Effect of Chronic Treatment With the Inducible Nitric Oxide Synthase Inhibitor N-Iminoethyl-L-Lysine or With L-Arginine on Progression of Coronary and Aortic Atherosclerosis in Hypercholesterolemic Rabbits

Delphine Behr-Roussel, PhD; Alain Rupin, PhD; Serge Simonet, PhD; Edith Bonhomme, RT; Sophie Coumailleau, RT; Alex Cordi, PhD; Bernard Serkiz, PhD; Jean-Noël Fabiani, MD; Tony J. Verbeuren, PhD

Background—We examined the implications of iNOS in atherosclerosis progression using the selective inducible NO synthase (iNOS) inhibitor N-iminoethyl-L-lysine (L-NIL) in hypercholesterolemic rabbits.

Methods and Results—Nine rabbits were fed a 0.3% cholesterol diet for 24 weeks (Baseline group); 25 animals were maintained on the diet and treated for 12 extra weeks with L-NIL (5 mg·kg⁻¹·d⁻¹, L-NIL group, n=8), vehicle (Saline group, n=9), or L-arginine (2.25%, L-Arg group, n=8). In abdominal aortas of Saline rabbits, the lesions (53.7±5.7%, Baseline) increased to 75.0±5.0% (P<0.05) but remained unaltered in the L-NIL group (63.4±6.6%). Similar results were obtained for the intima/media ratio in thoracic aortas. In coronary arteries, the intima/media ratio was comparable in Baseline (0.68±0.18) and Saline (0.96±0.19) rabbits but decreased to 0.34±0.19 (P<0.05) in L-NIL rabbits. L-Arginine had beneficial effects only in abdominal aortas. An increased thoracic aorta collagen content was found in Saline and L-Arg but not in L-NIL rabbits. In thoracic aortas of the Saline group, acetylcholine caused modest relaxations that slightly increased by L-arginine but not by L-NIL. Relaxations to nitroglycerin were ameliorated by L-NIL.

Conclusions—This is the first study showing that chronic treatment with an iNOS inhibitor, L-NIL, limits progression of preexisting atherosclerosis in hypercholesterolemic rabbits. Increased intimal collagen accumulation may participate in iNOS-induced atherosclerosis progression. (Circulation. 2000;102:1033-1038.)

Key Words: nitric oxide synthase ■ L-NIL ■ arginine ■ atherosclerosis ■ inhibitors

Received December 16, 1999; revision received March 29, 2000; accepted March 30, 2000.


Correspondence to Tony J. Verbeuren, PhD, Division of Angiology, Servier Research Institute, 11 Rue des Moulinaux, 92150 Suresnes, France. E-mail tover@netgrs.com

© 2000 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org

1033
of endothelium-dependent relaxations,\(^1\)\(^2\)\(^3\) whereas others suggested short-lasting effects of L-arginine.\(^4\)\(^5\)\(^6\) In humans, the beneficial effects of L-arginine may depend on the stage of coronary artery disease.\(^7\)\(^8\)\(^9\) Thus, L-arginine improved endothelium-dependent vasodilatation in young hypercholesterolemic humans\(^10\)\(^11\)\(^12\) but failed to improve cardiovascular performance in severely hypercholesterolemic patients.\(^13\) We therefore studied L-arginine treatment on atherosclerosis progression by a protocol similar to that for L-NIL.

Methods

Study Design

Male New Zealand White rabbits (8 weeks) started a 0.3% cholesterol diet as described previously.\(^1\)\(^2\)\(^3\)\(^4\)\(^5\)\(^9\)\(^10\) After 24 weeks, atherosclerosis was evaluated in 9 rabbits (Baseline group). The remaining rabbits were separated into 3 groups kept on the diet for 12 more weeks. Eight rabbits received treatment with L-NIL (5 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\)) (L-NIL group), administered by 2 subcutaneously implanted MLA miniosmotic pumps (Charles River) that delivered the drug in the jugular vein. Nine rabbits were treated with saline (Saline group), and 8 rabbits received L-arginine HCI (2.25%) in drinking water (L-Arg group), using conditions previously described.\(^2\)\(^2\) Experiments were conducted in accordance with institutional guidelines for animal studies.

