Increased Enzyme Activity and β-Adrenergic–Mediated Vasodilation in Subjects Expressing a Single-Nucleotide Variant of Human Adenylyl Cyclase 6

Robert Gros, Stan Van Uum, Adam Hutchinson-Jaffe, Qingming Ding, J. Geoffrey Pickering, Robert A. Hegele, Ross D. Feldman

Objective—cAMP is a critical regulator of metabolic and cardiovascular function. However, the role of genetic variability in the regulation of cAMP-mediated effects is unclear. Therefore, we assessed the effect of the expression of a recently identified missense genetic variant of adenylyl cyclase isoform 6 (ADCY6 S674).

Methods and Results—In rat vascular smooth muscle cells, gene transfer of ADCY6 S674 increased adenylyl cyclase activity and arborization to a greater extent than gene transfer of ADCY6 A674. Similarly, in adherent mononuclear leukocyte cells isolated from ADCY6 S674-expressing human subjects, both adenylyl cyclase activity and adenylyl cyclase–mediated cell retraction were significantly increased. Additionally, in dorsal hand vein LVDT studies, subjects expressing the hyper-functional ADCY6 S674 variant had significantly greater vascular sensitivity to the β-adrenergic agonist isoproterenol as assessed by both a greater potency and greater maximal effect than subjects expressing the ADCY6 A674 enzyme.

Conclusion—These data indicate that the expression of a novel, relatively common variant of ADCY6 parallels an increase in adenylyl cyclase activity and adenylyl cyclase–mediated function in humans. (Arterioscler Thromb Vasc Biol. 2007;27:2657-2663.)

Key Words: smooth muscle ■ adenylyl cyclase ■ vasodilation
of ADCY5 or ADCY6 improves cardiac function and survival. In transgenic mice, inhibition of adenyyl cyclase activity via activation of Gαs results in insulin resistance. However, the physiological or pathobiological significance of regulation of adenyyl cyclase function via “genetic” variability (ie, the expression of missense genetic variant enzymes) is unknown.

Given the critical role of ADCY6 in regulation of vascular smooth muscle contractility, the expression of a genetic variant of ADCY6 that significantly alters ADCY6 function could lead to altered vascular adenylyl cyclase-mediated effects. The identification of dysfunctional genetic variants of adenyyl cyclase is presently limited to those in ADCY6 and ADCY9, of which the latter has a much more restricted expression in normal subjects corresponding to residues 562 to 778, which include the C1b and IC4 regions of the molecule. This region was of particular interest because it includes domains critical in the regulation of catalytic activity. Additionally, our interest in this region was based on previous findings that serine to alanine alterations in this domain are associated with significant alterations in the regulation of ADCY6 function.

Consequently, the present studies were undertaken to examine the impact of the expression of ADCY6 S674 variant both on vascular reactivity and on cellular adenylyl cyclase activity/function. Our data demonstrate that the expression of the ADCY6 S674 variant is associated with enhanced adenylyl cyclase activity and enhanced cAMP-mediated regulation of contractile responses.

### Methods

#### Study Subjects

Recruitment of study subjects was based on mass advertising/emailing efforts within the Robarts Research Institute and the University of Western Ontario as part of an ongoing study of the phenotypic impact of the expression of the ADCY6 S674 variant. The age range of study subjects was 20 to 45 years. Subjects were normotensive- as determined by having a screening blood pressure measurement of less than 140/90 at the time of recruitment (based on the average of 5 readings, BPTru, Vancouver Canada) and healthy. Explicit exclusion criteria included: a history of cardiovascular events, average alcohol intake over 2 U per day, pregnancy, and use of antihypertensive/blood pressure altering drugs or anticoagulants.

Those subjects expressing the ADCY6 S674 variant allele (all were heterozygous) were subsequently invited to: (1) donate an additional blood sample for the in vitro assessment of adenylyl cyclase activity/function in adherent mononuclear leukocyte cultures or (2) participate in dorsal hand vein linear variable differential transformer studies assessing vascular reactivity. An otherwise similar population of subjects was recruited from among those expressing the ADCY6 A674 gene (Table 1). Recorded blood pressure was based on the average of the last 5 measurements taken. A 10 mL blood sample was taken to confirm the ADCY6 genotype.

