs in the arteries in atherosclerosis. The achieve this, we needed an animal model of atherosclerosis in which simvastatin does not alter lipid levels. Statins are reported to be ineffective in normal mice.14 A potential explanation for this is the very strong compensatory increase in HMG-CoA reductase that occurs in this species.15 To determine whether this resistance to plasma cholesterol lowering also occurs in hyperlipidemic mice, we tested simvastatin in apoE^−/− animals. The mice were fed a high-fat diet on weaning and were dosed with 10 mg/kg simvastatin (n=20), 100 mg/kg simvastatin (n=22), or vehicle control (n=20) for the last 6 weeks of the study (see Methods for details of the study). Simvastatin did not significantly alter plasma cholesterol and triglyceride levels in apoE^−/− mice when dosed at either 10 or 100 mg/kg (0.18<P<0.71) (Figure 2). To assess whether simvastatin caused subtle changes to the lipoprotein profile in the apoE^−/− mice, plasma lipoproteins were analyzed with FPLC. Neither the overall profile nor HDL levels were altered by simvastatin treatment (Figure 3). Simvastatin did decrease body weight in a dose-dependent manner in this study; the mean weights were 35±0.8 g for the control mice, 32±0.7 for the low-dose group (P=0.01 versus control), and 31±0.7 for the high-dose group (P=0.0002 versus control).

Simvastatin Decreases Aortic Cholesterol Accumulation in apoE^−/− Mice

The extent of atherosclerosis in the apoE^−/− mouse was quantified by measurement of the aortic content of cholesterol. This parameter has been used previously to quantify atherosclerosis in mice.16 In the rabbit model, many studies have found good correlations between aortic cholesterol content and atherosclerosis measured according to other criteria, including intima/media ratio17,18 and lesion area as percent of total aortic surface area.18–25 Furthermore, there are reports in both rabbits24,25 and apoE^−/− mice26 that drug interventions show larger effects in the thoracic aorta than in the arch, presumably because the area available for lesion development in the arch “saturates” quickly and therefore there is a smaller window available to detect changes. Aortic cholesterol content is less likely to saturate, as evidenced by the report27 of essentially linear accumulation of aortic cholesterol over 12 months in both cholesterol-fed and Watanabe heritable hyperlipidemic rabbits.
Simvastatin decreased aortic cholesterol content. Cholesterol was measured in aortic lipid extracts from mice in the control (n=20), low-dose (n=20), and high-dose (n=22) simvastatin groups described in the legend for Figure 2. Mean and SEM are shown. There were no statistically significant differences between the low-dose simvastatin group and either of the other groups (0.11 < P < 0.28). *Significantly different from control group (P < 0.01). †Significantly different from control group (P = 0.015).

The data in Figure 4 show that simvastatin decreased aortic cholesterol accumulation. Aortas from the control mice contained 56 ± 4 nmol/mg (mean ± SEM) total cholesterol, 38 ± 2 nmol/mg free cholesterol, and 17 ± 2 nmol/mg cholesteryl ester. Simvastatin at 100 mg/kg decreased total aortic cholesterol by 23%, free cholesterol by 19%, and cholesteryl ester by 34% (P < 0.015 for all 3 effects) (Figure 4). The decrease in aortic cholesterol was dose responsive, as the aortic cholesterol in the group of mice administered 10 mg/kg simvastatin was intermediate between the control group and the 100 mg/kg group. None of the values for the low-dose group were significantly different from those of either of the other 2 groups (0.11 < P < 0.28).

Aortic cholesterol content is only 1 possible measure of atherosclerosis. Aortic weight has been proposed as a valid surrogate for atherosclerosis. In the study shown in Figure 4, simvastatin decreased aortic weight in a dose-dependent manner; the values were 6.7 ± 0.5 mg in the control group, 6.4 ± 0.3 mg in the low-dose simvastatin group, and 6.2 ± 0.3 mg in the high-dose simvastatin group. Although aortic weights trended down in a dose-dependent fashion, neither the 10 mg/kg nor the 100 mg/kg data points alone attained statistical significance (0.21 < P < 0.37).

Simvastatin significantly decreased aortic cholesterol accumulation without significantly decreasing plasma cholesterol levels. Simvastatin did, however, tend to lower cholesterol levels, and it seemed possible that this tendency may have influenced the progression of disease. To test whether plasma cholesterol levels correlated with aortic cholesterol accumulation, we calculated Pearson correlation coefficients between these 2 parameters for each treatment group and found no significant correlation (P > 0.66). The correlation coefficients were also calculated by combining all 3 treatment groups, with residuals (ie, responses minus the group mean) used to remove the differences due to treatment effects. Again, no correlation was found (P > 0.71). The lack of correlation is shown graphically in Figure 5, which compares plasma cholesterol and aortic cholesteryl ester for each animal in the control and high-dose groups. There clearly is no correlation between these 2 parameters, even though the largest effect seen with simvastatin was for cholesteryl ester.