At monthly intervals, total cholesterol, triglycerides, and HDL cholesterol plasma levels were determined. Blood pressure and heart rate were measured before and at the end of the treatment.

Drugs

The following drugs were used: acetylcholine, ADP, phenylephrine, and L-arginine HCI (Sigma); L-NIL (A. Cordi); asymmetrical dimethyl arginine (ADMA) and symmetrical dimethyl arginine (SDMA) (Calbiochem); nitroglycerin (Besins-Iscovecco); and collagen and ATP (Coultronics).

Plasma L-Arginine, ADMA, SDMA, and L-NIL Concentrations

Plasma concentrations of L-arginine, ADMA, SDMA, and L-NIL were monitored as previously described.\(^3\)\(^1\) After acetonitrile extraction, samples were incubated with OPA reagent (5.4 mg/mL \(\text{Na}_2\text{CO}_3\), 2.64 g/L) containing 0.4% 2-mercaptoethanol and analyzed with high-performance liquid chromatography. Separation was obtained with a C\(_{18}\), Hypersil BDS-CPS 3-\(\mu\)m column, followed by analytical separation on a Kromasil 100-3.5 C18 column with a fluorescence detector. Samples were eluted with carbonate buffer. Drug concentrations were determined against internal standards.

Tissue Preparation

Animals were anesthetized with sodium pentobarbital (30 mg/kg IV), and carotid arteries were cannulated for blood sampling (10 mL). Thoracic aortas and hearts were harvested. Hearts were immersed in Bouin’s fixative before paraffin embedding; sections (4 \(\mu\)m) were cut to examine left common coronary arteries by histomorphometry after hemalun-eosin staining.

Aortic rings 3 mm wide were cut: the first ring, just below the aortic arch, was embedded in OCT compound (Ames), snap-frozen in liquid nitrogen, sectioned (7 \(\mu\)m), and stained with hemalun-eosin or orcein or stored after acetone fixation at \(-80^\circ\)C. The next ring was used for cGMP determination and the following rings for reactivity studies. Abdominal aortas were fixed in 10% buffered formalin, immersed in oil red O solution (Bio-optica) diluted in ethanol (1:1), and rinsed in water before being processed.

Tissue cGMP

Thoracic aortas were homogenized in a glass potter homogenizer (50 mmol/L phosphate buffer, 1 mmol/L theophylline, \(pH\) 7.4) and sonicated. An aliquot was used for DC protein assay (Bio-Rad). The remaining homogenate was centrifuged at 3000g for 15 minutes, and supernatants were frozen at \(-30^\circ\)C. cGMP was quantified with a competitive enzyme immunoassay (Cayman) as previously described.\(^6\)

Histomorphometry

Sections of thoracic aortas and coronary arteries were analyzed with an automated computerized image analyzer (Visiolar 1000, Bio-com). The intimal cross-sectional area (mm\(^2\)) was determined by subtracting the lumen area from the area enclosed by the internal elastic lamina. The medial area was determined by subtracting the area enclosed by the internal elastic lamina from that enclosed by the external elastic lamina. Mean areas were calculated to deduce intima/media (I/M) ratios.

Planimetry

Fixed abdominal aortas were incised longitudinally, pinned open, and placed flat on a Sylgard resin with transillumination for photography. The plaque surface was expressed as percentage of total surface quantified with the image analyzer.

Sirius Red Polarization Method for Collagen

Thoracic aortas were cut into sections, rinsed with distilled water, and incubated with 0.1% Sirius red F3BA (BDH) in saturated picric acid for 90 minutes. Sections were mounted in permanent nonaqueous medium (Kindler) and visualized by polarization microscopy.\(^3\)\(^2\) Intimal Sirius red-stained areas were measured with the image analyzer and calculated as percentage of total intimal area.

Immunohistochemistry

The following antibodies were used: anti-iNOS monoclonal antibody (1/50) (Transduction) and isotype-matched mouse IgG (Dako) as control antibodies and the specific cell markers (HHF-35) for smooth muscle cells (SMCs) (1/200), anti-rabbit macrophages (RAM-11) (1/250), and anti-CD45 RO (clone UCHL1) (1/100) for activated T cells.

