Inhibition of Sphingomyelin Synthesis Reduces Atherogenesis in Apolipoprotein E–Knockout Mice

Tae-Sik Park, PhD; Robert L. Panek, PhD; Sandra Bak Mueller, MS; Jeffrey C. Hanselman, BS; Wendy S. Rosebury, HT (ASCP); Andrew W. Robertson, HT (ASCP); Erick K. Kindt, BS, MBA; Reynold Homan, PhD; Sotirios K. Karathanasis, PhD; Mark D. Rekhter, MD, PhD

Background—In clinical studies, sphingomyelin (SM) plasma levels correlated with the occurrence of coronary heart disease independently of plasma cholesterol levels. We hypothesized that inhibition of SM synthesis would have antiatherogenic effects. To test this hypothesis, apolipoprotein E (apoE)–knockout (KO) mice were treated with myriocin, a potent inhibitor of serine palmitoyltransferase, the rate-limiting enzyme in SM biosynthesis.

Methods and Results—Diet-admix treatment of apoE-KO mice with myriocin in Western diet for 12 weeks lowered SM and sphinganine plasma levels. Decreases in sphinganine and SM concentrations were also observed in the liver and aorta of myriocin-treated animals compared with controls. Inhibition of de novo sphingolipid biosynthesis reduced total cholesterol and triglyceride plasma levels. Cholesterol distribution in lipoproteins demonstrated a decrease in β-VLDL and LDL cholesterol and an increase in HDL cholesterol. Oil red O staining of total aortas demonstrated reduction of atherosclerotic lesion coverage in the myriocin-treated group. Atherosclerotic plaque area was also reduced in the aortic root and brachiocephalic artery.

Conclusions—Inhibition of de novo SM biosynthesis in apoE-KO mice lowers plasma cholesterol and triglyceride levels, raises HDL cholesterol, and prevents development of atherosclerotic lesions. (Circulation. 2004;110:3465-3471.)

Key Words: sphingomyelin inhibitors cholesterol lipoproteins atherosclerosis

Sphingomyelin (SM) is one of the major lipids in plasma lipoproteins and cell membranes. In clinical studies, SM plasma levels correlated with the occurrence of coronary heart disease independently of plasma cholesterol levels. SM and its derivatives are accumulated in human and experimental atherosclerotic lesions.

See p 3400

Although direct mechanistic links between SM and atherosclerosis have not been established, available in vitro data suggest that SM might have the following proatherogenic properties. First, SM content of lipoprotein particles may influence lipid metabolism by affecting binding or activity of lecithin-cholesterol acyltransferase and lipoprotein lipase. Second, SM-rich lipoproteins can be converted to foam cell substrates by sphingomyelinase (SMase) in the artery wall, thereby promoting foam cell formation. Third, ceramide and related products of SM synthesis and breakdown are potent regulators of cell proliferation, activation, and apoptosis and hence may affect plaque growth and stability. Thus, inhibition of SM synthesis may have antiatherogenic effects.

Serine palmitoyltransferase (SPT) catalyzes the first committed step in sphingolipid synthesis. SPT condenses the palmitic acid of palmitoyl-coenzyme A with serine to produce ketosphinganine, the precursor to the unique aminolipid backbone that is characteristic of all sphingolipids. SPT is composed of 2 different subunits, LCB1 and LCB2. Myriocin is a potent and specific SPT inhibitor isolated from fungi and in high doses is known to have a potent immunosuppressive activity.

In current studies, we investigated the effect of SM biosynthesis on plasma cholesterol and triglycerides (TGs). We have demonstrated that myriocin, a potent SPT inhibitor, improved the lipid profiles and reduced atherogenesis in apolipoprotein E (apoE)–knockout (KO) mice.

