Oxidized Phospholipids Trigger Atherogenic Inflammation in Murine Arteries

Alexander Furnkranz, Andreas Schober, Valery N. Bochkov, Pavel Bashtrykov, Gerhard Kronke, Alexandra Kadl, Bernd R. Binder, Christian Weber, Norbert Leitinger

Objective—Lipoprotein-derived phospholipid oxidation products have been implicated as candidate triggers of the inflammatory process in atherosclerosis. However, in vivo evidence regarding the impact of oxidized phospholipids on the artery wall thus far has been elusive. Therefore, the aim of this study was to investigate if structurally defined oxidized phospholipids induce expression of atherogenic chemokines and monocyte adhesion in intact murine arteries.

Methods and Results—To model the accumulation of oxidized phospholipids in the arterial wall, oxidized 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine (OxPAPC) was topically applied to carotid arteries in mice using pluronic gel. Using quantitative reverse-transcriptase polymerase chain reaction (PCR) and immunohistochemistry, we show that OxPAPC induced a set of atherosclerosis-related genes, including monocyte chemotactic protein 1 (MCP-1) and keratinocyte-derived chemokine (KC), tissue factor (TF), interleukin 6 (IL-6), heme oxygenase 1 (HO-1), and early growth response 1 (EGR-1). OxPAPC-regulated chemokines were also expressed in atherosclerotic lesions of apolipoprotein E-deficient (ApoE−/−) mice. In isolated perfused carotid arteries, OxPAPC triggered rolling and firm adhesion of monocytes in a P-selectin and KC-dependent manner.

Conclusion—Oxidized phospholipids contribute to vascular inflammation in murine arteries in vivo, initiating atherogenic chemokine expression that leads to monocyte adhesion; therefore, they can be regarded as triggers of the inflammatory process in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2005;25:633-638.)

Key Words: atherosclerosis • oxidized phospholipids • inflammation • chemokines • leukocyte adhesion

Atherosclerosis is a chronic inflammatory disease involving accumulation of lipoproteins and mononuclear cells in the subendothelial space. Chemokines serve a vital role in supporting the inflammatory response of the arterial wall, leading to atherosclerotic plaque formation. In particular, genetic deletions of monocyte chemotactic protein 1 (MCP-1) or its receptor CCR2, as well as transplantation of bone marrow deficient in the IL-8 receptor homologue CXCR2,1–3 have been shown to decrease monocyte accumulation and lesion formation in mice susceptible to atherosclerosis. Although our knowledge about the mechanisms underlying atherosclerosis and its complications has dramatically increased, the question about the initiating factors or triggers of atherogenesis remains unsolved. Accumulating evidence suggests retention of low-density lipoprotein (LDL) particles in the subendothelial space with subsequent oxidative modification as key steps in beginning atherosclerosis. Oxidized LDL has been shown to induce chemokines such as MCP-1 in vascular cells, but direct evidence from suitable animal models is scarce and it has been questioned if lipoproteins oxidized in vitro yield similar biological responses as lipoproteins oxidized in the arterial wall.4 Recently, considerable advances have been made in dissecting the molecular components of oxidized LDL responsible for its pro-atherogenic effect, allowing for the experimental use of defined compounds rather than complex lipoproteins. Initially, LDL oxidation gives rise to minimally oxidized LDL,5 the biological activity of which results primarily from oxidation of phospholipids, such as 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine (PAPC), yielding a series of oxidation products (OxPAPC), some of which have been structurally identified and shown to accumulate in atherosclerotic lesions.6,7 The atherogenic potential of OxPAPC has been demonstrated in cell culture studies as shown by enhanced monocyte, but not neutrophil, binding to OxPAPC-stimulated endothelial cells, as well as induction of MCP-1 and IL-8.8,9 However, static coculture systems only incompletely model the complex cellular interactions in the vessel wall and provide no information as to whether monocyte–endothelial interactions occur under flow. Moreover, in vivo, inactivation