Thoracic aorta sections were rehydrated in Tris-buffered saline (TBS: 50 mmol/L Tris-HCl, 150 mmol/L NaCl, \(pH\) 7.6; Dako), then in TBS containing 0.5% BSA (Sigma); nonspecific immunoglobulin-binding sites were blocked by TBS containing 4% BSA. Sections were incubated with a monoclonal primary or a negative control antibody for 2 hours at 20°C. The presence of antigen was revealed with biotinylated goat anti-mouse antibodies followed by incubation with streptavidin–alkaline phosphatase complex (Vector). Alkaline phosphatase was visualized with Vector red alkaline phosphatase substrate incubation. Slides were rinsed with TBS, counterstained with hematoxylin (Gill’s formula; Vector), and mounted in permanent nonaqueous medium. SMCs, macrophages, T lymphocytes, and iNOS were quantified on the intima with the automated image analyzer. Positive areas (% of total intima) were determined with a predefined threshold.

Hematology and Platelet Aggregation

Platelets, red blood cells, leukocytes, hemoglobin, and hematocrit levels were measured on whole blood with a T-540 counter (Coultronics). Platelet aggregation to ADP or collagen and ATP release were measured in whole blood aggregometers (Chrono-log). For technical reasons, blood samples from 2 Baseline and 1 Saline rabbit could not be tested.

Vascular Reactivity

Segments (3 mm) of thoracic aortas were mounted in organ chambers as previously described.\(^5\)\(^3\)\(^0\) Segments were placed at the optimal resting tension (8 g) for atherosclerotic rabbit aortas.\(^5\)\(^3\)\(^0\) During contractions with phenylephrine (1 \(\mu\)mol/L), concentration-response curves to acetylcholine (0.01 to 3 \(\mu\)mol/L) or nitroglycerin (0.001 to 3 \(\mu\)mol/L) were performed.

Statistical Analysis

Results are expressed as mean\(\pm\)SEM. Statistical analysis was performed by 1-way ANOVA with Newman-Keuls complementary anal-
ysis or 2-way ANOVA (treatment versus time of treatment) with repeated measures. For vascular reactivity studies, IC50 values were determined by linear interpolation. Treatment-effect relationships were compared by 2-way ANOVA with repeated measures on factor concentration. A value of P<0.05 was considered statistically significant.

Results

Physiological and Biological Parameters
No differences in body weight, mean arterial pressure, or heart rate (Saline group: 4.2±0.1 kg, 86±2 mm Hg, and 213±10 bpm) or levels of total cholesterol, HDL cholesterol, or triglycerides (Saline group: 18.7±2.4, 0.39±0.09, and 1.05±0.20 mmol/L) were detected.

Plasma l-arginine concentrations in Baseline rabbits averaged 72.1±11.5 μmol/L and were similar in the 4 groups at 24 weeks; they increased 2-fold after 4 weeks of l-arginine supplementation and remained elevated throughout treatment (176±2.6 μmol/L; P<0.05, 2-way ANOVA); they were unaffected in Saline and L-NIL rabbits. ADMA and SDMA concentrations (0.64±0.07 and 0.27±0.06 μmol/L) were comparable in all groups at 24 weeks and remained stable throughout. L-NIL plasma concentrations in the L-NIL group averaged 11.4±2.3 μmol/L after 4 weeks, 7.8±0.8 μmol/L after 8 weeks, and 10.6±0.6 μmol/L after 12 weeks.

iNOS and Inflammatory Cell Staining
Immunohistochemistry of thoracic aorta sections with the anti-iNOS showed a strong positive signal in all groups (Figure 1); no quantitative differences existed between the groups (Table 1). Lesion infiltration by inflammatory components such as macrophages (RAM-11 antibody) and T lymphocytes (CD45RO antibody) averaged 85% and 6% of the total intimal area, respectively, and was similar in all groups (Table 1).

Planimetry
In the Baseline group, the surface area occupied by atherosclerosis averaged 53.7±5.7%. This was increased in the Saline group (P<0.05, 1-way ANOVA) but not in the L-Arg and the L-NIL groups (Figure 2).