#### Informed consent

Informed consent was obtained for all analyses, with approval from the University of Western Ontario Research Ethics Review Board. Genomic DNA was extracted from whole blood as previously described. Genotyping of ADCY6 A674S variants was performed using exon-specific DNA amplification followed by purification using shrimp alkaline phosphatase (Roche) and exonuclease I (Exon; New England Biolabs) and DNA sequencing as recently described.

#### Construction of Adenovirus Expressing S674 and A674 Adenylyl Cyclase 6

cDNAs encoding flag-tagged ADCY6 A674 and ADCY6 S674 or GFP were used to generate adenoaviral constructs (AdMax) as per manufacturer’s instructions (Microbix Biosystems Inc) as previously described.

#### Vascular Smooth Muscle Cell Primary Cultures

Rat aortic vascular smooth muscle cell (VSMC) primary cultures were isolated by a modification of the methods of Touyz et al. Briefly, freshly isolated thoracic aortae from Wistar rats (Harlan, Indianapolis, Indiana) were digested using collagenase and elastase incubations as previously described. Following digestion/isolation, vascular smooth muscle cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, gentamicin and fungizone. Vascular smooth muscle cells were used between passages 4 to 12 for all experiments. The rats were cared for in accordance with the Canadian Council on Animal Care guidelines.

#### Gene Transfer in Vascular Smooth Muscle Cells by Adenovirus

Vascular smooth muscle cells were infected with adenoaviral constructs (either adeno-GFP, adeno-ADCY6 A674, or adeno-ADCY6 S674) for 16 hours at 37°C after which infection media was replaced with fresh DMEM culture media. Cells were used for experimenta- tion 48 hours postinfection. Under these conditions, infection efficiency was greater than 95%, as assessed in GFP-infected cells.

#### Using Adherent Mononuclear Leukocyte Cultures to Assess Alterations in Adenylyl Cyclase-Mediated Responses With Variant ADCY6 Expression

Adherent mononuclear leukocyte fractions with fibrocyte characteristics can be separated from peripheral blood samples. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from human blood by centrifugation over Histopaque-1077 (Sigma-Aldrich) following the manufacturer’s protocol. PBMCs were washed twice with sterile 0.9% NaCl, resuspended in RPMI 1640 supplemented with 20% fetal bovine serum and gentamycin and seeded onto culture plates. This population of cells, isolated from adherent cell fractions, appears in a “spindle form” within 5 days after seeding onto culture plates and persist for several weeks (Figure 1A). Preliminary studies demonstrated that after one week of culture these spindle cells are “positive” for phalloidin, fibronectin, and CD68 but “negative” for α-smooth muscle actin—consistent with a “fibrocyte-type” cell of mononuclear leukocyte lineage (Figure 1B through 1D).

Adenyyl cyclase activity in permeabilized adherent mononuclear leukocytes and in vascular smooth muscle cells was assessed using our previously described methods that we have used in a range of cell types including mononuclear leukocytes and vascular smooth muscle cells.
cells. Briefly, digitonin-permeabilized cells were resuspended in a solution of Hanks’ Balanced Salt Solution with 33 mmol/L HEPES, 0.5 mmol/L EDTA and 1 mmol/L magnesium sulfate (pH 7.4 at 4°C) were added in an aliquot of 40 μL to give a final incubation volume of 100 μL with 1 μCi [α-32P] ATP, 0.3 mmol/L ATP, 2 mmol/L MgSO4, 0.1 mmol/L cAMP, 5 mmol/L phosphoenol pyruvate, 40 μg/mL pyruvate kinase and 20 μg/mL myokinase. Incubations with GTP (1 μmol/L), isoproterenol (μmol/L) or forskolin (100 μmol/L) were carried out at 37°C for 10 minutes and terminated by addition of 1 mL of a solution containing 100 μg ATP, 50 μg cAMP, and approximately 15 000 cpm [3H] cAMP. Cells were pelleted by centrifugation at 300g for 5 minutes. cAMP was isolated from the supernatant by sequential Dowex and alumina chromatography and was corrected for recovery using [3H] cAMP as the internal standard. Adenylyl cyclase activity was linear with time and cell number over the ranges used.

