Secretory Phospholipase A<sub>2</sub> Elicits Proinflammatory Changes and Upregulates the Surface Expression of Fas Ligand in Monocytic Cells

Potential Relevance for Atherogenesis

Marita Hernández, Lucía Fuentes, Francisco Javier Fernández Avilés, Mariano Sánchez Crespo, María Luisa Nieto

Abstract—Type IIA secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) is an acute-phase reactant that plays a role in atherogenesis and is expressed in atherosclerotic arterial walls displaying inflammatory features. This generates a relevant question addressing the biological effects of this enzyme on monocytic cells, in view of the role of these cells in the inflammatory process associated with atherosclerosis. sPLA<sub>2</sub> produced a mild activation of the p42 mitogen-activated protein module of the mitogen-activated protein kinase (MAPK) cascade and a prominent activation of c-Jun N-terminal kinase in THP-1 monocytes. This activation showed both an early and a late peak, different from that elicited by tumor necrosis factor-α (TNF-α), which only showed the first peak. This was accompanied by activation of arachidonate metabolism, as judged from both the activation of the cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) and the induction of cyclooxygenase-2 (COX-2) expression. sPLA<sub>2</sub> also elicited the production of monocyte chemoattractant protein-1 (MCP-1) and showed a synergistic effect with TNF-α on both COX-2 induction and MCP-1 production. sPLA<sub>2</sub> upregulated the expression of Fas ligand at the cell surface, but it did not influence Fas expression nor cell survival of monocytes. In summary, these data indicate that some of the atherogenic effects of sPLA<sub>2</sub> can be exerted by engagement of an sPLA<sub>2</sub>-binding structure on monocytic cells, most probably the M-type receptor for sPLA<sub>2</sub>, which produces the activation of the MAPK cascade, induces a proinflammatory phenotype, and upregulates the cell surface expression of Fas ligand. (Circ Res. 2002;90: 38-45.)

Key Words: apoptosis ■ atherosclerosis ■ chemokines ■ inflammation ■ lipid mediators

Type IIA secretory phospholipase A<sub>2</sub> (also called inflammatory-type sPLA<sub>2</sub>) is widely distributed in a variety of mammalian tissues, and its concentration increases manifold in many immunoinflammatory processes in response to proinflammatory cytokines and endotoxin. In fact, sPLA<sub>2</sub> levels in plasma increase to ≈1 μg/mL after intravenous injection of bacteria. Expression of sPLA<sub>2</sub> at very high levels has been found in human atherosclerotic arterial walls, where it has been regarded as an important molecule in the development of atherosclerotic plaques. The association of sPLA<sub>2</sub> to decorin, a proteoglycan that forms part of the collagen network in human arteries and links native LDL to collagen, has recently been reported. Thus, sPLA<sub>2</sub> may contribute to the pathogenesis of atherosclerosis by modifying lipoproteins and releasing lipid mediators at places of lipoprotein retention in the arterial wall. Additional atherogenic effects of sPLA<sub>2</sub> have been related to its ability to produce mitogenesis of human vascular smooth muscle cells. Interestingly, circulating levels of sPLA<sub>2</sub> have been shown as sensitive predictors of coronary events in patients with coronary arterial disease. The role of sPLA<sub>2</sub> in inflammation has been explained through several mechanisms: (1) generation as a result of its catalytic activity of both unesterified fatty acid and lysophospholipid; (2) interaction with membrane receptors analogous to those binding other sPLA<sub>2</sub>s; and (3) binding to acceptor heparan sulfate proteoglycans on the cell surface. The recent identification of different membrane proteins that bind sPLA<sub>2</sub> has highlighted biological effects of these enzymes that are independent of their catalytic activity. Previous studies have shown that sPLA<sub>2</sub> activates intracellular signaling pathways involving mitogen-activated protein kinase (MAPK) and cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), thus leading to the release of arachidonate and mitogenesis, and suggesting the existence of a receptor-like membrane structure in these cells. Studies in human macrophages have also stressed the signaling...
properties of sPLA₂, by showing the release of β-glucuronidase and IL-6 by a mechanism independent of its catalytic activity.¹⁷ In addition, mildly oxidized LDL induces expression of sPLA₂ in human macrophages,¹⁸ thus implicating these cells in the tissue damage associated with the release of sPLA₂ via autocrine/paracrine mechanisms. However, little is known regarding both the patterns of response and the distinct binding structures involved in the signaling effect of sPLA₂ among the different cell types. In the present study, we address the effect of sPLA₂ on monocytes in view of the prominent role of this cell type in the inflammatory setting of atherosclerosis.