Simvastatin Dramatically Decreased Accumulation of Aortic Cholesterol During the 6-Week Dosing Period

Atherosclerotic lesions in apoE−/− mice increase in size throughout the life span of the animals.8,9,16 The data in Figure 4 show that simvastatin decreased aortic cholesterol accumulation even though the mice were dosed for only ≈25% of their lifetime. To evaluate the suppression of atherosclerotic lesion formation during the 6 weeks of simvastatin dosing, we measured aortic cholesterol accumulation in 3 groups of apoE−/− mice: 20-week-old animals that were never dosed (baseline group), animals administered 50 mg/kg simvastatin for 6 weeks starting at 20 weeks of age (progression with simvastatin group), and animals dosed in parallel with vehicle only (progression control). Simvastatin did not alter plasma lipids or body weights in this study (0.35 < P < 0.87). The aortic total cholesterol, free cholesterol, and cholesteryl ester values in the vehicle control group were all approximately twice those found in the baseline group (P < 0.0002) (Figure 6). Simvastatin dramatically attenuated the increase in lesion size during the 6-week dosing period (66% decrease for total cholesterol, P = 0.005; 59% for free cholesterol, P = 0.02; and 77% for cholesteryl ester, P = 0.0008). Simvastatin treatment essentially halted the deposition of cholesteryl ester in the aorta, because this value was not different from the baseline group (P = 0.20).

The changes in aortic cholesterol content in the study were mirrored by changes in aortic weight. Aortic weight in the baseline group was 6.3 ± 0.5 mg, and this parameter increased during the 6 weeks of progression to 8.3 ± 0.8 mg (P = 0.04 versus baseline). The aortic weight in the simvastatin-treated group was 7.1 ± 0.3 mg (P = 0.16 versus progression control group). These data show that during the 6 weeks of dosing, simvastatin treatment slowed the increase in aortic weight by 60%. The magnitude of this effect is very similar to the 59% to 77% decrease in aortic cholesterol accumulation caused by simvastatin.
Histology of Aortic Root Lesions From Simvastatin-Treated and Control Animals Reveals Similar Morphology

The data that show simvastatin prevented an increase in aortic cholesterol content implied that atherosclerotic lesion size was diminished. To determine whether simvastatin altered the morphology of the lesions, the aortic root areas of apoE2/2 mice treated with 100 mg/kg simvastatin were examined histologically and compared with those from untreated animals (Figure 7). The overall morphology of lesions was very similar with or without simvastatin treatment, with fibrous areas, areas rich in foam cells, and necrotic areas present in both (Figures 7A and 7B). Oil red O staining showed a similar localization of lipid in lesions from untreated or simvastatin-treated animals (Figures 7C and 7D). Macrophage-derived foam cells were identified through immunohistochemical staining of sections for the macrophage marker CD18 (Figures 7E and 7F). Macrophages were present both beneath overlying fibrous caps and at the surface of complex lesions from both simvastatin-treated and control animals. The effect of simvastatin treatment on lesion morphology in the aortic root was therefore less apparent than the effect on the overall deposition of cholesteryl esters within the aorta. This is perhaps due to the lesions reaching a near-maximal size in the aortic root during the course of the study, because the time for analysis was chosen to obtain optimal biochemical measurements of aortic cholesterol. Many studies have shown that lesions develop faster in the aortic arch, and lesion development proceeds more slowly in the more distal portions of the aorta, in both rabbits24,25,29 and apoE2/2 mice.26,30,31

Discussion

Here, we show that simvastatin has anti-inflammatory activity in a well-characterized acute animal model: carrageenan-induced foot pad edema. The potency and effectiveness of simvastatin in this model were comparable to that of indomethacin, a classic anti-inflammatory agent. Both simvastatin
and indomethacin were effective acutely (ie, when administered 1 hour before carrageenan injection), and neither compound was effective when administered 24 hours before injection. This anti-inflammatory effect could not have been caused by plasma lipid lowering, because simvastatin did not change lipid levels in these mice. Importantly, the strong effect of statins in causing changes in footpad swelling in mice occurred within 4 hours, well before any lipid changes could possibly occur. These observations suggest that although plasma lipids may affect inflammation, they cannot account for all of the anti-inflammatory effects observed for statins. These data strongly support the hypothesis that simvastatin has anti-inflammatory activity beyond its cholesterol-lowering activity.

