Reversal of Thrombin-Induced Deactivation of CD39/ATPDase in Endothelial Cells by HMG-CoA Reductase Inhibition
Effects on Rho-GTPase and Adenosine Nucleotide Metabolism

Nicole C. Kaneider, Petra Egger, Stefan Dunzendorfer, Patrizia Noris, Carlo L. Balduini, Donatella Gritti, Giovanni Ricevuti, Christian J. Wiedermann

Abstract—Adenosine triphosphate and diphosphate that activate platelet, leukocyte, and endothelium functions are hydrolyzed by endothelial CD39/ATPDase. Because CD39/ATPDase is downregulated in endothelial cells by inflammation and this may be affected by HMG-CoA reductase inhibitors, we examined the role of cerivastatin and simvastatin in regulation of endothelial CD39/ATPDase expression, metabolism of ATP/ADP, and function in platelets. Thrombin-stimulated endothelial cells in vitro were treated with the statins, and hydrolysis of exogenous ADP and ATP was assessed by high-performance liquid chromatography and malachite green assay. Platelet aggregation studies were performed with endothelial cell supernatants as triggers. CD39/ATPDase surface expression by endothelial cells was determined immunologically by fluorescence-activated cell sorter, mRNA expression by RT-PCR, and thrombin-induced dissociation of Rho-GTPases by Western blotting. Treatment by simvastatin or cerivastatin restored impaired metabolism of exogenous ATP and ADP in thrombin-activated endothelial cells by preventing thrombin-induced downregulation of CD39/ATPDase. In platelet aggregation studies, ATP and ADP supernatants of thrombin-activated endothelial cells were less stimulatory in the presence of statins than in their absence. Data show that statins preserve CD39/ATPDase activity in thrombin-treated endothelial cells involving alterations by statins of Rho-GTPase function and CD39/ATPDase expression. Preservation of adenine nucleotide metabolism may directly contribute to the observed anti-thrombotic and anti-inflammatory actions of statins. (Arterioscler Thromb Vasc Biol. 2002;22:lll–lll.)

Key Words:
Methods

Materials
Human thrombin, ADP, and gelatin were from Sigma. Cervicitin (CVN) was from Bayer AG (Pharma Research) and simvastatin (SVN) from Merck (Research Laboratories). The high-performance liquid chromatography (HPLC)-grade solvents (methanol and water), NaOH, NaH₂PO₄, malachite green, ammoniumheptamolybdate tetrahydrate, and sulfuric acid were from Merck. Hanks’ balanced salt solution without phenol red was from Life Technologies Ltd. ATP and ADP were from Roche Molecular Biochemicals Corporation. EC growth medium (ECCM) was from PromoCell, and fetal calf serum (FCS) was from PAA Laboratories GmbH. Monoclonal antibody to cytosolic guanine nucleotide dissociation inhibitor (Rho-GDI) and anti-mouse IgG-HRPO were from Transduction Laboratories. Protein transfer membrane was from Amersham Pharmacia Biotech. Monoclonal antibody to CD39 was from Ancell Corp. Phycoerythrin-conjugated anti-mouse IgG, human IgG, and anti-human mouse IgG were purchased from Sigma.

EC Culture
HUVECs from fresh placenta cords were isolated and grown to confluence at 37°C (humidified atmosphere). The growth medium was ECGM with 10% FCS. Culture flasks and 96-well plates were coated with 0.2% gelatin before seeding of cells. Cells were passaged by treatment with 500 μg/mL collagenase for 3 minutes. HUVECs of passages 1 and 2 were used.

Platelet Rich Plasma (PRP)
Human platelets were obtained from peripheral blood of healthy volunteers, anticoagulated with acid citrate, by centrifugation at 110g. Platelets were counted, and platelet-poor plasma was centrifuged at 1500g. Plastic or siliconized glassware was used for all platelet manipulations.