Methods

Animal Experiments

Male C57Bl/6J and apoE-KO mice on C57Bl/6J background were obtained from the Jackson Laboratory (Bar Harbor, Me). Myriocin was mixed with Western diet containing 0.21% cholesterol and 21% fat. Eight- to 12-week-old apoE-KO (n = 16) mice received myriocin 0.3 mg · kg⁻¹ · d⁻¹ for 12 weeks. A myriocin dose was chosen from a previous dose-dependent experiment with apoE-KO mice (M.D.R., unpublished data, 2002). Control groups consisted of apoE-KO mice fed standard chow or Western diet without myriocin and standard chow–fed C57Bl/6J mice (n = 16). Body weight and chow feeding were measured every week to examine the food consumption. All procedures and protocols involving the use of animals were approved by the Pfizer Animal Care and Use Committee and conformed with the “Guide for the Care and Use of Laboratory Animals” published...
by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

**SPT Expression and Activity**

Total RNA was isolated from liver and aorta with TriZol (Invitrogen). LCB1 and LCB2 mRNA levels were measured by real-time polymerase chain reaction on ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The following primers and probe sets were used: LCB1, forward primer 5'-CCGGCTCTCTCCTGGTTGTA-3', reverse primer 5'-GAGGTAACGAGCAGAA-AAGCAG-3', probe 5'-6FAM-TACACGGCTCTCGGTCAAAG-GAT-TAMRA-3'; LCB2, forward primer 5'-CTGGATGAGGCTCAGCGATT-3', reverse primer 5'-CCTCAGGATCCAGGCCAA-3', probe, 5'-6FAM-CCCTCAGGCGAGGCCGTGATGATG-3'. The optimum number of cycles was set for each gene product with uniform amplification. Each mRNA level was expressed as a ratio to GAPDH RNA. Liver tissues from each group were homogenized, and SPT activity was measured with 14C-serine and palmitoyl-coenzyme A as substrates and thin-layer chromatography analysis as described previously.11

**Analysis of Sphingolipids and Phospholipids by LC/MS/MS and High-Performance Liquid Chromatography**

Total lipids were extracted by the modified method of Bligh-Dyer extraction12 as described previously.13 A Micromass (Manchester, United Kingdom) Quattro II tandem quadrupole mass spectrometer with a standard Z-spray ion source, set to electrospray positive ionization mode, with MassLynx version 3.4 operating software, was used for all quantitative determinations. Precursor-to-product ion transitions were established through direct infusion of each compound into the mass spectrometer. The following ion transitions were used for quantification: SM (704→284 m/z), sphinganine (302→284 m/z), ceramide (566→264 m/z), and psychosine (462→282 m/z) as an internal standard. For our instrument, at a collision cell pressure of 2×10⁻¹ mbar of argon, cone and collision voltages were as follows: SM 40 V and 25 eV, sphinganine 40 V and 15 eV, ceramide 45 V and 25 eV, and psychosine 35 V and 25 eV. A sample volume of 2 μL was injected into the LC/MS/MS system. Final chromatographic retention times for SM, sphinganine, and psychosine (internal standard) were 1.40, 1.43, and 1.25 minutes, respectively. Lipid extracts were analyzed by high-performance liquid chromatography to determine plasma phosphatidylcholine (PC) levels as described previously.14

**Plasma Lipids**

Mice were euthanized by CO₂ inhalation, and blood was collected through cardiac puncture. Plasma concentrations of total cholesterol and triglyceride were determined enzymatically on a Cobas Mira (Roche Diagnostics). Colorimetric changes were measured at 500 nm. Lipoproteins were separated from mouse plasma by fast-protein liquid chromatography with a Superose 6HR column. Cholesterol distribution among lipoproteins was determined by in-line post column analysis as described previously.15

**Vascular Pathology**

For quantitative analysis of atherosclerotic lesion coverage, euthanized mice were perfused with saline, and the aorta was isolated from the heart to the iliac bifurcation by severing of the minor branching arteries and dissection of the adventitia. After 24 hours of fixation with 10% buffered formalin, the aorta was opened longitudinally and pinned down on black wax. Lipids were stained with oil red O, and photographs were taken. The percentage of aorta stained with oil red O was determined by image analysis software (Image Pro Plus).

For histological analysis, the mice were perfused and fixed in zinc-Tris fixative. Paraffin-embedded sections were stained with Masson’s trichrome. Macrophages were immunohistochemically stained with MAC-2 antibody (clone M6/38 from Cedar lane Laboratories Limited) counterstained with Verhoeff elastic stain. T lymphocytes were immunohistochemically stained with rat CD3 antibody (clone CD3–12, Serotec). Lesion thickness and area occupied by macrophages were determined with Image Pro Plus software.