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From the Department of Vascular Biology and Thrombosis Research (A.F., V.N.B., G.K., A.K., B.R.B., N.L.), University of Vienna, Austria; Wilhelminen Hospital (A.F.), Vienna, Austria; the Department of Cardiovascular Molecular Biology (A.S., C.W.), University of Aachen, Germany; and the Cardiology Research Center (P.B.), Moscow, Russia.
Current affiliation for G.K., A.K., and N.L.: Cardiovascular Research Center, University of Virginia, Charlottesville.
Correspondence to Norbert Leitinger, Cardiovascular Research Center, University of Virginia, PO Box 801394, Charlottesville, VA 22908. E-mail nl2q@virginia.edu
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of oxidized phospholipids by protective enzymes such as paraoxonase or platelet-activating factor acetyl-hydrolase may occur, limiting the proinflammatory potential of these lipids. Therefore, the purpose of this study was to investigate if OxPAPC induces atherogenic chemokines or other inflammatory genes in the arterial wall in vivo and whether this would entail monocyte adhesion to the arterial endothelium.

**Methods**

**Animals**

C57BL/6J mice were purchased from the Institut fuer Versuchstierzucht und haltung (Himberg, Austria). ApoE−/− mice on a C57BL/6J genetic background were acquired from the Proefdierencentrum (Leuven, Belgium). Mice were kept on a 12-hour dark/light cycle and received water and regular chow ad libitum.

**Application of OxPAPC to Carotid Arteries**

OxPAPC was obtained by air oxidation of dry PAPC (Sigma-Aldrich) as described previously.2 Immediately before surgical application, dry OxPAPC or PAPC was dissolved in cold 1% (wt/vol) F-127 pluronic gel (Sigma-Aldrich) in sterile water, followed by addition of 5 volumes of 50% (wt/vol) F-127. Sixty µL of F-127 with or without 50 µg OxPAPC, 50 µg PAPC, or 6 µg lipopolysaccharide (LPS) (Escherichia coli serotype 055:B5; Sigma-Aldrich) was applied to carotid vessels. At indicated time points, animals were euthanized, perfused for 5 minutes with PBS via the left ventricle, and the treated part of the common carotid artery was removed. For comparison of gene expression levels between ApoE−/− and wild-type animals, mice were used at 12 months of age, at which time common carotid arteries of ApoE−/− mice showed typical macrophage-rich lesions at the proximal and distal bifurcation.10 Relative quantification of gene expression was performed as described.11 Details about surgical procedures, tissue harvesting, RNA isolation, quantitative reverse-transcriptase polymerase chain reaction (RT-PCR), used PCR primers (Table I), and immunohistochemistry are given in the online supplement (please see http://atvb.ahajournals.org).

**Ex Vivo Perfusion Model**

Cell rolling and arrest of calcine-labeled monocytic MM6 cells (1×10⁶/mL) on endothelium of common carotid arteries from 10- to 12-week-old C57BL/6J mice were determined by epifluorescence videomicroscopy as described,12 after preperfusion with OxPAPC or native PAPC (100 µg/mL, in sterile filtered MOPS-buffered physiological salt solution with 0.5% human serum albumin), for 4 hours at 37°C. Some carotid arteries were perfused with blocking antibody to KC (20 µg/mL, clone 124014; R&D Systems, Minneapolis, Minn) or P-selectin (30 µg/mL, RB40.34; Pharmingen, San Diego, Calif) for 20 minutes after OxPAPC treatment. Rolling flux was determined by counting the number of cells that rolled on the vessel wall for at least 1 second during an 8-minute period.

**Statistical Analysis**

Data are expressed as mean±SEM. Results were analyzed using unpaired Student t test (gene expression data) or 1-way ANOVA with Newman–Keuls post-test (ex vivo perfusion model). Differences were considered statistically significant at a value of P<0.05.