**Materials and Methods**

**Reagents and Cell Culture**

sPLA₂ was purified from plasma of patients diagnosed of sepsis syndrome according to the criteria of Bone.¹⁹ Written informed consent was provided by patients. Mouse anti-MAPK monoclonal antibody (mAb) reacting with both p42 and p44 MAPK/ERK (extracellular signal–regulated kinase) was from Zymed Laboratories (San Francisco, Calif). Rabbit phosphospecific antibody against p42/p44 MAPK was from New England Biolabs, Inc (Beverly, Mass). Rabbit phosphospecific antibodies against cPLA₂ (sc-454), and against inhibitors of NF-κB activity (IkB) IκBα (sc-847), IκBβ (sc-945), and IκBe (sc-7155) were from Santa Cruz Biotechnology Inc (Santa Cruz, Calif). Oligonucleotide primers for the detection of sPLA₂ M-type receptor mRNA by RT-PCR were designed from human gene sequence and were 5′-AAAGAAAACCCACTG-AATGCC-3′ (sense) and 5′-TCTTGAAGTCCATACCC-3′ (antisense), corresponding to nucleotides 626 to 645 and 1025 to 1044 of human sPLA₂ M-type receptor encoding sequence.²⁰ Rabbit antibody for the detection of human cyclooxygenase-2 (COX-2) was from Cayman Chemical Co, Inc (Ann Arbor, Mich). THP-1 and Jurkat cells were cultured in RPMI 1640 medium supplemented with 2 mmol/L glutamine and 10% heat-inactivated FBS. Human monocytes were isolated from peripheral blood by centrifugation into Ficoll cushions and adherence to Primaria 6-well dishes. After removal of nonadherent cells, monocytes were cultured overnight in the presence of 10% pooled human sera. The detection of monocyte chemoattractant protein-1 (MCP-1) was carried out by ELISA with reagents from R&D Systems, Inc, as described.²¹

**Synthesis of First-Strand cDNA and PCR of sPLA₂ M-Type Receptor**

Total cellular RNA was extracted from culture plates by the TRIzol method (Life Technologies). cDNA first strand was synthesized from total RNA by reverse-transcription reaction. cDNA was amplified by PCR according to the following amplification profile: 1 cycle of initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 63°C for 30 seconds, and extension at 72°C for 30 seconds; 1 cycle of final extension at 72°C for 7 minutes. The expression of β-actin was used as control for the assay of a constitutively expressed gene. PCR products were identified by automatic sequencing of the DNA eluted from the agarose gel by excision of the band under UV light followed by purification using a GeneClean kit (Bio 101, Inc).

**Immunoblot of cPLA₂, p42 MAPK, COX-2, and IκB Proteins**

Cell lysates were loaded into a 10% SDS/PAGE and transferred to nitrocellulose membranes. The membranes were blocked with dry milk for 2 hours and used for immunoblot using the cognate antibody. This was followed by incubation with sheep anti-rabbit IgG HRP-conjugated antibody and detection with the Amersham ECL system.

**Assay of c-Jun N-terminal Kinase (JNK) Activity**

To obtain the substrate for the kinase assay as a glutathione-S-transferase (GST) c-Jun fusion protein, the procedure of Smith and Corcoran²³ was followed. The cytosolic extracts for the kinase assay were obtained from the lysis of 5×10⁶ cells. After centrifugation at 12,000 rpm at 4°C, the supernatant was mixed with 10 μg of GST-c-Jun protein and glutathione agarose beads. The mixture was incubated under continuous shaking for 3 to 5 hours at 4°C and then washed to remove the fraction nonassociated to the glutathione-agarose beads. Phosphorylated GST-c-Jun was resolved by SDS/PAGE and immunodetected by Western blot using rabbit phosphospecific c-Jun (Ser³²) antibody. Quantification of the phosphorylation was carried out by densitometric scanning.