Adenylyl cyclase-mediated arborization response in rat vascular smooth muscle cells was assessed by video-microscopy, using our recently published techniques. Dishes were mounted on a temperature-controlled chamber (Bionomic controller, 20/20 Technology, Inc) on an inverted microscope (Zeiss, Axiovert S100). Arborization was induced by the addition of forskolin (10 μmol/L). Progression of arborization was evaluated using time-lapse video microscopy with a digital recording system. Images were obtained every minute and the extent of arborization was determined by the change in image intensity (Northern Eclipse 6.0, Empix Imaging). The change in image intensity was expressed as a percent of basal intensity ie, before the addition of drug. The change in image intensity was plotted against time and slopes were determined from linear regression analysis using Prism 4.0 (GraphPad Software).

Adenylyl cyclase-mediated cell retraction as a manifestation of arborization in adherent mononuclear leukocyte fractions was assessed by video-microscopy as described above for vascular smooth muscle cells. However, in adherent mononuclear leukocytes, the “arborization” effect was assessed by measurement of change in cell perimeter during 15 minutes of basal recording and after 15 minutes of forskolin (100 μmol/L) stimulation. This mode of analysis was chosen based on the much lower extent of optical density changes associated with comparable extents of retraction seen in the arborization response in cultured mononuclear leukocytes (versus those seen in vascular smooth muscle cells). Cell perimeter was obtained by using the trace tool within the analysis software (Northern Eclipse 6.0). Typically 6 to 8 cells in the field of view (1 or 2 cells in each quadrant randomly chosen and prospectively) were analyzed under basal and forskolin-treated conditions. Change in cell perimeter was expressed as a percentage of the initial cell perimeter.

Linear Variable Differential Transformer Studies

Assessment of Vascular Sensitivity to Isoproterenol by Dorsal Hand Vein Linear Variable Differential Transformer Technique

Studies using the linear variable differential transformer (LVDT) technique in dorsal hand veins were performed according to our previously described methods. Baseline venous distension was assessed after compression of the ipsilateral arm with a sphygmomanometer cuff inflated to 50 mm Hg. The extent of this distension at baseline was defined as 100%. Phenylephrine-mediated vasoconstriction was assessed by infusion of increasing doses from 16 to 20 000 ng/min (in normal saline at an infusion rate of 0.1 mL/min). The maximal extent of phenylephrine-mediated constriction and the potency of phenylephrine (as defined by the dose that produced half-maximal effect [ED50]) were determined by computerized nonlinear curve fitting (Sigmoid Plot, Subroutine, Prism 4.0, GraphPad Software). To assess the extent of isoproterenol-mediated attenuation of phenylephrine-mediated vasoconstriction, veins were preconstricted with phenylephrine at a dose that achieved approximately 80% of the maximum phenylephrine-induced effect; the dose was individualized for each subject in each study. In the assessment of vasodilator responses, the extent of venous distension achieved with this dose of phenylephrine was defined as 0% vasoconstriction. Isoproterenol was then concurrently infused at a dose of 0.32 to 200 ng/min in normal saline at an infusion rate of 0.1 mL/min. Maximum isoproterenol-mediated vasoconstriction and ED50 for isoproterenol were determined by analysis of the data by curve fitting techniques, as previously described. Maximal nitroglycerin-mediated vasorelaxation was determined at a dose of 100 ng/min.

Data Analysis

The nominal probability value for significance was <0.05. For 2-group comparisons, the statistical significance of differences was determined by student t test for unpaired data with Welch’s correction when necessary. For column statistics, the significance of differences from control was determined by 1-sample t tests. P<0.05 on a 2-sided test was taken as a minimum level of significance (Prism 4.0, GraphPad Software).
expressed as their geometric means.

Therefore, these parameters are not normally distributed. However, after log transformation these data are normally distributed. Therefore, these parameters are expressed as their geometric means.