**Results**

**Induction of Chemokine Expression by OxPAPC In Vivo**

To investigate effects of oxidized phospholipids on the arterial wall in vivo, we applied F-127 pluronic gel with or without 50 µg OxPAPC to surgically exposed carotid arteries of C57BL/6J mice. This corresponds to a concentration of the bioactive phospholipids 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine of ~30 µg/mL in the gel,9 which is lower than the concentrations measured in rabbit atherosclerotic lesions.13 F-127 dissolves within several hours and releases trapped lipids, allowing for topical exposure of arteries while minimizing systemic effects.14 We used this system to investigate differential expression between OxPAPC, native PAPC and mock-treated arteries of a set of atherosclerosis-related chemokines, including MCP-1 and KC (keratinocyte-derived chemokine, CXCL1), the murine chemokine closest related to human IL-8. Quantitative RT-PCR showed that treatment of carotid arteries for 6 hours with 50 µg OxPAPC in vivo increased vascular expression of MCP-1 (3.3±0.68-fold) and KC (4.8±0.22-fold), as compared with mock-treated arteries (Figure 1a). Immunohistochemistry of carotid arteries treated for 24 hours with OxPAPC confirmed these findings (Figure 1b), showing homogenous chemokine distribution throughout the vessel wall, a pattern also reported in atherosclerotic carotid arteries of ApoE−/− mice.12 In addition, treatment of carotid arteries with OxPAPC induced MIP-1α (3.3±0.57-fold) and MIP-1β (3.6±0.59-fold), whereas RANTES, serum-derived factor-1, and eotaxin were not induced (Figure 1a). To confirm previous in vitro observations that nonoxidized PAPC is not biologically active,6,13 we included a PAPC group in 1 experiment representing 3 animals. Application of 50 µg nonoxidized PAPC to carotid arteries did not influence gene expression levels as compared with mock-treated arteries (Figure 1c), demonstrating that oxidative modification of phospholipids was necessary to form pro-inflammatory agonists. Investigation of untreated contralateral carotid arteries, as well as other organs, revealed that systemic effects of OxPAPC were negligible (data not shown).

**Figure 1.** Chemokine induction by OxPAPC. a, OxPAPC induces MCP-1, KC, MIP-1α, and MIP-1β mRNA after 6 hours in murine carotid arteries, P<0.05 (n=4). b, Vehicle- or OxPAPC-treated (24 hours) carotid arteries stained with antibody against MCP-1 (upper panel) or with antibody against KC (lower panel). Scale bar represents 20 µm. c, Native PAPC does not influence arterial gene expression after 6 hours. One experiment is shown representing pooled arteries from 3 animals. d, Chemokine mRNA expression in atherosclerotic carotids of ApoE−/− mice versus wild-type (WT) control animals. P<0.05 (n=4).
OxPAPC-Induced Monocyte Rolling and Arrest Are Mediated by P-Selectin and KC, Respectively

Among the chemokines found to be upregulated by OxPAPC in the artery wall, KC has been shown to play a dominant role in triggering monocyte arrest on early atherosclerotic endothelium in ex vivo perfused carotid arteries of ApoE<sup>−/−</sup> mice. We hypothesized that KC serves a similar function in OxPAPC-stimulated arteries. Preperfusion of a blocking KC antibody in OxPAPC-treated carotid arteries reduced MM6 cell arrest to levels seen in control arteries (Figure 2a), indicating that OxPAPC-induced monocyte arrest was critically dependent on KC.

Next, we were interested if OxPAPC-induced monocyte rolling would also involve mechanisms analogous to those in murine atherosclerosis. The selectin family of adhesion molecules mediates initial attachment and rolling of leukocytes on vascular endothelium, and functional blocking of P-selectin has been shown to abrogate monocyte rolling on atherosclerotic endothelium in isolated murine carotid arteries. Here, we found that preperfusion with a blocking P-selectin antibody abolished MM6 cell rolling in OxPAPC-treated arteries (Figure 2b), demonstrating a crucial role for P-selectin in OxPAPC-triggered monocyte rolling. However, preperfusion with a blocking KC antibody enhanced rolling flux (Figure 2b). In accord with this observation, continuous P-selectin–dependent monocyte rolling without arrest has been observed in atherosclerotic carotid arteries when blocking α4β1 (VLA-4), the integrin on monocytes that mediates KC-triggered cell arrest.