**Fas and Fas Ligand (FasL) Immunodetection**

THP-1 cells (5×10⁶) were suspended in PBS containing 1% BSA and incubated with 1 μg/mL anti-human FasL NOK-1 IgG₁ mAb (Pharmingen, San Diego, Calif) or 500 ng/mL anti-human Fas DX2 IgG₁ mAb (Pharmingen) for 1 hour at 4°C. The cells were then washed in PBS, incubated with 1:100 FITC-conjugated goat anti-mouse IgG (Sigma, Saint Louis, Mo) for 30 minutes at 4°C, and subsequently, washed, resuspended in 500 μL of PBS, and analyzed by immunofluorescence flow cytometry in a FACScan cytofluorometer (Becton Dickinson). To increase the levels of membrane-bound FasL by blocking FasL cleavage before the stimulation with the agonists, cells were treated with 10 μmol/L of the matrix metalloproteinase inhibitor K8301.

**Assay of Apoptosis**

Cells were starved of serum for 48 hours and then washed and incubated in fresh medium with the different additions. Cells were analyzed for cell-cycle distribution at 48 hours by flow cytometry by staining with propidium iodide using a FACScan cytofluorometer. The measurement of apoptosis was also carried out with the annexin-V–FITC apoptosis detection kit of Pharmingen.

**Results**

**THP-1 Monocytic Cells and Human Monocytes Express the M-Type sPLA₂ Receptor**

Because sPLA₂ elicits functional responses in both 1321N1 cells⁵,¹⁶ and THP-1 monocytic cells (discussed later), the expression of M-type sPLA₂ receptor was searched by RT-PCR in both cell types. Unlike astrocytoma cells of the 1321N1 line, expression of mRNA encoding the receptor was observed in THP-1 cells, as judged from the appearance of a unique cDNA band of the expected size (Figure 1A), which was identified as the M-type sPLA₂ receptor by automatic sequencing on the two strands by the dideoxynucleotide technique. Interestingly, this band was also detected in human monocytes. This finding suggests, although it does not prove, that M-type sPLA₂ receptor might mediate some of the effects of sPLA₂ on THP-1 cells.

**sPLA₂ Mainly Activates the JNK Module of MAPKs**

sPLA₂ produces in THP-1 cells a minimal activation of p42 MAPK/ERK as judged from the appearance of a mobility shift due to the phosphorylation of the enzyme involving about a 10% of all the p42 MAPK protein, which was only detectable at 5 minutes after addition of the stimulus and returned to the prestimulation state by 10 minutes (Figure 2A). This mobility shift was inhibited by the ERK inhibitor PD98059 (Figure 2B) and was weaker than that elicited by platelet-activating factor (PAF), a mediator that acts through a G protein–coupled receptor and is a potent stimulus for
monocytic cells. The phosphorylation of p42 MAPK was confirmed with anti–phospho-MAPK antibody and was also observed in monocytes (Figure 2D). To compare this response with that elicited by a prototypic proinflammatory agonist, tumor necrosis factor-α (TNF-α) was tested at the usual concentration of 100 U/mL. As shown in the far right lanes of the left panel of Figure 2A, TNF-α failed to activate p42 MAPK, which agrees with current views assigning to this proinflammatory cytokine an action mostly impinging on the stress module of MAPKs.24,25 sPLA2 also produced a significant activation of JNK showing a biphasic pattern of increase of activity defined by a 3-fold increase at 5 minutes, a decrease to prestimulation values at 1 hour, and a new increase from 4 to 6 hours (Figure 3A). Interestingly, the temporal pattern of activation differed somewhat from that elicited by TNF-α (Figure 3B), since the response to TNF-α was most prominent at early times, showed a 13-fold increase at 5 minutes, and decreased after this time without further evidence of significant activation thereafter. Human monocytes showed a response to sPLA2 similar to that elicited on THP-1 cells (Figure 3C). Downstream consequences of the activation of MAPKs were searched at different levels, including activation of cPLA2 and COX-2 induction. As shown in the bottom panel of Figure 2A, both sPLA2 and TNF-α produced the activation of cPLA2, as judged again by the appearance of a mobility shift that is characteristic of the phosphorylation of this enzyme by MAPKs.26 Assessment of downstream events related to arachidonate metabolism showed that both sPLA2 and TNF-α induced a mild expression of COX-2 protein after 8 to 24 hours of incubation both in THP-1 cells and monocytes (Figure 4A). Interestingly, the combined addition of both stimuli produced a response stronger than that elicited by each agonist alone, thus suggesting that different mechanisms might be involved in the induction of COX-2 elicited by each of the agonists. Note- worthy, COX-2 induction was blocked by the ERK inhibitor PD98059, thus indicating the functional coupling of this induction to the ERK route (Figure 2C).

