Transcriptional Regulation of Apolipoprotein A5 Gene Expression by the Nuclear Receptor RORα

Annelise Genoux, Hélène Dehondt, Audrey Helleboid-Chapman, Christian Duhem, Dean W. Hum, Geneviève Martin, Len A. Pennacchio, Bart Staels, Jamila Fruchart-Najib, Jean-Charles Fruchart

Objective—The newly identified apolipoprotein A5 (APOA5), selectively expressed in the liver, is a crucial determinant of plasma triglyceride levels. Because elevated plasma triglyceride concentrations constitute an independent risk factor for cardiovascular diseases, it is important to understand how the expression of this gene is regulated. In the present study, we identified the orphan nuclear receptor retinoic acid receptor-related orphan receptor-α (RORα) as a regulator of human APOA5 gene expression.

Methods and Results—Using electromobility shift assays, we first demonstrated that RORα1 and RORα4 proteins can bind specifically to a Direct Repeat 1 site present at the position –272/–260 in the APOA5 gene promoter. In addition, using transient cotransfection experiments in HepG2 and HuH7 cells, we demonstrated that both RORα1 and RORα4 strongly increase APOA5 promoter transcriptional activity in a dose-dependent manner. Finally, adenoviral overexpression of hRORα in HepG2 cells led to enhanced hAPOA5 mRNA accumulation. We show that the homologous region in mouse apoa5 promoter is not functional. Moreover, we show that in staggerer mice, apoa5 gene is not affected by RORα.

Conclusions—These findings identify RORα1 and RORα4 as transcriptional activators of human APOA5 gene expression. These data suggest an additional important physiological role for RORα in the regulation of genes involved in lipid homeostasis and probably in the development of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2005;25:1-7.)

Key Words: apolipoprotein A5  ■  nuclear receptor RORα  ■  triglyceride homeostasis  ■  transcriptional regulation
reported to downregulate APOA5 gene expression via a direct binding to the functional E-box present in human APOA5 promoter.12

The retinoic acid receptor-related orphan receptors (RORs) constitute a subfamily of orphan receptors encoded by 3 different genes, RORα, ROR-β, and ROR-γ.13–15 RORs were initially reported to bind to response elements (retinoic acid receptor-related orphan receptor response element [RORE]) consisting of a 6-bp A/T-rich sequence preceding the half-core PuGGTCA motif.15–17 However, more complex RORE have also been described such as direct repeats of the PuGGTCA motifs preceded by a 6-bp A/T-rich sequence.18 Because of alternative splicing and promoter usage, the RORα gene gives rise to 4 isoforms: α1, α2, α3, and α4 (or RZRa),14,15,19 which differ in their N-terminal domains and display distinct DNA sequences and transcriptional activities.14 In contrast to RORβ, only expressed in brain, retina, and pineal gland,20 both RORα and RORγ are widely expressed in peripheral tissues.15,15,16,19 Staggerer mice (sg/sg) carry a natural deletion in the RORα gene that prevents the translation of its putative ligand-binding domain, thereby presumably disrupting the normal function of this transcription factor.21 Interestingly, in addition to severe neurological disorders,22 these mice display metabolic abnormalities.23–25 These mice, maintained on an atherogenic diet, have severe hypoalphaproteinemia and atherosclerosis develop.25 Furthermore, RORα has already been involved in apolipoprotein A1 and C3 gene transcription,23,24 These data suggest an important role for RORα in lipoprotein metabolism and cardiovascular diseases.

In the present study, we demonstrated that both RORα1 and RORα4 isoforms are positive regulators of human APOA5 gene transcription. Our data identify APOA5 as a new target gene for RORα and suggest a role for RORα in plasma triglyceride homeostasis.