Treatment of HUVECs
HUVECs were passaged and plated at a density of 2.5×10⁵ cells/1000 μL medium into 12-well culture plates. When cells were grown to confluence, media were removed and cells washed with Hanks’ balanced salt solution without Ca²⁺ and Mg²⁺ and incubated with ECGM/0.2% FCS for 48 hours. HUVECs were treated with thrombin (0.1 mU/mL) and CVN or SVN (1 μmol/L to 100 μmol/L) for 45 minutes. Medium- or thrombin-treated cells served as controls. Then cells were washed twice and incubated with RPMI 1640 containing ADP (100 μmol/L) and ATP (100 μmol/L) for 20 minutes. To analyze distinct metabolism of ADP and ATP, HUVECs were treated with either 100 μmol/L ADP or ATP. Supernatants were then harvested and subjected to either HPLC analysis or malachite green or platelet aggregation assays. Time course experiments were performed with SVN (1 μmol/L) and either ADP (100 μmol/L) or ATP (100 μmol/L). Supernatants in time course experiments were collected every 10 minutes for a total of 1 hour and analyzed by HPLC (ADP-treated) or malachite green assay (ATP-treated).

Measurement of Adenine Compounds in Platelet and HUVECs Supernatants by HPLC
HUVEC supernatants, 300-μL aliquots, were subjected to HPLC analysis. Separation of adenine nucleotides was performed on a Lichrosphere RP-18 column (200×4.6 mm; 5 μm; Agilent Technologies) connected to a ZORBAX SB-C₁₈-precolumn (12.5×4.6 mm; 5 μm; Agilent Technologies). Signals were detected with a UV-visible detector (HP series 1100, Hewlett-Packard). The chromatographic conditions were as follows: isocratic elution at room temperature with 0.1 mol/L NaH₂PO₄ (pH 6.0 NaOH/methanol (96%/4%); flow rate, 0.3 mL/min; pressure, ~122 bar; UV-visible detector wavelength, 254 nm; injection volume, 30 μL. ATPDase Activity
Inorganic phosphate and pyrophosphate generated by CD39/ATP-Dase in culture supernatants were measured by the malachite green colorimetric assay.

Platelet Aggregation
Platelet aggregation was studied in PRP containing 250×10⁵ platelets/mL by Born’s method with a Lumi-Aggregometer (ChronoLog Corporation). PRP was preincubated at 37°C in an aggregometer cuvette. Platelet activation was started by addition of HUVEC supernatants. Addition of ADP served as positive control. Platelet aggregation was monitored by the increase of light transmission. Platelet-poor plasma served as negative control. Platelet aggregation experiments were performed with ECs incubated with ADP, β-S, a mediator of platelet aggregation, that is not hydrolyzed.

Western Blot Analysis of Rho-GTPases
HUVECs were incubated with thrombin (0.1 U/mL) and CVN or SVN (1 μmol/L to 100 μmol/L) for 20 minutes. Thrombin- or medium-treated cells served as controls. Cells were lysed in Nonidet-P40 buffer (Roche). Proteins were separated on 7.5% SDS polycrylamide gels and blotted onto polyvinylidenefluoride membranes. After blocking, the antibody was diluted to a final concentration of 0.05 μg/mL. Blots were incubated overnight at room temperature. Immunoreactivity was determined by using peroxidase-conjugated goat-anti-mouse IgG and the enhanced chemiluminescence reaction (Amersham). Intensity of the Western blot bands was quantified by using the Fluor-S Multimager System and the Quantity One Software (BioRad Laboratories).

Fluorescence-Activated Cell Sorter (FACS) Analysis of CD39
Confluent cells were treated with medium or thrombin or co-incubated with SVN. A total of 5×10⁵ HUVECs were washed in phosphate-buffered saline/0.5% bovine serum albumin and resuspended in 150 μg/mL human IgG for 20 minutes at 4°C. After pelleting, HUVECs were incubated with 10 μg/mL anti-CD39 or with the respective isotype-matched control IgG for 30 minutes at 4°C followed by a 1:40 dilution of anti-mouse R-phycoerythrin-conjugated IgG for 30 minutes. Then cells were analyzed by FACS and Cellquest software (Becton-Dickinson FACScan).

Soluble CD39/ATPase in EC Culture Supernatants
Sandy ELISA was performed according to standard protocols with supernatants of cells treated with medium, thrombin (0.1 μmol/L), SVN (1 μmol/L), and the combination of SVN (1 μmol/L) and thrombin (1 μmol/L) that were collected after 10, 20, 30, and 60 minutes (please see http://atvb.ahajournals.org). To investigate whether the soluble ATPDase is enzymatic active, ADP and ATP was added to the supernatants of thrombin- or thrombin- and statin-treated cells. HPLC measurement of adenine nucleotides in supernatants containing shedded CD39 was then performed.