Histomorphometry
The I/M ratio of thoracic aortas of the Baseline group averaged 0.98±0.04. This ratio was increased in the Saline and L-Arg groups (P<0.05, 1-way ANOVA) but not in the L-NIL group (Table 2; Figure 3). Cross-sectional areas of medias were not different between the groups (Table 2).

The I/M ratio of coronary arteries of the Baseline group averaged 0.68±0.18. This ratio was not altered in the Saline and L-Arg groups but decreased in the L-NIL group (P<0.05, 1-way ANOVA) (Table 2; Figure 3).

![Image](https://example.com/image1.png)

**Figure 1.** Immunostaining of iNOS on cross sections of thoracic aortas of rabbits from Baseline (a), Saline (b), L-NIL (c), and L-Arg (d) groups. L indicates lumen. Magnification ×10.

![Image](https://example.com/image2.png)

**Figure 2.** Surface (expressed as % of total intimal area) occupied by atherosclerotic plaques in abdominal aortas of 4 groups of rabbits. In Saline but not in L-Arg or L-NIL groups, lesion area was significantly increased (*P<0.05 Baseline vs Saline, 1-way ANOVA).
ANOVA with Newman Keuls.

A decreased coronary I/M ratio was noted in L-NIL group (*P<0.05 and **P<0.01 vs Baseline). Aments per rabbit. Increased aortic I/M ratios were noted in Saline and L-Arg groups (*P<0.05 and **P<0.01 vs Baseline).

Table 2. Histomorphometric Measurements on Thoracic Aortas and Coronary Arteries of Hypercholesterolemic Rabbits

<table>
<thead>
<tr>
<th>Thoracic aorta</th>
<th>Media, mm²±SEM</th>
<th>Intima, mm²±SEM</th>
<th>I/M Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>3.41±0.08</td>
<td>3.50±0.21</td>
<td>0.98±0.04</td>
</tr>
<tr>
<td>Saline</td>
<td>3.55±0.07</td>
<td>5.75±0.23</td>
<td>1.63±0.17†</td>
</tr>
<tr>
<td>L-NIL</td>
<td>3.46±0.08</td>
<td>4.40±0.25</td>
<td>1.32±0.23</td>
</tr>
<tr>
<td>L-Arg</td>
<td>3.18±0.20</td>
<td>5.59±0.42</td>
<td>1.81±0.19†</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Media, 10⁻³ mm²</td>
<td>Intima, 10⁻³ mm²</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>105.6±20.6</td>
<td>67.7±26.0</td>
<td>0.68±0.18</td>
</tr>
<tr>
<td>Saline</td>
<td>108.7±19.1</td>
<td>105.4±22.0</td>
<td>0.96±0.19</td>
</tr>
<tr>
<td>L-NIL</td>
<td>92.7±19.1</td>
<td>31.8±18.3§</td>
<td>0.34±0.19§</td>
</tr>
<tr>
<td>L-Arg</td>
<td>96.8±13.9</td>
<td>96.2±33.3</td>
<td>1.00±0.29</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P=NS, †P<0.05, and ‡P<0.01 vs baseline; §P<0.05 vs saline, one-way ANOVA with Newman Keuls.

SMC and Collagen Staining
In the Baseline group, the percentage of total intimal surface of thoracic aortas stained by SMCs averaged 48.1±7.4%. This area was not altered in the Saline, L-Arg, or L-NIL group (Table 1).

In the Baseline group, the percentage of collagen-stained areas in thoracic aortas averaged 21.3±4.5%; this was increased in the Saline and L-Arg groups (P<0.05, 1-way ANOVA) but not in the L-NIL group (Figure 4; Table 1).

Tissue cGMP Determination
Tissue cGMP in thoracic aortas averaged 0.45±0.10 pmol/mg protein in the Baseline group; it was increased in the

Hematological Analysis and Platelet Aggregation
Levels of platelets (418±32×10³/μL), white (6.9±0.5×10³/μL) and red (4.8±0.1×10³/μL) blood cells, hemoglobin (10.9±3 g/dL), and hematocrit (31.9±0.9%) were comparable in all rabbits (values from Saline group). Collagen (10 μg/mL) induced aggregation (26.5±1.9 Ω) and ATP release (7.8±0.8 mmol), and ADP (3 μmol/L) induced aggregation (6.6±1.3 Ω) of platelets in whole blood. These responses were comparable in all groups.