**sPLA2 Produces a Proinflammatory Phenotype in THP-1 Cells That Is Accompanied by Upregulation of FasL**

sPLA2 synergistically enhanced the production of MCP-1 elicited by TNF-α both in THP-1 cells (Figure 4B) and monocytes (Figure 1B). Interestingly, mannose-BSA also induced MCP-1 production in human monocytes, thus indicating that it behaves as a noncatalytic ligand of the M-type receptor, which elicits productive binding to the receptor, as it has been previously shown in mast cells.27 Because κB-dependent transcription is involved in the regulation of both COX-2 and MCP-1 expression,28 the possible effect of sPLA2 as an activator of NF-κB was searched by looking at both the appearance of κB-binding activity in the nuclear extracts and the degradation of IκB proteins. In contrast to TNF-α, sPLA2 did not induce the appearance of κB-binding activity in the nuclear extract as judged from electrophoretic

---

**Figure 1.** A, sPLA2 receptor is expressed in THP-1 cells and human monocytes. Total mRNA was used for RT-PCR reactions. B, Production of MCP-1 by human monocytes incubated in the presence of different additions for 24 hours. Data represent mean±SE of 3 experiments. C, Effect of sPLA2 on the expression of FasL in monocytes identified by the surface expression of CD11b.

**Figure 2.** Effect of sPLA2 and TNF-α on the activation of p42 MAPK and cPLA2. A, THP-1 cells were incubated with 1 μg/mL sPLA2 and 100 U/mL TNF-α for the times indicated. Cell lysates were then collected to assay the band-shift characteristic of the phosphorylation of both p42 MAPK and cPLA2. THP-1 cells stimulated with PAF were used as a positive control. B, Effect of PD98059 on the phosphorylation of p42 MAPK induced by sPLA2. C, Effect of PD98059 on the induction of COX-2 elicited by sPLA2. D, Effect of sPLA2 on the phosphorylation of p42 MAPK was assayed in standard gels, which do not allow the appearance of band shift, using phosphospecific antibody reacting with p42/p44 MAPK. Blots were stripped and reprobed with antibody to MAPK to ensure equal loading (bottom panels). P indicates phosphorylated.
mobility-shift assay studies (Figure 4C), nor did it produce any significant degradation of IκB proteins (Figure 4D). Taken together, these data indicate that sPLA₂ behaves as a pleiotropic proinflammatory agent that enhances the response to TNF-α by a mechanism not directly related to the activation of the NF-κB/Rel system.