Semi-quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)
Total RNA was isolated from 8×10⁶ cells by phenol-chloroform-isoamylalcohol extraction (RNACleanTM; Hybaid-AGS). RT-PCR was performed on 1 μg of RNA by using random hexamers reverse-transcriptase (Gibco BRL, Life Technologies). Ten microliters of the RT reaction mixture was then subjected to 35 cycles of PCR in a 50-μL reaction mixture containing 1.0 pmol of sense and anti-sense primer pairs in a Perkin-Elmer thermocycler: 95°C for 30 seconds (denaturation), 53°C for 60 seconds (annealing), 72°C for 30 seconds (extension). Hot Start Taq polymerase was from QIagen Inc. Primers (MWG Biotech) were designed to amplify a 428-bp coding sequence of CD39; sense, GCC CCA AGA ATA TCC TAG; anti-sense, CTT TCC ATC CTG AAC AAG. The PCR products were subjected to agarose gel analysis.
Statistical Analysis
Data are expressed as mean and SEM. Means were compared by using the Mann-Whitney U-test after Kruskal-Wallis ANOVA and Wilcoxon signed rank test. A P value <0.05 was considered significant.

Results
Effects of Statins on Thrombin-Inhibited Metabolism of ATP and ADP
Treatment of HUVECs with thrombin led to a decrease in free inorganic phosphate and pyrophosphate that was reversed in a concentration-dependent manner by treatment with either statin (Figure 1).

During incubation periods of up to 65 minutes, no measurable amounts of endogenous adenine nucleotides were released from HUVECs (data not shown). Under resting conditions (45 minutes of pretreatment with medium only), HUVEC monolayers metabolized exogenous ATP from 100 μmol/L to 30±12.4 μmol/L (n=7). Incubation of HUVECs for 45 minutes with thrombin, before addition of exogenous ATP, diminished this rate of ATP metabolism to 71±6.06 μmol/L (versus pretreatment with medium, n=7, P<0.01) (Figure 2). After addition of 100 μmol/L of ADP, ADP was hydrolyzed to 52.3±5.6 μmol/L, whereas pretreatment with thrombin diminished this rate to 86.9±4.3 μmol/L (versus pretreatment with medium, n=7, P<0.01) (Figure 2).

Because the simultaneous presence of exogenous ADP may affect the rate of ATP hydrolysis by CD39/ATPDase, enzymatic activities were also tested after addition of a combination of ATP and ADP. As compared with the untreated control, there were increased ATP and ADP levels in HUVEC supernatants by ≈240% and ≈180%, respec-

**Figure 1.** Effects of CVN or SVN with and without MVA on thrombin-induced free phosphate and pyrophosphate in ATP- and ADP-supplemented EC culture supernatants. Cells were incubated with thrombin (0.1 U/mL) and CVN or SVN (1 pmol/L to 100 pmol/L) in RPMI 1640 for 45 minutes; ADP (100 μmol/L) and ATP (100 μmol/L) were added for 20 minutes. Results given as percent of control of untreated culture supernatants obtained at a wavelength of 630 nm (1.775±0.103). Data was corrected for the amount of phosphorus ions (2.135 mmol/L) of medium; in control supernatants, 11.7±3.8 mmol/L of Pi/PPi found (supernatant volume 100 μL/well, 100 000 cells/well corresponding to 0.117 mg protein). Statistical analysis: Mann-Whitney U-test after Kruskal Wallis (P<0.01); *P<0.05, **P<0.01; (n=15).

**Figure 2.** Hydrolysis of adenosine nucleotides by thrombin-activated ECs. HUVECs were treated with thrombin (0.1 μU/mL) with or without SVN (1 pmol/L to 100 pmol/L) for 45 minutes. ATP and ATP (100 pmol/L) were added; supernatants were harvested after 20 minutes for HPLC analysis. Bars show degradation of ATP by medium-treated cells (n=7). Lines show recovery rate of ATP by thrombin-treated cells with and without pretreatment with SVN (n=7, +P<0.01 thrombin versus medium; *P<0.05; **P<0.01 SVN versus thrombin alone; Mann Whitney U-test). ADP and ADP (100 pmol/L) were added and supernatants analyzed (see above). Bars show degradation of ADP by medium-treated cells (n=7). Lines show recovery rate of ADP by thrombin-treated cells with and without pretreatment with SVN (n=7, +P<0.01 thrombin versus medium; *P<0.05; **P<0.01 SVN versus thrombin alone; Mann Whitney U-test).
tively, and a concentration-dependent increase of adenosine and a decrease of ATP and ADP levels when HUVECs were treated with SVN (please see http://atvb.ahajournals.org).