OxPAPC-Induced Expression of Other Atherosclerosis-Related Genes In Vivo

In addition to chemokines, OxPAPC induces several other atherosclerosis-related genes in cells of the artery wall in vitro. Recently, we showed induction of tissue factor (TF) by OxPAPC in human endothelial cells, accompanied by and dependent on the expression of early growth response-1 (EGR-1). Both genes have been implicated in human and experimental murine atherosclerosis. To investigate if these effects would also be observed in the arterial wall in vivo, TF and EGR-1 mRNA levels were determined by quantitative RT-PCR in murine carotid arteries treated for 6 hours with OxPAPC or vehicle alone. OxPAPC treatment led to upregulation of both TF (2.6±0.62-fold) and EGR-1 (2.0±0.35-fold) transcripts in carotid arteries (Figure 3).

IL-6 has been shown to be expressed in atherosclerotic lesions in mice. Furthermore, IL-6 has been reported to mediate effects of OxPAPC such as decreased hepatic paraoxonase expression in mice. Here, OxPAPC treatment induced IL-6 mRNA 3.5±0.6-fold compared with vehicle-treated arteries (Figure 3). In addition, OxPAPC has been shown to induce the protective gene heme oxygenase-1 (HO-1) in vascular cells in vitro. Here, treatment of carotid arteries with OxPAPC induced HO-1 message 3.6±0.34-fold (Figure 3).

OxPAPC-Induced Inflammation Versus LPS-Induced Inflammation

Target gene expression induced by OxPAPC differs from that induced by other inflammatory mediators such as LPS or...
tumor necrosis factor-\(\alpha\).\(^{25-27}\) We have shown previously that expression of the adhesion molecules E-selectin, vascular cell adhesion molecule-1 (VCAM-1), or intercellular adhesion molecule-1 is not induced by OxPAPC in vitro.\(^{9}\) Consistently, quantitative RT-PCR, as well as immunohistochemistry, showed that treatment of carotid arteries with LPS effectively upregulated expression of E-selectin, VCAM-1, or intercellular adhesion molecule-1, whereas treatment with OxPAPC had no effect (Figure 4a and 4b). In contrast to LPS, OxPAPC did not upregulate P-selectin mRNA in murine arteries (Figure 4a), suggesting that monocyte rolling after OxPAPC treatment (Figure 2b) was mediated by surface translocation of P-selectin from Weibel–Palade bodies.\(^{28}\)

**Discussion**

A growing body of evidence suggests oxidized phospholipids as triggers of vascular inflammation in early atherosclerosis. Most importantly, component lipids of OxPAPC have been detected in atherosclerotic lesions in concentrations sufficient to stimulate vascular cells.\(^{8,13}\) However, evidence for a direct contribution of oxidized phospholipids to vascular inflammation in vivo has not yet been obtained. Thus, to model the accumulation of oxidized phospholipids in the arterial wall during atherogenesis, we applied OxPAPC to the adventitia of carotid arteries of C57BL/6 mice using a slow-release preparation. The murine carotid artery consists of only 4 to 5 cell layers, facilitating penetration of the lipids through the vessel wall. The high sensitivity offered by real-time PCR allows quantifying gene expression from such minute samples. In this model, OxPAPC induced several chemokines in the carotid artery wall that are also expressed in human or experimental atherosclerosis.\(^{29}\) Accordingly, investigation of atherosclerotic carotid arteries from \(\text{ApoE}^{-/-}\) mice revealed that OxPAPC-induced chemokines are expressed in atherosclerotic lesions in mice. Interestingly, the chemokines RANTES, eotaxin, and serum-derived factor-1 were not regulated by OxPAPC in murine arteries, nor were they differentially expressed in atherosclerotic versus normal arteries in \(\text{ApoE}^{-/-}\) mice. However, a nontranscriptional mechanism of RANTES deposition by activated platelets has been described.\(^{30}\)