Results

Effect of ADCY6 S674 Expression on Adherent Mononuclear Leukocyte Adenylyl Cyclase Activity

To determine whether the ADCY6 S674 variant was associated with any functional alterations in enzymatic activity, we examined adenylyl cyclase activity in adherent cell cultures derived from circulating mononuclear leukocytes isolated from whole blood samples. Basal (unstimulated) adenylyl cyclase activity did not differ significantly between subjects with ADCY6 A674 or the ADCY6 S674 variant (ADCY6 A674: 25 ± 5 vs. ADCY6 S674: 37 ± 7 pmol/min/mg of protein, n = 12 for both, P = 0.2). However, both GTP- and forskolin-stimulated adenylyl cyclase activities were significantly increased in adherent mononuclear leukocytes obtained from ADCY6 S674 variant subjects as compared with ADCY6 A674 subjects (Figure 2A). Isoproterenol-stimulated adenylyl cyclase activity was also significantly increased in ADCY6 S674 adherent mononuclear leukocytes as compared with cells from subjects expressing WT ADCY6 (Figure 2A). However, isoproterenol-stimulated adenylyl cyclase activity actually represents isoproterenol + GTP-stimulated adenylyl cyclase activity. Therefore, we examined the proportional increase in isoproterenol-stimulated adenylyl cyclase activation. The proportional increase of isoproterenol-stimulated over GTP-stimulated adenylyl cyclase activity was not significantly different between ADCY6 S674- and ADCY6 A674-expressing adherent mononuclear leukocytes (141 ± 15% versus 136 ± 11% of GTP-ACA, n = 12, both for the ADCY6 S674 variant and ADCY6 A674 respectively, P = 0.75). Therefore, the elevation in forskolin-stimulated activity suggests an increase in the intrinsic activity of the enzyme, whereas the similar proportional increase in isoproterenol-stimulated activity suggests that the efficiency of GPCR-G protein coupling was not altered by the expression of the ADCY6 S674 variant.

Effect of ADCY6 S674 Expression on Adenylyl Cyclase–Mediated Adherent Mononuclear Leukocyte Retraction

To determine whether the increase in forskolin-stimulated adenylyl cyclase activity in adherent mononuclear leukocytes from subjects with the ADCY6 S674 variant resulted in increased functional adenylyl cyclase-mediated responses, we performed adherent mononuclear leukocyte retraction assays. Forskolin treatment mediated both time- and concentration-dependent increases in cellular retraction (data not shown). Similar to the results obtained for the adenylyl cyclase activity assay, forskolin-mediated retraction was significantly increased in ADCY6 S674 variant-expressing adherent mononuclear leukocytes as compared with the ADCY6 A674 expressing cells (Figure 2B).

Assessment of the Impact of Expression of the ADCY6 Variant on Vascular Reactivity: LVDT Studies

In ADCY6 A674- expressing subjects, phenylephrine mediated a dose-dependent reduction in vascular distension with an ED50 of 553 ng/min to a nadir of 36 ± 13% of baseline distension. With infusion of a dose of phenylephrine sufficient to mediate approximately 80% of its maximal effect, concurrent infusion of isoproterenol caused dose-dependent vasodilation with an ED50 of 8 ng/min reaching a maximum of 97 ± 6% of initial baseline (Table 2). Subsequent infusion of nitroglycerin at a dose of 100 ng/min resulted in a further increase in distension to 109 ± 9% of baseline (Table 2). These findings are similar to those we have previously reported in normal populations using this protocol.

In subjects expressing ADCY6 S674, vascular sensitivity to isoproterenol was increased on average by approximately 10-fold (ie, the ED50 isoproterenol was decreased by more than 90%, Table 2). Further, there was a significant increase in maximal isoproterenol-mediated vasodilation (Table 2). In contrast, neither the extent of baseline distension nor maximal nitroglycerin-mediated relaxation differed between groups (Table 2). Additionally, indices of vascular vasoconstrictor responses to phenylephrine (ED50 phenylephrine, maximal phenylephrine-mediated vasoconstriction) did not differ between groups (Table 2).
Assessment of Adenyl Cyclase–Mediated Responses in Variant and ADCY6 S674–Expressing Vascular Smooth Muscle Cells