Similar patterns of enzymatic activities were seen with their combined addition. To test whether this effect is independent of cholesterol synthesis–inhibiting properties of the statins, we incubated HUVECs concomitantly with thrombin, statins, and mevalonic acid lactone (MVA). Statin-induced effects on thrombin-activated HUVECs were reversed by mevalonic acid. In this particular set of experiments \( n = 3 \), rates of ATP recovered in HUVEC monolayer supernatants after activation with 0.1 \( \mu \text{mol/L} \) thrombin were 136±26.0% compared with untreated cells (\( P < 0.05 \)), which was reduced by the presence of 100 \( \mu \text{mol/L} \) SVN to 11±1.6% (\( P < 0.05 \) versus thrombin); this reduction was affected by simultaneous presence of 500 \( \mu \text{mol/L} \) MVA resulting in a recovery rate of 101±24.9% (\( P < 0.05 \) versus SVN). Corresponding numbers were 165±30.9%, 23±1.1%, and 155±7.1% for ADP; 133±11.6%, 105±13.3%, and 101±22.1% for AMP; and 173±26.1%, 446±97.0%, and 153±23.8% for adenosine, respectively. These effects of MVA on adenine nucleotide metabolism was correspondingly confirmed in the malachite green assay of supernatants (Figure 1).

**Figure 3.** Kinetics of ADP metabolism by ECs exposed to thrombin and statin. HUVEC monolayers were incubated with either medium, thrombin (0.1 \( \mu \text{U/mL} \)), SVN (1 \( \mu \text{mol/L} \)) or thrombin (0.1 \( \mu \text{U/mL} \)) and SVN (1 \( \mu \text{mol/L} \)); after 45 minutes cells were washed and ADP (100 \( \mu \text{mol/L} \)) added; supernatants were harvested every 10 minutes for 60 minutes and subjected to HPLC analysis (ADP-supplemented medium under cell culture conditions but without cells also measured). Recovery rates for ADP, AMP, and adenosine shown. Statistical analysis: Mann-Whitney \( U \)-test after Kruskal Wallis (\( P < 0.01 \)); *\( P < 0.05 \), **\( P < 0.01 \); \( n = 7 \).

**Time Course Experiments and Kinetics of CD39/ATPDase Activity**

ADP, 100 \( \mu \text{mol/L} \), was added to ECs, and supernatants were taken every 10 minutes for a total of 60 minutes. Already after 5 minutes of incubation, a significant increase of phosphate production was seen in supernatants of medium-, SVN-, or SVN- and thrombin-treated cells, whereas in thrombin-treated ECs, no such effect was observed (data not shown). HPLC measurement of ADP hydrolysis to AMP and adenosine revealed corresponding results (Figure 3). Enzymatic activities were calculated from rates of ADP degradation and phosphate generation (please see http://atvb.ahajournals.org). Data demonstrate that SVN does not affect \( V_{\text{max}} \) of CD39/ATPDase, whereas thrombin reduced \( V_{\text{max}} \) significantly, which was partly reversed by addition of SVN.

**Shedding of CD39 From the ECs Induced by Thrombin**

Soluble CD39 was detected in supernatants of thrombin-treated ECs (please see http://atvb.ahajournals.org).

**Statin-Dependent Effects of HUVEC Supernatants on Platelet Aggregation**

Aliquots of 100 \( \mu \text{L} \) of statin- and thrombin-treated supernatants supplemented with ADP and ATP were added to 1
mL of PRP and aggregation was measured. Supernatants of thrombin-treated HUVECs led to strong platelet aggregation (41.25 ± 1.25%), whereas statin-treated cell supernatants diminished this response in a concentration-dependent manner (Figure 4). As a positive control, platelet aggregation was triggered with ADP (100 μmol/L), where 75 ± 2.5% of aggregation was observed. When exogenous ADP and ATP were replaced with ADP-β-S, the effects of the supernatants on platelet aggregation were unaltered (Figure 4).