**Statistical Analysis**

Results are expressed as mean±SEM. Comparisons among several groups were determined by 1-way ANOVA with Dunnett’s post hoc analysis using PRISM 2.01. If a significant difference was found among groups, distribution-free multiple comparisons were performed to find significance among groups. When SEMs were unequal, a nonparametric test (Mann-Whitney) was used to calculate the level of significance. Results were considered significant at P<0.05.

**Results**

**SPT Gene Expression and Enzyme Activity**

Real-time polymerase chain reaction analysis demonstrated that myriocin had no effect on expression of LCB1 and LCB2 mRNA (Figures 1A and 1B). Compared with C57BL/6J mice, SPT activity was increased in apoE-KO mice fed a Western diet and standard chow, by 60% (n=3, P<0.05) and
times higher than C57Bl/6J and apoE-KO mice displayed the highest level of plasma SM, 33 apoE-KO mice (Figure 2C). Moreover, Western diet–fedimulation in the liver by 45% compared with Western diet–fed standard chow–fed alike). Myriocin treatment lowered SM accu-
smhance in the liver of C57Bl/6J mice were 65% lower than those in
apoE-KO group, standard chow–fed apoE-KO group, and C57Bl/6J group were decreased by 45%, 54%, and 63%, respectively, compared with the Western diet–fed group (Figure 2A).

In aorta, sphinganine levels in the myriocin-treated apoE-KO group, standard chow–fed apoE-KO group, and C57Bl/6J group were decreased by 45%, 54%, and 63%, respectively, compared with the Western diet–fed group (Figure 2B). Sphinganine in plasma was below detectable levels. Thus, myriocin treatment inhibited the SM synthetic pathway in both liver and aorta.

Although SM levels are determined by both synthesis and degradation, in the present experimental system, SM changes were generally associated with changes in SPT activity and sphinganine production, which emphasizes the role of the SPT-dependent synthetic pathway. Specifically, SM levels in the liver of C57Bl/6J mice were 65% lower than those in Western diet–fed apoE-KO mice (myriocin-treated and standard chow–fed alike). Myriocin treatment lowered SM accumulation in the liver by 45% compared with Western diet–fed apoE-KO mice (Figure 2C). Moreover, Western diet–fed apoE-KO mice displayed the highest level of plasma SM, 33 times higher than C57Bl/6J and >2 times higher than the standard chow–fed apoE-KO mice (Figure 2D). Myriocin treatment lowered plasma SM in Western diet–fed apoE-KO mice by 64%, bringing it to the level of their standard chow–fed counterparts. Small differences were observed among aortas after the various treatments; however, there were statistically significant differences between Western diet–fed apoE-KO and control mice. Myriocin decreased SM levels by 20% (Figure 2E). Thus, SPT inhibition by myriocin drastically affected SM production and accumulation.

SM Synthesis, Accumulation, and Characteristics
To determine whether inhibition of SPT activity was translated into inhibition of de novo SM production, we measured the quantity of sphinganine, a close downstream marker of SPT activity that cannot be influenced by SM degradation via SMase. Sphinganine levels were significantly increased in Western diet–fed and chow-fed apoE-KO mice compared with control C57Bl/6J mice, which indicates an increased rate of SM synthesis in this model of atherosclerosis. Myriocin treatment lowered sphinganine levels in liver by 42% compared with the Western diet–fed group (Figure 2A).

In aorta, sphinganine levels in the myriocin-treated apoE-KO group, standard chow–fed apoE-KO group, and C57Bl/6J group were decreased by 45%, 54%, and 63%, respectively, compared with the Western diet–fed group (Figure 2B). Sphinganine in plasma was below detectable levels. Thus, myriocin treatment inhibited the SM synthetic pathway in both liver and aorta.