To determine whether the difference in adenyl cyclase activity observed in adherent mononuclear leukocytes obtained from subjects expressing the ADCY6 S674 variant was a property of the adherent mononuclear leukocytes or of the intrinsic adenyl cyclase activity, we examined the effect of ADCY6 S674 expression on adenyl cyclase activity in another cell type, rat vascular smooth muscle cells, which express a highly homologous (93% compared with human) endogenous ADCY6.31 With comparable expression of ADCY6 S674 and ADCY6 A674 in vascular smooth muscle cells (Figure 3A), gene transfer of the ADCY6 S674 variant resulted in a significant increase in forskolin-stimulated adenyl cyclase activity as compared with ADCY6 A674-expressing smooth muscle cells (Figure 3B).

To determine whether increased ADCY6 S674-mediated enzymatic activity in vascular smooth muscle cells paralleled an increase in adenyl cyclase-mediated function we examined forskolin-stimulated arborization in vascular smooth muscle cells. With gene transfer of ADCY6 S674 variant, forskolin-mediated arborization responses were significantly increased as compared with responses in ADCY6 A674-infected smooth muscle cells (Figure 3C).

Discussion

Although our most recent studies had identified a relatively common genetic variant of ADCY6, namely ADCY6 S674, the significance of its expression was unknown, either in mammalian systems or in humans. The present studies demonstrate that the expression of the ADCY6 S674 variant is associated with an increase in both adenyl cyclase and β-adrenergic–mediated vascular reactivity.

The mechanism of the increase in adenyl cyclase function in circulating adherent mononuclear leukocytes derived from whole blood samples taken from subjects expressing the ADCY6 S674 variant would appear to be best explained by an enhancement of ADCY6 function. This conclusion is supported by our findings that gene transfer of the ADCY6 S674 variant into rat vascular smooth muscle cells increased adenyl cyclase effects, as assessed both enzymatically and functionally, to a significantly greater extent than expression of the WT ADCY6. It is important to note that in our prior evaluation of this variant we reported that, in the baculovirus/S9 insect cell system, the expression of ADCY6 S674 demonstrated reduced activity as compared with activity in cells expressing ADCY6 A674.4 The reason for this discrepancy with our current findings is speculative. However, for membrane proteins, the “functional readouts” from insect cell systems may not predict their impact in mammalian systems.32 This has been related to differences in functional responses of nonglycosylated protein forms as seen in insect models or to differences in intracellular scaffolding cytoskeletal structure that are critical for the functional effect of a number of membrane-associated proteins.33 However, regardless of the explanation for the differences in effect of ADCY6 S674 in insect versus mammalian cells, our current studies indicate that increased adenyl cyclase activity associated with expression of the ADCY6 S674 variant in a more relevant mammalian cell system (and at a much lower extent of overexpression) parallels the increase in “global” adenyl cyclase activity and in adenyl cyclase-mediated function in ADCY6 S674 adherent mononuclear leukocytes.

In the hypothetical case that ALL adenyl cyclase isoforms would contribute comparably to regulation of contractile function in vascular smooth muscle cells, a 2-fold increase in adenyl cyclase activity between variants of a single isoform (ie, as seen for the genetic variant of ADCY6 S674 versus the more common ADCY6 A674 when expressed in rat vascular smooth muscle cells) would NOT be expected ultimately to impact on “global” adenyl cyclase-mediated function. However, ADCY6 has been identified as a predominant isoform expressed in a range of tissues important in cardiovascular regulation.33 Further, our recent studies identified that among the AC isoforms, ADCY6 was selectively coupled to regulation of vascular smooth muscle contractile responses,11 suggesting that the regulation of ADCY6 function would have an impact on contractile regulation far exceeding the proportional contribution of ADCY6 to total adenyl cyclase expression. Our current findings support the hypothesis that a significant alteration of ADCY6 function leads to increased adenyl cyclase–mediated contractile effects, both at a single–cell level as well as in vivo in humans.