The footpad swelling affected by simvastatin represents an acute inflammatory response characterized by the influx of polymorphonuclear leukocytes. Other workers have also observed an effect of simvastatin on acute inflammatory responses. Lefer et al. demonstrated that a single administration of simvastatin (25 μg/rat) blocked the influx of polymorphonuclear leukocytes into heart muscle after ischemia and reperfusion. Furthermore, Endress et al. showed that the administration of simvastatin to mice induces aortic endothelial NO synthase (eNOS) activity and renders animals resistant to cerebral ischemia/reperfusion injury. Additional observations in the literature suggest that simvastatin affects not only acute inflammation and the movement of cells of the innate immune system but also chronic inflammation and the action of cells of the adaptive immune system. Stanislaus et al. showed that the long-term administration of 2 mg/kg lovastatin blocked neuroinflammation after challenge of the rats with myelin basic protein, a response that requires T lymphocytes. Moreover, additional work has shown that statins block the rejection of islet transplants in animals. Importantly, clinical studies have shown that both simvastatin and pravastatin decrease the incidence of cardiac allograft vasculopathy in cardiac transplant patients. These observations suggest a broad range of anti-inflammatory activities of statins.

Atherosclerosis is clearly an inflammatory process, and interruption of the function of inflammatory mediators can decrease atherosclerotic lesion size in mice. This has been shown for interferon-γ as well as for monocyte chemotractant protein-1 and its receptor, CCR2. In humans, serum levels of C-reactive protein predict vascular disease, implying that low-level inflammation accelerates atherosclerosis. The anti-inflammatory properties of statins may thus contribute to the observed effects on coronary heart disease. However, determination of the magnitude of the potential contribution is made difficult by the strong effects of statins on LDL cholesterol, an undisputed contributor to atherogenesis. To overcome this difficulty, we tested simvastatin in a model of atherosclerosis that is resistant to statin-mediated plasma cholesterol lowering. Using the apoE−/− mouse model, we show that simvastatin decreased aortic cholesterol accumulation in mice without lowering cholesterol levels or altering lipoprotein profile. These data strongly support the hypothesis that simvastatin has antiatherosclerotic activity beyond its cholesterol-lowering activity.

In our studies of apoE−/− mice, the extent of atherosclerosis did not correlate with plasma cholesterol levels. This lack of correlation has been previously observed in this model. Danksy et al. showed that plasma cholesterol did not correlate with atherosclerotic lesion area in the F2 progeny of C57BL6J apoE−/− × FVB/n apoE−/− mice. Despite the failure of small changes in plasma cholesterol levels to correlate with atherosclerosis in apoE−/− mice, interventions that dramatically lower cholesterol levels can decrease atherosclerosis in apoE−/− mice. Therefore, plasma cholesterol does contribute to atherosclerosis in this model.

What is the mechanism by which statins achieve blockade of inflammation leading to decreased atherosclerotic lesion size? The most frequently proposed model is that statins interrupt proinflammatory signaling by blocking the geranylgeranylation of proteins such as the GTPase Rho-A. Members of the Rho family and related proteins have well-documented roles in signaling a variety of cellular functions, including the cytoskeletal rearrangements required for cell migration. Interruption of geranylgeranylation is plausible, because statins block the synthesis of mevalonate, the precursor of farnesol, geranyl-geraniol, and cholesterol. Simvastatin-induced changes in protein prenylation might have their greatest influence on vascular biology by altering the production of NO. Statins have been shown to increase both the expression and the activity of eNOS in vitro and in vivo. This increase may be related to changes in prenylation, because the in vitro effects could be overcome by the addition of mevalonate or geranylgeranyl pyrophosphate to the media. NO from endothelial cells is thought to be anti-inflammatory. For example, Fox-Robichaud et al. observed that inflammatory cells move into tissues on inhibition of eNOS, suggesting an anti-inflammatory tone produced by eNOS action. The NO produced from eNOS is probably also antiatherosclerotic, as evidenced by the favorable effects of arginine on atherosclerosis. This hypothesis is also consistent with the observation that fluvasstatin decreased atherosclerosis and increased eNOS mRNA in rabbit aorta without altering plasma lipids.

Our results with simvastatin support the hypothesis that simvastatin has anti-inflammatory activity that is relevant to the prevention of atherosclerosis by this drug. Although the mechanism is not yet established, further research may lead to new understanding of the actions of statins and new therapeutic interventions for atherosclerosis.

References


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doi: 10.1161/01.ATV.21.1.115

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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