Because the SM content of lipoproteins affects the activities of enzymes involved in lipid metabolism in vitro, we questioned whether the inhibition of sphingolipid biosynthesis affected cholesterol and TG levels in plasma. As expected, plasma cholesterol and TGs were highest in the Western diet–fed apoE-KO mice and lowest in C57Bl/6J mice, with standard chow–fed apoE-KO mice positioned in between. Myriocin exhibited significant lipid-lowering activity by bringing both parameters to the levels of standard chow–fed apoE-KO mice. Myriocin lowered plasma cholesterol and TGs by 41% and 45%, respectively (Figure 4). In addition, myriocin lowered β-VLDL and LDL cholesterol levels by 51% and 35%, respectively. In contrast, HDL cholesterol content was increased by 54% (Figure 5). Cholesterol distribution in lipoproteins of standard chow–fed apoE-KO mice was comparable to that in myriocin-treated mice. Compared

**Figure 2.** Sphinganine and sphingomyelin concentrations in liver, plasma, and aorta. After 12 weeks of myriocin treatment, mice were euthanized, and plasma, liver, and aorta were isolated. Total lipids were extracted by Bier-Dyer method. Sphinganine levels in liver (A) and aorta (B) and SM levels in liver (C), plasma (D), and aorta (E) were determined by LC/MS as described in Methods. Values are mean±SEM (n=5, **P<0.05**). WD indicates Western diet chow; WD+myr, Western diet chow plus myriocin; and STD, standard chow.
with apoE-KO mice, the WT C57Bl/6J mice showed very low total cholesterol in plasma. Most of the cholesterol content in C57Bl/6J was found in HDL. In addition, myriocin lowered plasma apoB levels, which were comparable to those in the standard chow–fed group (T.-S. Park, unpublished data, 2004). Because plasma apoB levels, especially apoB100 levels, in LDL correlate with atherogenesis, the apoB-lowering effect might contribute to prevention of atherogenesis by myriocin. Thus, myriocin exerted profound lipid-lowering effects.

**Atherogenesis**

Oil red O staining of en face aortas revealed that myriocin treatment reduced atherosclerotic lesion coverage in Western diet–fed apoE-KO mice by 93% (Figure 6). Growth of atherosclerotic lesions in the brachiocephalic artery and aortic valve area was also significantly inhibited (Figure 7). In the brachiocephalic artery, myriocin treatment led to a 76% decrease in lesion area and a 74% decrease in macrophage area. Lesions in myriocin-treated, Western diet–fed apoE-KO mice did not develop a necrotic core. In the aortic root, lesion area was decreased by 44% and macrophage area was decreased by 31% with myriocin treatment. Accumulation of T cells was not affected by myriocin treatment (Figure 8). Thus, SPT inhibition had substantial antiatherogenic effects.

**Discussion**

We have demonstrated that SM content and production were proportionally increased in plasma, liver, and aorta of the Western diet–fed apoE-KO mice compared with standard chow–fed apoE-KO and C57Bl/6J control mice. Myriocin, a specific inhibitor of SPT, inhibited de novo SM synthesis in the liver and aorta, and this was associated with reductions of
plasma SM and ceramide that were not accompanied by changes in the SM/PC ratio. Inhibition of SM synthesis led to the lowering of plasma cholesterol and TGs. These changes were associated with dramatic antiatherosclerotic effects.

Increased plasma SM levels previously have been linked to enhanced SPT activity in the liver of apoE-KO mice. Increased plasma SM levels previously have been linked to enhanced SPT activity in the liver of apoE-KO mice.17 The present data support this observation and provide further evidence that SPT inhibition by myriocin leads to a reduction of plasma SM. Plasma SM is a component of lipoprotein particles and has been suggested to be proatherogenic.1,18 Intermediates of SM synthesis, in particular ceramide, also possess independent proatherogenic properties. Ceramide plays an important role in lipoprotein aggregation and may promote foam cell formation.18 It is a potent regulator of cell proliferation, activation, and apoptosis6 and hence may affect plaque growth and stability. We demonstrated the ceramide-lowering effects of myriocin. It is not absolutely certain that these effects are driven by inhibition of ceramide production. An alternative source of ceramide is SM hydrolysis by SMase; however, the present data suggest that the synthetic arm of the pathway is more likely to be affected by myriocin. At physiological pH, recombinant human secretory SMase

Figure 6. Lipid deposition in aortas of Western diet–fed apoE-KO mice. ApoE-KO mice were fed with Western diet in presence (A) or absence (B) of myriocin for 12 weeks. Mice were euthanized and fixed with 10% buffered formalin for 24 hours. Aorta from heart to iliac bifurcation was dissected, opened along ventral surface, and pinned down on black wax background. Accumulated lipids were visualized by oil red O staining. Areas of atherosclerotic lesion were quantified by Image Pro Plus (C) and represented as percentage of lesion area to total aorta area. Values are mean±SEM (n=4; *P<0.01, Western diet vs Western diet plus myriocin or standard chow; #P<0.01, standard chow vs Western diet plus myriocin). Bar represents 1 cm. WD indicates Western diet chow; WD+myr; Western diet chow plus myriocin; and STD, standard chow.