Methods

Cloning and Construction of Recombinant Plasmids

The human APOA5 promoter fragment (−305/+62) was amplified by polymerase chain reaction (PCR) using an APOA5 genomic BAC clone5.10 as template and cloned in the pGL3 luciferase vector. The forward oligonucleotide 5'-TCTGTTTGGGGCCACCCAG-3' and reverse oligonucleotide 5'-AAGGATCCCTTTAAGCTGTGAC-3' primers were used for the PCR reaction. Site-directed mutagenesis of the construct (−305/+62) (−2705−3), −2635−2) was accomplished according to the recommendations of the manufacturer with the QuikChange site-directed mutagenesis kit (Stratagene). Site-directed mutagenesis of the promoter with the following oligonucleotides: forward 5'-ATGCGAGGTAAAGAACGTCGAC-3' and reverse 5'-AACCCATGATGTTTCAGTCTTACTTTACGTCA-3' was accomplished according to the recommendations of the manufacturer with the QuikChange site-directed mutagenesis kit. The wild type promoter fragment (−305/+62) was cloned in 4 copies into the SV40-pGL3. The pCDNA3-hROR expression vectors have been previously described.26 The constructs Ad-GFP and Ad-RORα1, as well as the corresponding adenoviral particles, were prepared as described previously.26 pAd-RORα4 and pAd-LacZ adenoviruses were obtained by the Vira Power Adenoviral Expression system (Invitrogen, Paisley, UK). Briefly, RORα4 and control LacZ cDNA were cloned in pAd DEST-based expression vector (Bett et al. 1994); 293 A cells were transfected with pAd vectors to produce an adenoviral stock. This stock was amplified, purified on cesium chloride, and titered by a plaque assay in 96-well plates to define the titer in pfu/mL.

Cell Culture, Viral Infection, and Transient Transfection Assays

Human hematopoietic HepG2 and HuH7 cells were obtained from the European Collection of Animal Cell Culture (Porton Down, Salisbury, UK). Cell lines were maintained in standard culture conditions (Dulbecco’s modified Eagle’s minimal essential medium) supplemented with 10% fetal calf serum, 5% nonessential amino acids, and 5% sodium pyruvate (Invitrogen) at 37°C, 95% air. Medium was changed every 2 days.

For adenovirus experiments, HepG2 cells were seeded in 6-well plates at a density of 5×104 cells/well and incubated at 37°C before viral infection. Viral particles were added at a multiplicity of infection of 100 and incubated for 3 hours. HepG2 cells were infected with adenovirus coding, respectively, for RORα1 or RORα4 (Ad-RORα1, Ad-RORα4) or with control adenovirus coding for GFP (Ad-GFP, green fluorescent protein) or LacZ (Ad-LacZ, β-galactosidase). Thereafter, cells were washed with phosphate-buffered saline and incubated in culture medium for the indicated times. At the end of the experiment, cells were washed once with ice-cold phosphate-buffered saline, lysed, and scraped in 0.5 mL of ice-cold Trizol reagent.

For transfection experiments, HepG2 and HuH7 cells were seeded in 24-well plates at a density of 103 cells per well and incubated at 37°C for 16 hours before transfection. Cells were transfected by the calcium phosphate coprecipitation procedure using 300 ng of reporter vector and different quantities (10, 30, 100, and 300 ng) of pCDNA3-hRORα1, α2, α3, α4, and γ expression vectors, or empty pCDNA3 plasmid vector as control. The total amount of DNA was kept constant by complementation with pCDNA3 empty vector. At the end of the experiment, the cells were washed with ice-cold phosphate-buffered saline, and the luciferase activity was measured using a luciferase assay system (Promega Corp, Madison, Wis).

Mice

Staggerer mutant mice were obtained by crossing heterozygote (+/sg) mice maintained in a C57BL/6 genetic background and maintained on chow diet as previously described.24 Wild-type littermates of the same age as the homozygous mutants were used as control.