**Effect of Statins on Rho-GTPases**

Western blot analysis with a monoclonal antibody directed against free (unbound) Rho-GDI was performed. Quantification of monoclonal antibody binding to free Rho-GDI showed an increase in thrombin-treated (0.01 and 0.1 mU/mL) HUVECs (which means a decrease in membrane bound Rho-GTPases) and a concentration-dependent decrease (increase of Rho-GDI-bound) of activated Rho-GTPase in statin-treated cells (Figure 5).

**Effect of Statins on CD39 Expression**

Thrombin-treated (0.1 mU/mL) cells exhibited a decrease in anti-CD39 fluorescence activity compared with untreated quiescent cells (Figure 6A); treatment of thrombin-activated HUVECs with SVN restored anti-CD39 fluorescence activity (Figure 6B). After a 45-minute incubation period of HUVECs with thrombin (0.1 mU/mL), CD39 mRNA expression was reduced, but cotreatment with SVN (1 μmol/L) restored CD39 mRNA transcription (Figure 6C).

**Discussion**

CD39/ATPDase is the dominant vascular nucleoside triphosphate diphosphohydrolase that exerts major effects on platelet reactivity by regulating hydrolysis of extracellular adenine nucleotides. Purinergic signaling influences hemostasis and inflammatory responses. Therefore, hydrolysis of extracellular ATP and ADP by the CD39/ATPdase would inhibit inflammatory and thrombotic processes. Statins may directly affect cells known to play key roles in the pathogenesis of atherothrombosis, including ECs and preservation of their anti-coagulatory properties. Because endothelial CD39/ATPdase plays an important role in preventing vascular clot formation, we investigated possible roles of two lipophilic statins, CVN and SVN in endothelium-platelet interactions. We confirmed that inflammatory stimulation of ECs leads to decreased ATP/ADP hydrolysis via downregulation of CD39/ATPdase using thrombin, which was previously not tested in this regard. This reduced adenine nucleotide metabolism led to an increase of platelet aggregation when PRP was supplemented with supernatants from thrombin-activated ECs. In contrast, with supernatants of cells incubated with ADP-β-S that is not hydrolyzed by CD39/ATPdase, platelet aggregation was not regulated. Effects of thrombin and adenine nucleotide metabolism were significantly diminished...
when HUVECs were cotreated with SVN or CVN; adenosine levels showed a concentration-dependent increase, whereas adenosine nucleotide levels were decreased. An increase of phosphate and pyrophosphate was correspondingly demonstrated.

Platelet reactivity most likely was decreased as a result of decreasing amounts of ADP and the increase of the adenosine level. Direct effects of statins or thrombin on platelets are unlikely because these agents were removed from HUVEC cultures by aspiration of supernatants followed by washing of cells, before adenine nucleotides were added.

ATPDases of quiescent ECs act synergistically to disperse platelet aggregates through degradation of ADP and ATP, and cells activated with a pro-inflammatory stimulus or by exposure to polyunsaturated fatty acids, lose this function. Our data suggest that thrombin may affect anti-thrombotic properties of ECs by deactivation of endothelial CD39/ATPDase, which can be prevented by statins. Via this mechanism, statins may reduce extracellular free ADP and ATP and, as a consequence, increase extracellular adenosine. Significant differences between thrombin- and thrombin-plus statin-treated cells were observed after time periods as short as 20 minutes, suggesting that this adenosine metabolism might underlie rapid alterations. Adenosine has previously been shown to inhibit a variety of cellular functions, including platelet aggregation, expression of tissue factor or adhesion molecules, and cytokine release by activated ECs. Our observation of decreased ATP and ADP concomitant with an increase of adenosine correlates with the simultaneous measurement of reduced in vitro aggregation in response to the culture supernatants.