Figure 7. Formation of atherosclerotic lesions in brachiocephalic artery and aortic root. Cross section of brachiocephalic artery was stained by Masson's trichrome (A, C) and MAC-2 antibody counterstained with Verhoeff elastic stain (B, D). Atherosclerotic lesion (black bars) and macrophage size (gray bars) in brachiocephalic artery (E) and in aortic root (F) were quantified by Image Pro Plus (E). Values are mean±SEM (n=5; *P<0.01, Western diet vs Western diet plus myriocin; #P<0.01, standard chow vs Western diet plus myriocin). Bar represents 100 μm. WD indicates Western diet chow; WD+myr; Western diet chow plus myriocin; and STD, standard chow.
lipoprotein lipase–mediated lipolysis in lipid emulsions. 4

compound.

be partially responsible for the antiatherogenic activity of this

Western diet–fed and myriocin-treated groups. Thus, myri-

cates Western diet chow; WD

that we observed in myriocin-treated mice. The precise

observations from the present study; however, further studies

are needed to explore effects of SM depletion on reverse

cholesterol transport and, ultimately, atherogenesis.

We have demonstrated that inhibition of SM synthesis was

associated with a significant reduction in atherosclerotic

lesion formation in apoE-KO mice. Because plaque formation

in apoE-KO mice is lipid-driven,22 the observed antiathero-

genic effects were likely indirect, due to normalization of

plasma lipids as a result of the inhibition of SM synthesis by

liver; however, we have also shown local inhibition of SM

production in aorta. Myriocin-treated, Western diet–fed

apoE-KO mice showed a plasma lipid profile similar to that

in the standard chow–fed apoE-KO mice, but their lesions

were significantly smaller. Taken together, these findings

suggest that the antiatherogenic effects of myriocin could be

attributed in part to local inhibition of SPT in the arterial wall.

Myriocin may potentially influence atherogenesis in an

SPT-independent manner, eg, by inhibition of lipid absorp-

tion from the gut or by exertion of its immunomodulatory

properties.9,10 The potential role of myriocin or sphingolipids

on lipid absorption has not been examined, and additional

studies are warranted. Nonspecific immunomodulatory ef-

fects of myriocin also cannot be ruled out completely.

Indeed, a myriocin analogue (FTY720) devoid of SPT

activity is a potent immunosuppressor.23 The predominant

mechanism of action of FTY720 is immunosuppression via

sphingosine-1-phosphate (S1P) receptor agonism.23 How-

ever, we suggest that S1P agonism does not play a leading

role in the observed atheroprotective phenomena for the

following reasons. First, to the best of our knowledge, there

are no published data suggesting an influence of S1P on

plasma SM and lipoproteins. To the contrary, SM- and

lipid-lowering effects were correlated with inhibition of SM

synthesis, as shown by significant lowering of sphinganine

concentrations. Second, S1P agonism has diverse effects on

various aspects of atherosclerosis, but an overall proathero-

genic and prothrombogenic activity is suggested.24 Specifi-

cally, it activates macrophages.25 In contrast, the present data

demonstrate a significant decrease in both total plaque and

macrophage area. Third, immunosuppressive activity of

FTY720 is driven by alteration of lymphocyte trafficking.23

Although we did not count peripheral blood lymphocytes in

the present study, we performed immunostaining of arterial

sections with CD3, a T-cell–specific antibody. We have

demonstrated that CD3-positive cell staining in this model of

atherosclerosis was minimal and was not affected by myri-

ocin. These data, together with the generally accepted fact

that atherosclerotic lesion development in apoE-KO mice is

preferentially acts on lipoproteins with a high SM/PC ratio5; however, we found no difference in SM/PC ratio between the Western diet–fed and myriocin-treated groups. Thus, myriocin is likely to inhibit ceramide synthesis, which in turn can be partially responsible for the antiatherogenic activity of this compound.