Gel Retardation Assays

The pCDNA3-hRORα1, α2, α3, α4, and γ expression plasmids were transcribed in vitro using T7 polymerase and subsequently translated using rabbit reticulocyte lysate as recommended by the manufacturer (Promega). DNA-protein binding assays were conducted as described27 using the following binding buffer: Hepes 10 mmol/L, KCl 60 mmol/L, glycerol 10%, MgCl2 5 mmol/L, EDTA 0.5 mmol/L, dithiothreitol 1 mmol/L, PMSF 1 mmol/L, poly(dIdC) 0.1 μg/mL, and herring sperm DNA 50 ng/μL containing 3 μL of programmed or unprogrammed reticulocyte lysate. Double-stranded oligonucleotides corresponding to the wild-type (forward 5'-GGGAGTAAAAGGTCAAGG-3' and reverse 5'-CCCCATGACCTTTACCTCCCC-3') or mutated (forward 5'-AGTGAGGAGATTTAAAAGTACGGGTT-3' and reverse 5'-AACCCCTATCTTTAAAGGTTCCCCTT-3') were labeled with [32P]ATP using T4 polynucleotide kinase and used as probes. For competition experiments, 25−, 100−, and 400-fold excess of cold wild-type or mutated probe was included 20 minutes before adding labeled probe. For supershift assays, 5 μL of 1X polyclonal RORα antibody (Santa Cruz Biotechnology, La Perray en Yvelines, France) were added to the binding reaction. DNA-binding complexes were resolved by 5% native polyacrylamide gel electrophoresis (PAGE) in 0.25X TBE. Gels were dried and exposed at −80°C to XOMAT-AR film (Eastman Kodak Co, Rochester, New York).
Results

RORα1 and RORα4 Proteins Specifically Bind to a DR1 Site in the Human APOA5 Gene Promoter

It was previously described that peroxisome proliferator-activated receptor-α/retinoid X receptor dimers bind to a DR1 site present at the position −272/−260 in the human APOA5 gene promoter.10,11 Because this site presents structural homologies with the binding site for the orphan nuclear receptor hRORα, we evaluated whether hROR proteins translated in vitro could bind to this site by electromobility shift assay. As shown in Figure 1A, only in vitro translated hRORα1 and RORα4 proteins bound to the DR1 site, whereas RORα2, α3, and γ failed to bind this site (a SDS-PAGE analysis was performed with the translated products in the presence of 35S methionine and demonstrated that all the ROR isoforms, α1,α2, α3, α4, and γ, are synthesized in equivalent amounts [data not shown]). The specificity of both hRORα1 and RORα4 protein bindings to the hAPOA5 promoter ROR response element-Direct Repeat 1 (RORE-DR1), was determined first by using a specific antibody against RORα, which results in a supershift of the binding for both RORα1 and RORα4 (Figure 1A). Next, the mutated (−270G→C, −263G→A) radiolabeled DR1 site probe in electromobility shift assay failed to bind both RORα1 and RORα4 (Figure 1A). Furthermore, a competition with unlabelled wild-type RORE-DR1 site resulted in a complete inhibition of the binding. However, the unlabeled mutated RORE-DR1 (Figure 1B) failed to compete with the binding of both RORα1 and RORα4 proteins to the RORE-DR1 site. These data indicate that the RORE-DR1 site present in the human APOA5 gene promoter is a binding site for both RORα1 and RORα4 nuclear receptors.

Using a labeled consensus probe that binds in vitro monomers and dimers as reference, we determined that RORα1 and RORα4 bind as monomers to the DR1 located at the position (−272/−260) of the human APOA5 gene promoter. Gel retardation assays were performed with the indicated end-labeled wild-type (wt) (−276/−256) (A and B) and mutant (mt) (−279/−253) (B) fragment of the human APOA5 gene promoter in the presence of in vitro transcribed/translated human RORα1 and 4 (A and B), human RORα2, α3, and RORγ (A), or unprogrammed reticulocyte lysate as control (5 μL of each). Super-shift assays were performed using 5 μL of a 1X polyclonal RORα specific antibody (A). DNA-protein complexes were resolved by non-denaturing PAGE. Specific complexes not observed with unprogrammed lysate are indicated by arrows. Competition experiments (B) were performed in the presence (+) or absence (−) of unlabelled wt (competition wt, 25-, 100-, 200-, and 400-fold molar excess) or mt (competition mt, 25-, 100-, 200-, and 400-fold molar excess) oligonucleotides.