Vascular Reactivity
In thoracic aortas, phenylephrine (1 μmol/L) caused contractions (5.4±0.5 g, Saline group) that were comparable between the groups. During such contractions, acetylcholine (0.01 to 3 μmol/L) evoked weak relaxations in the Baseline group (maximum, 35.5±10.6%) that were decreased in the Saline group (maximum, 21.3±7.1%, P<0.001; 2-way ANOVA), in the L-Arg group (P<0.05; 2-way ANOVA) and partly restored in the L-NIL group (P<0.01; 2-way ANOVA) but not in the L-NIL group (Figure 6).

In the Baseline group, nitroglycerin (0.001 to 3 μmol/L) evoked relaxations (IC⁵₀, 41±10 μmol/L) that were attenuated in the Saline group (P<0.001; 2-way ANOVA), in which the IC⁵₀ averaged 113±32 μmol/L (P<0.05; 1-way ANOVA). The relaxations to nitroglycerin were not altered by L-arginine treatment (IC⁵₀: 86±19 μmol/L) remained increased; P<0.05; 1-way ANOVA) but improved by L-NIL treatment (P<0.01; 2-way ANOVA), with an IC⁵₀ value not different from Baseline (Figure 6).

Discussion
The principal findings of our study show that the selective iNOS inhibitor L-NIL limits progression of preexisting ath-

Figure 3. I/M ratio in (a) aortic and (b) coronary cross sections of 4 groups of rabbits. Values are mean±SEM of 4 measurements per rabbit. Increased aortic I/M ratios were noted in Saline and L-Arg groups (*P<0.05 and **P<0.01 vs Baseline). A decreased coronary I/M ratio was noted in L-NIL group (#P<0.05 vs Saline, 1-way ANOVA).

Figure 4. Interstitial thoracic aorta collagen accumulation (% of total intimal areas stained by Sirius red) in 4 groups of rabbits. Significant increases were noted in Saline and L-Arg groups (*P<0.05 and **P<0.01 vs Baseline, 1-way ANOVA). Saline (P<0.01, 1-way ANOVA) but not in the L-NIL or L-Arg group (Figure 5).

Figure 5. Tissue cGMP concentrations in thoracic aortas of 4 groups of rabbits. A significant increase was noted only in Saline group (**P<0.01, Baseline vs Saline, 1-way ANOVA).
Coronary arteries; iNOS is present in the neointima, some-

data thus suggest that iNOS expression may aggravate devel-

erosclerosis in cholesterol-fed rabbits. L-Arginine influences

Figure 6. Acetylcholine (Ach, top) or nitroglycerin (NTG, bottom)
relaxations during contractions by phenylephrine (1 μmol/L) on
rabbit thoracic aortas of Baseline (•), Saline (□), L-Arg (○), and
L-NIL (△) groups. Data are mean±SEM (n=8 or 9). Relaxations
to Ach and NTG were decreased in Saline rabbits and partly
restored in L-Arg group for Ach and in L-NIL group for NTG
(*P<0.001, Baseline vs Saline, $P<0.05, L-Arg vs Saline, top, or
L-NIL vs Saline, bottom, 2-way ANOVA with repeated
measures).

SCM infiltration occurred between 24 and 36 weeks of
hypercholesterolemia or in L-NIL–treated rabbits. However,
increases in collagen accumulation were noted in saline-
treated but not in L-NIL–treated rabbits. Collagen is an
essential factor in plaque evolution, and L-NIL may decrease
its synthesis; indeed, increased expression of iNOS in wounds
precedes collagen synthesis. Activation of MMPs by iNOS
seems unlikely, because NO activates MMPs. A decreased
collagen accumulation by L-NIL without modification of
SMC infiltration suggests changes in SMC phenotype that
can be influenced by collagen through adhesion receptors.