Because sPLA₂ elicits cell proliferation and mitogenesis in several cell types in view of its capacity to activate the MAPK cascade, its effect on proliferation and survival was addressed. Incubation of THP-1 cells with sPLA₂ produced nonsignificant changes regarding distribution of cells into the different phases of the cell cycle after release from the quiescent state (Figure 5A), thus indicating that there was neither an increased number of cells in mitosis nor an increased number of cells within the subdiploid peak that encloses cells undergoing apoptotic DNA fragmentation. Moreover, the absence of mitogenic effect was confirmed by [³H]thymidine incorporation studies, since the radioactivity incorporated into the trichloroacetic acid–precipitable fraction was analogous in serum-starved cells and in sPLA₂-treated cells that were measured (143 900±9630 versus 144 100±9780 [³H]dpm, n=3). TNF-α treatment increased the fraction of cells in the subdiploid peak to 16±4% at 48 hours compared with 3.9±0.6% in control cells and 3.7±1.1% in sPLA₂-treated cells (mean±SE, n=9). Similar results were also observed when apoptosis was assessed with the annexin-V assay, since unlike TNF-α, treatment with sPLA₂ failed to increase the expression of FasL at the cell surface, thus suggesting that the apoptotic death produced by TNF-α in THP-1 cells is independent from Fas/FasL interactions. When resting cells were permeated before addition of anti–human FasL mAb, positive immunofluorescence was observed regardless of the stimuli (Figure 6B, left panel), thus suggesting that sPLA₂ influences exposition of FasL at the cell surface irrespective of the stimulation by both sPLA₂ and TNF-α. Interestingly, sPLA₂ upregulated FasL expression at the cell surface of THP-1 monocytes, since this protein was not observed in resting cells and appeared after incubation with sPLA₂ for several hours (Figure 6B, right panel). In contrast, treatment with TNF-α did not increase the expression of FasL at the cell surface, thus suggesting that the apoptotic death produced by TNF-α in THP-1 cells is independent from Fas/FasL interactions.
cell surface rather than FasL protein expression. This was further confirmed by Western blot, because similar amounts of FasL protein were observed in cell lysates from both control and sPLA2-treated cells (Figure 7, inset), as well as by the effect of the protein synthesis inhibitor cycloheximide, which failed to block the effect of sPLA2 (Figure 7). Since sPLA2 did not affect the overall distribution of THP-1 cells into the different phases of the cell cycle, these data suggest that the increased surface expression of FasL elicited by sPLA2 is not linked to an autocrine signaling mechanism leading to programmed cell death of monocytic cells, but rather might represent a juxtacrine mechanism of signaling involving other cell types. In this connection, the resistance of THP-1 cells to Fas-induced death was confirmed by treatment with an anti–human Fas antibody (clone CH11, IgM, from Upstate Biotechnology), which triggers Fas-dependent apoptosis in sensitive cells.

Figure 5. Distribution of THP-1 cells in the cell cycle and assay of apoptotic cell death. Cells were treated with the indicated additions for 48 hours and then fixed with ethanol and stained with propidium iodide. Numbers below histogram marker indicate the percentage of apoptotic nuclei. A, Representative experiment of 9. B, Apoptosis was also measured using annexin-V–FITC in control (shown in gray) and in cells treated under the aforementioned experimental conditions. Clone CH11 antibody indicates cells treated with this mAb, which triggers Fas-dependent apoptosis in sensitive cells.

Figure 6. Effect of both sPLA2 and TNF-α on the surface expression of Fas/FasL in THP-1 cells. THP-1 cells were incubated with 100 U/mL TNF-α and 1 μg/mL sPLA2 for 18 hours and immunolabeled with 500 ng/mL anti–human Fas DX2 IgG1 mAb, followed by FITC-conjugated goat anti-mouse IgG for analysis by flow cytometry. P3×63 myeloma supernatant was used as a negative control of and isotype-matched control antibody (shown in gray). A, Representative histograms of 6 experiments. To address the effect of these treatments on the expression of FasL, THP-1 cells were incubated with TNF-α and sPLA2 and were immunolabeled with anti–human FasL NOK-1 IgG1 mAb. To address the intracellular expression of FasL, cells were fixed with ice-cold 4% formaldehyde and permeabilized with 0.1% Tween 20. B, Representative histograms of 7 experiments.

Figure 7. Effect of different treatments on the expression of FasL induced by sPLA2. THP-1 cells were incubated with 1 μg/mL sPLA2 for 18 hours in the presence and absence of the following additions: 25 μmol/L mannose-BSA, 1 μmol/L cycloheximide, and 25 μmol/L PD98059. At the end of this period, the expression of FasL was assayed. Inset. Assay of FasL protein by Western blot in both control and sPLA2-treated cells.
sPLA₂ triggers a signaling cascade in monocytic cells, which displays some similarities to those reported in other cell types, and seems unrelated to the generation of the products of its catalytic activity on both membrane- and lipoprotein-associated phospholipids. Conversely, these effects could be mediated by direct triggering of a plasma membrane signaling structure, for instance, the M-type receptor, because both THP-1 cells and human monocytes express this receptor. Moreover, overexpression of the M-type receptor in murine bone marrow–derived mast cells increases the response to sPLA₂. However, our data do not exclude the involvement of other structures, for instance, glycosylphosphatidylinositol-anchored heparan sulfate proteoglycans, the binding of which has been associated to physiological effects by sPLA₂. The response observed in monocytes is pleiotropic and includes a transient but functionally relevant activation of the ERK module of MAPK and an increase of JNK activity displaying a biphasic pattern, which is somewhat different from that elicited by TNF-α. As to the downstream functional consequences of this activation, sPLA₂ activates arachidonate metabolism via phosphorylation of the cPLA₂, ie, the enzyme involved in the release of arachidonic acid from phospholipids, and through the induction of COX-2, the enzyme involved in the delayed production of prostanoids. Interestingly, sPLA₂ induces COX-2 through a mechanism different from that used by TNF-α, since the effect observed after a combination of agonists is higher than that observed by each of the stimuli. In keeping with these findings is the recently reported upregulation of TNF-α–induced COX-2 protein expression by sPLA₂ in human rheumatoid synoviocytes.