Figure 1. RORα1 and RORα4 proteins bind specifically to the DR1 located at the position (−272/−260) of the human APOA5 gene promoter. Gel retardation assays were performed with the indicated end-labeled wild-type (wt) (−276/−256) (A and B) and mutant (mt) (−279/−253) (B) fragment of the human APOA5 gene promoter in the presence of in vitro transcribed/translated human RORα1 and 4 (A and B), human RORα2, α3, and RORγ (A), or unprogrammed reticulocyte lysate as control (5 μL of each). Super-shift assays were performed using 5 μL of a 1X polyclonal RORα specific antibody (A). DNA-protein complexes were resolved by non-denaturing PAGE. Specific complexes not observed with unprogrammed lysate are indicated by arrows. Competition experiments (B) were performed in the presence (+) or absence (−) of unlabelled wt (competition wt, 25-, 100-, 200-, and 400-fold molar excess) or mt (competition mt, 25-, 100-, 200-, and 400-fold molar excess) oligonucleotides.

RNA Analysis Experiments

Total RNA extraction was performed with Trizol reagent (Invitrogen) as indicated by the manufacturer. RNA expression of human and mouse apolipoprotein A5 and 36B4 genes was analyzed by real-time quantitative PCR using SYBR Green technology on a MX4000 apparatus (Stratagene Europe, Amsterdam, the Netherlands). PCR was performed with oligonucleotides forward 5′-ACGCACGCATCCAGCAGAAC-3′ and reverse 5′-GAGCATCTGGGGGTC-3′ for human APOA5, forward 5′-CTGTTCCACAAACTCACAGG-3′ and reverse 5′-AGGTAGGTGTACATGCCGAAAAG-3′ for mouse apoA5, and forward 5′-CATGTCAACATCTCCCTTCTCC-3′ and reverse 5′-GGGAAGGTGTAATCCGTCTCCACAG-3′ for 36B4. Quantification of APOA5 and apoA5 mRNA levels was normalized using 36B4 as housekeeping gene.

Statistical Analysis

Results are presented as mean ± SD. Statistical analysis was performed using t tests and P<0.05 was considered statistically significant. Statistical differences from controls are P<0.01, and P<0.001.

Next, we investigated whether RORα1, α2, α3, α4, and γ can modulate APOA5 gene promoter transcriptional activity. HepG2 and HuH7 human hepatocytes were transiently co-transfected with a luciferase reporter vector driven by the human promoter fragment (−305/+62) (Figure 3A) and the

Genoux et al Gene Regulation of Apolipoprotein A5 by RORα

Figure 3. RORα1 and RORα4 enhance the activity of the human APOA5 gene promoter. The wild-type (wt) APOA5 promoter fragment (−305/+62) was co-transfected with a RORα or RORα4 expressing plasmid. The activities were normalized to the activity of the empty vector.
different isoforms of ROR. The results in Figure 3B (HepG2 cells) and 3C (HuH7 cells) show that only RORα1 and RORα4 increased human APOA5 promoter activity, whereas RORα2, α3, and γ had no effect. These data are in agreement with the gel shift assay findings and confirm that only RORα1 and RORα4 are functional. Then, we showed that the transcriptional activity of the APOA5 reporter construct was strongly increased in a dose-dependent manner by the co-transfection of either RORα1 or RORα4 expression vectors in HepG2 cells (Figure 3D) and in HuH7 cells (Figure 3E). To demonstrate that the increase of human promoter activity by RORα1 and RORα4 is caused by their binding to the identified RORE-DR1 at the position −272/−260, HepG2 and HuH7 cells were transiently transfected with the luciferase reporter vector driven by the mutated human APOA5 promoter fragment −305/+62 (−270G→C, −263G→A). The response of the mutated construct to RORα1 or RORα4 was strongly decreased compared with the wild-type construct in HepG2 (Figure 4A) and in HuH7 cells (Figure 4B). These results suggest that the identified RORE-DR1 plays a key role in the regulation of the human APOA5 transcriptional activity by RORα. However, because 18% residual increase is still