As thrombin is a potent agonist for a number of biological responses that may mediate inflammatory and reparative responses to vascular injury, patients with increased thrombin generation are considered to be at enhanced cardiovascular risk. It was shown that thrombin levels in hypercholesteremic patients are enhanced. Protease-activating receptor (PAR) signaling pathways that mediate cellular effects of thrombin involve the activation of Rho-GTPases; PAR-1 receptors are not only found on platelets but also on ECs. Protease-activating receptor (PAR) signaling pathways that mediate cellular effects of thrombin involve the activation of Rho-GTPases; PAR-1 receptors are not only found on platelets but also on ECs. There is evidence that statins may affect cells via interference with signaling pathway proteins such as nuclear laminin B, ras proto-oncogen, Rho-related proteins, and the γ subunit of heterodimeric GTP-binding proteins. ECs are potentially exposed to a variety of extracellular proteases, particularly during vascular injury or inflammation. Thrombin is among these. Thrombin mediates increased endothelial retraction resulting in increased endothelial permeability to macromolecules. Activation of PAR by thrombin dissociates Rho-GTPases. As we found a decrease by statin treatment of ECs in thrombin-dissociated Rho-GTPases by detection of free cytosolic Rho-GDI, the effects of statins on adenosine metabolism as described here may be results of the inhibition of thrombin signaling. Preservation of CD39/ATPDase might be the effect of inhibition of thrombin signaling by inhibition of HMG-CoA reductase–dependent pathways.

Exogeneous MVA, a product of the mevalonate pathway, results in a redistribution of these GTPases to the membrane. Correspondingly, we observed MVA-induced reversal of statin-induced modulation of Rho-GTPase–dependent effects of thrombin.

A decrease of CD39 surface expression was observed. This may be due to a decrease of CD39 synthesis as detected by reduced mRNA in RT-PCR experiments and an increase of shedding of CD39 in thrombin-treated cells. These observa-
tions were made after exposure of cells to thrombin and were to varying degrees affected by statins. Shedding of CD39/ATPdase was already previously reported, which may remain enzymatically active. 36 It might also be that thrombin cleaves CD39/ATPdase at the apyrase-conserved region-1, resulting in substantive loss of biochemical activity. 37 Quantitative contributions of thrombin-affected shedding and mRNA synthesis of CD39/ATPdase in ECs to altered adenine nucleotide metabolism awaits further study.

Adenosine protects tissue from hypoxic or ischemic damage and has been postulated to trigger preconditioning by increasing mitochondrial K-ATP channel activity; preconditioning through adenosine can protect tissue from ischemia/reperfusion damage when added during the reperfusion period. 38 As we found an increase of free adenine nucleosides in statin-treated EC supernatants, it might be that via this mechanism statins also exert beneficial effects in patients who have ischemia/reperfusion injury. 39 It is known that thrombin—via PAR receptors—activates the transcription factor NF-κB and, hence, is a prototypical pro-inflammatory mediator that inhibits endothelial CD39/ATPdase, the regulation of which is NF-κB-dependent. 20 Statins are able to prevent endothelial dysfunction and NF-κB activation in response to inflammatory stimuli by disrupting the Rho-GTPase—dependent signal transduction pathways. 39 In conclusion, our data suggest that treatment of cells with thrombin suppresses endothelial CD39/ATPdase activity which can be completely preserved by the concomitant presence of CVN or SVN at doses that are achieved clinically. Adenosine nucleotides, which modulate platelet adhesion, recruitment to injured subendothelium, and formation of occlusive thrombi, are affected by statins in vitro. CD39/ATPdase is the major enzyme that converts ADP and ATP to AMP and adenosine; inhibition of CD39/ATPdase by thrombin suggests that its regulation during activation of coagulation may play a central role in the switch from an anti-thrombotic to a pro-thrombotic endothelial state. Functional antagonism of thrombin-triggered increase of ADP and ATP by statins represents a novel mechanism of action that may be of clinical relevance.

References


Reversal of Thrombin-Induced Deactivation of CD39/ATPase in Endothelial Cells by HMG-CoA Reductase Inhibition. Effects on Rho-GTPase and Adenosine Nucleotide Metabolism

Nicole C. Kaneider, Petra Egger, Stefan Dunzendorfer, Patrizia Noris, Carlo L. Balduini, Donatella Gritti, Giovanni Ricevuti and Christian J. Wiedermann

Arterioscler Thromb Vasc Biol. published online April 11, 2002;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2002/04/11/01.ATV.0000018305.95943.F7.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/03/31/22.6.894.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology 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 Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/