Since the effect of TNF-α and lipopolysaccharide on COX-2 induction is currently explained by an upregulation of NF-κB-dependent transcription in macrophage cell lines, the effect of sPLA₂ should be exerted through another regulatory element(s), because sPLA₂ failed to produce the activation of NF-κB. The possibility of an effect linked to the MAPK route is suggested by the inhibitory effect of PD98059 and by the induction of COX-2 expression by mitogenic stimuli in different cell types.

Another finding from this study is that the scope of proinflammatory events elicited by sPLA₂ is not restricted to the activation of enzymes involved in arachidonate metabolism and the ensuing production of lipid mediators, since sPLA₂ induces the production of the chemokine MCP-1. This suggests a widespread proinflammatory effect of sPLA₂, overlapping some of the effects of TNF-α and involving the recruitment of mononuclear cells. sPLA₂ also produces effects on cell proliferation and/or apoptosis that are different from those elicited by TNF-α. In fact, TNF-α enhances the apoptosis of THP-1 cells, whereas sPLA₂ displays an irrelevant effect on the survival of THP-1 cells. sPLA₂ exerts a clear activation of FasL on THP-1 cells, which in view of the lack of apoptotic cell death elicited by both sPLA₂ and anti–Fas antibody, would point to the activation of a juxtacrine mechanism of signaling involving FasL expressed and/or released on monocytes and Fas expressed on other cells such as infiltrating leukocytes and endothelial
cells. This might have pathophysiological implications in view of the increased expression of sPLA₂ in clinical conditions such as atherosclerosis and rheumatoid arthritis, where the Fas-signaling pathway has been proposed to play a role in their pathogenesis. Because apoptosis mediated by FasL release from mononuclear phagocytes is an important mechanism of resolution of inflammation under nonphlogistic conditions, it is possible to propose a programmed sequence of functions for sPLA₂ on mononuclear phagocytes; (1) a set of proinflammatory changes including activation of the MAPK cascade, induction of COX-2, and mobilization of monocytes; (2) a contribution to the safe clearance of infiltrating leukocytes through the triggering of the Fas/FasL system. Taken together, these findings enlarge the scope of biological functions for sPLA₂ and stress the variety of effects elicited on different cell types. The distinct pattern of expression of sPLA₂ receptors could explain, at least in part, this variety of biological effects.

Acknowledgments

M.H. was supported by a grant from Sociedad Española de Cardiología. This work was supported by grants from Plan Nacional de Salud y Farmacia (grant No. SAP98/0176), Comisión Interministerial de Ciencia y Tecnología and European Comission (grant No. 1FD97-0590), and Fondo de Investigación Sanitaria (grant No. FIS00/0393). We are grateful for expert technical assistance by María del Carmen Valle and Cristina Gómez.

References

secreted phospholipase A₂ but not the group IB, IIA, and V enzymes efficiently release arachidonic acid from adherent mammalian cells.


Secretory Phospholipase A₂ Elicits Proinflammatory Changes and Upregulates the Surface Expression of Fas Ligand in Monocytic Cells: Potential Relevance for Atherogenesis
Marita Hernández, Lucía Fuentes, Francisco Javier Fernández Avilés, Mariano Sánchez Crespo and María Luisa Nieto

Circ Res. 2002;90:38-45; originally published online December 6, 2001;
doi: 10.1161/hh0102.102978

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/90/1/38

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

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

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