Figure 2. RORα proteins do not bind to the mouse promoter homologous sequence. A, A gel retardation assay was performed with the wild-type fragment of the mouse APOA5 gene promoter homologous to the human RORE-DR1 in the presence of in vitro transcribed/translated RORα1, RORα2, RORα3, RORα4, and RORγ or unprogrammed reticulocyte lysate as control (3 µL of each). DNA–protein complexes were resolved by nondenaturing PAGE. No specific complex was observed. B, The human and the other species promoter sequences homologous to the human RORE-DR1 were aligned. The sequence corresponding to the human PPRE-DR1 site AGGGTTAACGTCATGGGTGGA in bold and the sequence corresponding to the human RORE half-core motif AGGTCAT is underlined.
observed with the mutated construct, we cannot exclude that another mechanism independent of the binding to this site could exist. To further demonstrate that the APOA5 RORE-DR1 is functional, we cloned it in 4 tandem copies in front of an heterologous promoter and examined its response to ROR1 or ROR4 cotransfection in HepG2 and HuH7 cells. We found that this site is able to transmit ROR1 and ROR4 activator effect in a dose-dependent manner in HepG2 (Figure 4C) and in HuH7 cells (Figure 4D). Taken together, these results demonstrate that ROR1 and ROR4 increase APOA5 promoter transcriptional activity mainly by a specific binding to the RORE-DR1 at position -272/-260.

Furthermore, we determined that none of the RORα isoforms had any effect on the mouse promoter activity (data not shown). This is in agreement with the absence of binding in gel shift assay.

Figure 4. The (-305/+62) region of the human APOA5 gene promoter is activated by ROR1 and ROR4 mainly via the functional RORE-DR1 present at the position (-272/-260). HepG2 (A) and HuH7 (B) cells were transiently cotransfected with the mutated APOA5 promoter fragment reporter plasmid -305/+62 (-270/-263G A) cloned in front of the luciferase reporter gene (300 ng) and the expression plasmids pCDNA3-hROR1, pCDNA3-hROR4 (100 and 300 ng), or the empty pCDNA3 vector as control (300 ng) for 24 hours. HepG2 (C) and HuH7 (D) cells were transiently cotransfected with the reporter construct containing four tandem copies of the human APOA5 RORE-DR1 inserted in front of an heterologous promoter cloned upstream of the luciferase reporter gene (300 ng) and the pCDNA3-hROR1 and pCDNA3-hROR4 expression plasmids (100 and 300 ng), or the empty pCDNA3 vector as control (300 ng) for 24 hours.

The Adenovirus-Mediated Overexpression of hRORα1 and hRORα4 Lead to Increase hAPOA5 mRNA Accumulation

To evaluate whether the overexpression of hRORα1 and hRORα4 could lead to an increase of hAPOA5 mRNA levels, HepG2 cells were infected with the Ad-RORα1 or the Ad-RORα4 adenoviral expression vector. As control, HepG2 cells were infected with the same vector, allowing expression of GFP or LacZ. Total RNA was extracted and analyzed by real-time quantitative PCR using hAPOA5-specific oligonucleotides and 36B4 as an internal control. As shown in Figure 5A, an increase of hAPOA5 mRNA was observed in cells infected with the hRORα1 adenoviral expression vector compared with control cells infected with Ad-GFP. In addition, the adenovirus-induced hAPOA5 mRNA accumulation was shown to be time-dependent to reach a 6-fold increase.
after 48-hour incubation. A significant increase of hAPOA5 mRNA was also observed in HepG2 cells infected with Ad-RORα4 compared with their control cells infected with Ad-LacZ (Figure 5B). These results confirm that hRORα1 and hRORα4 play a key role in the physiological control of APOA5 gene expression in human HepG2 cells.

Furthermore, we analyzed the hepatic apo5 gene expression in staggerer mice, which carry a natural deletion in the RORα gene (Figure 5C). No effect has been observed indicating that mouse apo5 is not affected by RORα.

Discussion

It is well-established that hypertriglyceridemia constitutes an independent risk factor for cardiovascular diseases. Because APOA5 has been described as a crucial determinant of plasma triglyceride levels, the identification of factors that regulate its expression is of major medical importance for the treatment of hypertriglyceridemia and associated coronary heart disease. It has been reported that hAPOA5 gene expression is upregulated by the heterodimer peroxisome proliferator-activated receptor-α/retinoid X receptor by a direct binding to the Direct Repeat 1.10,11 Because this DR1 site presents structural homologies with the binding site for the orphan nuclear receptor ROR, we investigated the role of RORα in the control of hAPOA5 gene expression. Several studies attributed an important role to RORα in the regulation of lipid homeostasis by upregulating some apolipoprotein gene expression as APOA1 and APOC3 at the transcriptional level23,24 and recently caveolin-3 and CPT-1 in skeletal muscle.29 Moreover, it has been shown that RORα has been shown to play a role in lipid homeostasis in the vascular wall.30 Besnard et al demonstrated that RORα expression is significantly decreased in human atherosclerotic plaques.30 In addition, sg/sg mice have an increased susceptibility to atherosclerosis22 attributed to lower high-density lipoprotein plasma levels and to heightened inflammatory response.26