SM has been shown to negatively affect the rate of lipoprotein lipase–mediated lipolysis in lipid emulsions.4 Extrapolation of this phenomenon to the in vivo situation is suggestive of accelerated lipolysis associated with SM depletion. This mechanism could be involved in the TG lowering that we observed in myriocin-treated mice. The precise mechanism for total cholesterol lowering in myriocin-treated mice is unclear. We suggest that lipid lowering may be driven by SM-dependent inhibition of cholesterol synthesis, at least in part. SM, a major plasma membrane component, has a high affinity for free cholesterol.19 It is conceivable that inhibition of SM synthesis leads to a reduced SM concentration in the plasma membrane of hepatocytes, thereby inducing release of free cholesterol into the cytoplasm. Increased intracellular concentrations of free cholesterol could be sensed as a signal to inhibit cholesterol synthesis. Recent findings demonstrated that inhibition of sphingolipid biosynthesis caused suppression of lipogenic gene expression in Chinese hamster ovary cells.20 This hypothesis, however, needs further in vivo testing.

SM depletion was also associated with an elevation of HDL. In vitro data suggest that increased SM content in lipoproteins can inhibit key enzymes involved in lipoprotein metabolism.3,4 It has also been demonstrated that SM in macrophage membranes interfered with reverse cholesterol transport.21 It is conceivable that SM depletion would lead to activation of reverse cholesterol transport and contribute to elevation of HDL cholesterol, which is consistent with observations from the present study; however, further studies are needed to explore effects of SM depletion on reverse cholesterol transport and, ultimately, atherogenesis.

Figure 8. Incorporation of T lymphocytes into lesion of aortic root. Cross section of aortic root was stained by rat CD3 antibody and developed by diaminobenzidine (brown color) to detect incorporated T lymphocytes (A, B). Sections were counterstained with Harris hematoxylin (blue). T-lymphocyte incorporation was quantified by measuring number of intimal T lymphocytes in aortic root (C). Values are mean ± SEM (n=5; *P<0.05, Western diet vs standard chow; #P<0.05, Western diet plus myriocin vs standard chow). Bar represents 50 μm. WD indicates Western diet chow; WD+myr; Western diet chow plus myriocin; and STD, standard chow.
lipid driven, indicate that dramatic lipid-lowering effects of myriocin are likely to be responsible for its antiatherogenic activity. It is also likely that direct inhibition of SPT in the vascular wall may be responsible for some additional effects, whereas SPT-independent mechanisms play a minor role.

Although inhibition of SM synthesis may be beneficial for treatment of atherosclerosis, it is still unknown whether the targeting of SPT would be associated with any toxicological issues. LCBl and LCBl2 genes are essential for cell survival, and the changes in SPT activity result in a defective development of the fruit fly and filamentous fungi, as well as hereditary sensory neuropathy type I in humans. In the present study, however, no overt toxicity was associated with chronic administration of myriocin.

In conclusion, SPT inhibition by myriocin in apoE-KO mice effectively inhibited SM synthesis, an effect that was associated with an improved plasma lipid profile and significant antiatherogenic activity. Consistent with these observations are clinical reports indicating that SM is an independent risk factor for coronary heart disease and a plasma marker of atherosclerosis. LCBl and LCBl2 genes are essential for cell survival, and the changes in SPT activity result in a defective development of the fruit fly and filamentous fungi, as well as hereditary sensory neuropathy type I in humans. In the present study, however, no overt toxicity was associated with chronic administration of myriocin.

Acknowledgments
We gratefully acknowledge Dr Mark Kowala and Dr Robert Leadley for helpful discussion and critical reading of the manuscript.

References
Inhibition of Sphingomyelin Synthesis Reduces Atherogenesis in Apolipoprotein E–Knockout Mice

Tae-Sik Park, Robert L. Panek, Sandra Bak Mueller, Jeffrey C. Hanselman, Wendy S. Rosebury, Andrew W. Robertson, Erick K. Kindt, Reynold Homan, Sotirios K. Karathanasis and Mark D. Rekhter

Circulation. 2004;110:3465-3471; originally published online November 15, 2004; doi: 10.1161/01.CIR.0000148370.60535.22

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/22/3465

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/