The cardiovascular significance of the expression of this hyper-functional ADCY6 is supported by our LVDT studies demonstrating enhanced β-adrenergic–mediated vasodilatory responses in subjects expressing the S674 variant of ADCY6. Notably, the heritability of vasodilatory responses has been

Table 2. Venous Responsiveness in ADCY6 A674 and ADCY6 S674 Subjects

<table>
<thead>
<tr>
<th></th>
<th>A674 n=31</th>
<th>S674 n=32</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline distension, mm</td>
<td>1.165±0.199</td>
<td>0.956±0.119</td>
<td>0.39</td>
</tr>
<tr>
<td>Phenylephrine ED50, ng/min</td>
<td>553 (+995/−321)</td>
<td>384 (+681/−216)</td>
<td>0.65</td>
</tr>
<tr>
<td>Max PE-mediated constriction, %</td>
<td>64±13</td>
<td>62±9</td>
<td>0.93</td>
</tr>
<tr>
<td>Max NTG-mediated dilation, %</td>
<td>109±8</td>
<td>97±5</td>
<td>0.22</td>
</tr>
<tr>
<td>Isoproterenol ED50, ng/min</td>
<td>8.1 (+15.5/−4.2)</td>
<td>0.6 (+0.9/−0.4)</td>
<td>0.009*</td>
</tr>
<tr>
<td>Max ISO-mediated dilation, % of NTG</td>
<td>89±2</td>
<td>106±3</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

Data represent the mean±SEM. For ED50 values data are expressed as the geometric means and errors. Log transformations of individual ED50 values were performed, and means and standard errors were determined and expressed as their antilogs. ng/min. PE indicates phenylephrine; NTG, nitroglycerine; ISO, isoproterenol. P value obtained for 2 group comparisons by 2-tailed Student t test.
associated with genetic variants of both the beta-2-adrenoceptor (Ile164 and Gln27 variants), as well as the G protein beta subunit GNB3 C825T. However, genetic variability in vasodilatory responses related to expression of an AC isoform genetic variant has not previously been reported.

The expression of missense genetic variants of proteins regulating adenylyl cyclase function have been linked to both hypertension and obesity. The expression of beta-adrenoceptor variants and variants of GRK4 (an enzyme that regulates G protein–coupled receptors linked to AC activation) have been associated with variation in blood pressure and development of hypertension. Further, hyper-functional G protein variants have been associated with variation in the development of obesity as well as hypertension (reviewed in). Expression of this ADCY6 variant and the consequent enhancement of ADCY6-mediated effects would be predicted to be associated with a "hyperdynamic" cardiovascular phenotype, demonstrating increased pulse pressure, systolic blood pressure, or increased pulse rate. Further, expression of this adenylyl cyclase variant would be predicted to be associated with a "leaner" phenotype, marked by decreased abdominal obesity.

In summary, our data indicate that the expression of a novel, relatively common variant of ADCY6 parallels an increase in adenylyl cyclase activity and adenylyl cyclase mediated function in humans. Whether there might be an altered frequency of the expression of the A674 allele in patients with metabolic syndrome, diabetes, and hypertension, ie, whether this genetic variant might prove to be a predictive marker for cardiovascular disease, is the focus of ongoing studies.

Acknowledgments

We gratefully acknowledge the important contributions made by Nancy Schmidt in assisting in the performance of the LVDT studies.

Sources of Funding

These studies were supported by grants-in-aid to R.D.F. from the Heart and Stroke Foundation of Ontario. R.G. is supported by a New Investigator Award from the Heart and Stroke Foundation of Canada.

Disclosures

None.

References

11. Gros R, Ding Q, Chorazycewski J, Pickering JG, Limbird LE, Feldman RD. Adenylyl cyclase isoform-selective regulation of vascular smooth...
Gros et al Increased Function of an ADCY6 Genetic Variant

2663


Increased Enzyme Activity and β-Adrenergic–Mediated Vasodilation in Subjects Expressing a Single-Nucleotide Variant of Human Adenylyl Cyclase 6
Robert Gros, Stan Van Uum, Adam Hutchinson-Jaffe, Qingming Ding, J. Geoffrey Pickering, Robert A. Hegele and Ross D. Feldman

Arterioscler Thromb Vasc Biol. 2007;27:2657-2663; originally published online October 4, 2007;
doi: 10.1161/ATVBAHA.107.145557
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 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/27/12/2657

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/