We demonstrated initially that both RORα1 and RORα4 can specifically bind to the identified RORE site —272/—260 present in the human APOA5 gene promoter, which consists of a perfect AGGTCA half-site core motif. The functional evaluation of this site, using transient cotransfection experiments with RORα and wild-type or mutated RORE in the context of the hAPOA5 promoter, demonstrated that only RORα1 and RORα4 increase human APOA5 promoter activity in HepG2, as well as in Huh7 cells. In addition, in vitro experiments with Ad-RORα1 adenoviral expression vectors revealed that RORα1 and RORα4 overexpression lead to APOA5 mRNA accumulation in HepG2 cells. Our data demonstrate that human APOA5 is a new target gene for RORα1 and RORα4. These data could strengthen the role of RORα in lipid homeostasis.

The second objective of our study was to evaluate whether our results obtained in human could be extended to mouse. The sg/sg, which carry a natural mutation in the RORα gene, exhibit an aberrant blood lipid profile associated with changes in plasma apolipoprotein levels. In particular, sg/sg mice display decreased apoC-III and triglyceride plasma levels.22 Because the triglyceride metabolism was affected in this mouse model, we addressed the question whether apo5 gene is involved in this mechanism. However, we show that the apo5 gene expression is not altered in the sg/sg mice. In addition, EMSA and transient transfection experiments (data not shown) results suggested that the homologous site of human APOA5 RORE-DR1, identified at the position —272/ —260, is not functional in mice. The alignment of the human and mouse promoter sequences revealed that the mouse deviates from the human APOA5 RORE-DR1 core by 1 nucleotide and this could probably explain the nonfunctionality of this site. These data indicated that RORα cannot modulate apo5 gene expression in mice and that APOA5 is a RORα target gene only in humans.

In a recent study, it was demonstrated that apoA-V and apoC-III independently influence plasma triglyceride concentrations in an opposite manner.31 It is well-known that apoC-III, which is a component of VLDL, increases triglyceride levels via an inhibitory effect on lipoprotein lipase activity and thus constitutes a risk factor for atherosclerosis.32 The apoC-III has been reported to be upregulated by RORα and these data rather argue against the potential protective effect of RORα in atherosclerosis. Interestingly, in our study, we demonstrate that RORα strongly increases hAPOA5 gene expression in human hepatocytes. This new finding confers to RORα a beneficial role in the regulation of triglyceride metabolism that could counteract apoC-III effect. By regulating the gene expression of both apolipoproteins, RORα may play a determinantal role, at least in maintaining plasma triglyceride levels in humans. In mice, a different approach may be expected because apoC-3 is increased, whereas apoA5 seems to be unaffected. This hypothesis correlates with the lower circulating plasma triglyceride levels observed in sg/sg mice, which could be caused by the repression of apoC-3 by the deleted RORα but not by the downregulation of apoA5 in this animal model.

In conclusion, we speculate that the upregulation of hAPOA5 expression might counteract the negative effect of the upregulation of APOC3 by RORα in humans and could avoid the elevation of plasma triglyceride levels. These new findings make RORα a more attractive therapeutic target in the treatment of dyslipidemia and atherosclerosis.

Acknowledgments

This work was supported by the Leducq Foundation.

References


Transcriptional Regulation of Apolipoprotein A5 Gene Expression by the Nuclear Receptor ROR α

Annelise Genoux, Hélène Dehondt, Audrey Helleboid-Chapman, Christian Duhem, Dean W. Hum, Geneviève Martin, Len A. Pennacchio, Bart Staels, Jamila Fruchart-Najib and Jean-Charles Fruchart

Arterioscler Thromb Vasc Biol. published online March 24, 2005;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 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/2005/03/24/01.ATV.0000163841.85333.83.citation

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/