L-NIL treatment had only modest effects on relaxations in
hypercholesterolemic rabbit aortas. It did not influence endo-
thelium-dependent relaxations, which contradicts evidence
that iNOS induction in injured arteries inhibits platelet
adhesion and restores blood flow. In the latter study, iNOS
may influence the acute response to endothelial denudation,
or else platelets see less NO than do the SMCs in which iNOS
is induced. L-NIL treatment prevents reduced nitroglycerin
relaxations; the receptor for NO, soluble guanylate cyclase,
becomes desensitized by excessive activation occurring after
iNOS induction, especially when this occurs in SMCs, and
this may explain the reduced activity for NO donors.

Thus, the selective iNOS inhibitor L-NIL possesses benefi-
cial antiatherogenic effects, limiting progression of athero-
sclerosis in both advanced (thoracic aorta) and less developed
( abdominal aorta) lesions of hypercholesterolemic rabbits.

Several studies in atherosclerosis showed beneficial effects
of L-arginine treatment on lesion development or endothelial
dysfunction. When the effects of L-arginine treat-
ment on preexisting lesions were studied, positive
effects depended on the protocol used: Böger et al and
Wang et al reported decreased intimal arterial growth,
augmented apoptosis, and restoration of endothelium-
dependent relaxations, whereas Candipan et al, who fed
rabbits a cholesterol diet for 23 weeks with L-arginine
supplementation for the last 13 weeks, reported nonsustained
effects of L-arginine, as also suggested by Jeremy et al.

In our study, dietary L-arginine, which increased plasma
L-arginine levels 2-fold, failed to influence lesion pro-
gression and composition in thoracic aortas and coronary
arteries but decreased it in abdominal aortas. Lesion progres-
sion is more important in thoracic than in abdominal aor-
tas, and studies in cholesterol-fed rabbits illustrated a
greater benefit of L-arginine on plaque formation in distal
parts of the aorta; regional variation of eNOS activity was
described in rat aortas, and iNOS expression is more
pronounced in more developed lesions of thoracic aortas and
coronary arteries. These findings may explain the regional
difference in L-arginine activity. The lack of antiatherogenic
effect of L-arginine on severe lesions may be related to the
duration of hypercholesterolemia or to unexpected effects,
such as blockade of the increased cGMP production. The
latter may be due to transformation of L-arginine by iNOS
into oxygen-derived free radicals. Yet L-arginine treatment
had a modest, beneficial effect on endothelium-dependent
relaxations in thoracic aortas.

Our data with L-arginine contribute to the debate on proathero-
genic or atherogenic functions for NO. L-Arginine ameliorates
manifestations of cardiovascular disease, but our data suggest that this effect may be limited, at least in the case of hypercholesterolemia, as previously shown in patients. Further investigations should clarify this issue as well as that of the hypercholesterolemic rabbit model to study NO in atherosclerosis.

In conclusion, although iNOS activity within developed atherosclerotic plaques does not influence infiltration and proliferation of cellular components, it accelerates progression of plaque development, which may be related to intimal collagen accumulation. Thus, contrary to eNOS activity, which exerts antiatherogenic effects on early lesions, iNOS activity may aggravate atherosclerotic lesion progression. Selective inhibitors of iNOS, e.g., L-NIL, can interrupt this proatherogenic action. Our study is the first to illustrate negative effects of iNOS induction in rabbit atherosclerosis. If predictive for human atherosclerosis, the data suggest iNOS inhibition as a target for disease treatment. The results stress the role of inflammatory mechanisms on atherosclerosis progression.

Acknowledgments
This work was supported by a grant from the Institut de Recherches Servier. We thank Nathalie Durand, Camille Decaux, Nathalie Da Costa, Jean Féchas, Pierre Bougneux, Loïc Vasseur, Régis Meulnotte, Catherine De Montrion, and Karine Baudelocq for assistance.

References
Effect of Chronic Treatment With the Inducible Nitric Oxide Synthase Inhibitor N-Iminoethyl-l-Lysine or With l-Arginine on Progression of Coronary and Aortic Atherosclerosis in Hypercholesterolemic Rabbits

Delphine Behr-Roussel, Alain Rupin, Serge Simonet, Edith Bonhomme, Sophie Coumailleau, Alex Cordi, Bernard Serkiz, Jean-Noël Fabiani and Tony J. Verbeuren

_Circulation_. 2000;102:1033-1038
doi: 10.1161/01.CIR.102.9.1033

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 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/102/9/1033