Importantly, tissue composition differs between native arteries and established atherosclerotic lesions as indicated by high expression of the macrophage marker CD68 in arteries of \(\text{ApoE}^{-/-}\) mice. Thus, although not being representative of the complex cellular interactions in atherosclerotic lesions, our data indicate that OxPAPC accumulation triggers atherogenic chemokine expression and monocyte adhesion in the normal artery wall, underscoring the role of oxidized phospholipids in early lesion formation.\(^{5}\) Besides MCP-1 and KC, whose role in atherogenesis is firmly established, we found MIP-1\(\alpha\) and \(\beta\) to be expressed in OxPAPC-stimulated, as well as in atherosclerotic, arteries. MIP-1\(\alpha\) and \(\beta\) are members of the CC chemokine subfamily that attract monocytes and lymphocytes.\(^{29}\) In addition, MIP-1\(\beta\) induces TF activity in vascular smooth muscle cells.\(^{31}\)

To investigate whether OxPAPC-induced chemokine expression resulted in arterial monocyte adhesion, a hallmark of early atherogenesis, we used ex vivo perfused carotid arteries, a model that has been used extensively to study monocyte–endothelial interactions in atherosclerosis.\(^{12,16,17}\) In our study, OxPAPC stimulation led to monocyte rolling and firm adhesion in normal murine arteries. Firm adhesion of monocytes is mediated by arrest chemokines immobilized on the endothelial surface, leading to integrin activation or clustering on rolling leukocytes.\(^{15}\) It is not known which chemokines serve this function in OxPAPC-stimulated arteries; however, it has been shown that KC, but not MCP-1, triggers monocyte arrest on early atherosclerotic endothelium in isolated carotid arteries of \(\text{ApoE}^{-/-}\) mice.\(^{12}\) Thus, we functionally blocked KC in OxPAPC-stimulated arteries, and the results demonstrated that OxPAPC-induced monocyte arrest was completely dependent on KC.

In addition, it has been shown that functional blocking of P-selectin abrogates monocyte rolling on atherosclerotic endothelium in mice in vivo,\(^{32}\) as well as in isolated carotid arteries.\(^{16}\) Here, we found that OxPAPC-induced monocyte rolling was also dependent on P-selectin in isolated carotid arteries. Although we did not find induction of P-selectin mRNA in OxPAPC-treated carotid arteries in vivo, oxidized LDL has been shown to induce surface translocation of preformed P-selectin from Weibel–Palade bodies.\(^{28}\)

Together, our data support a mechanism of OxPAPC-induced monocyte adhesion in which P-selectin mediates initial attachment and rolling on arterial endothelium, with subsequent activation and arrest triggered by immobilized KC. Although playing an important and nonredundant independent role in atherogenesis, MCP-1 is not involved in
initial monocyte arrest on early atherosclerotic endothelium in murine carotid arteries. Similarly, OxPAPC-induced MCP-1 could be rather involved in subsequent transmigration of adherent monocytes. Thus, our data are in accordance with observations on atherosclerotic vessels in mice and strongly suggestive of a role for oxidized phospholipids as triggers of monocyte recruitment to atherosclerotic lesions.

In ApoE⁻/⁻ mice, KC has been shown to act via VLA-4 on monocytes, which binds to VCAM-1 and fibronectin containing the CS-1 region. We did not observe induction of VCAM-1 by OxPAPC; however, at sites of atherosclerosis predilection, such as the lesser curvature of the aortic arch, VCAM-1 is expressed in C57Bl/6J mice, possibly because of hemodynamic influences. However, binding of monocytes to OxPAPC-stimulated endothelial cells is mediated by CS-1 fibronectin and blocking CS-1 reduced atherosclerotic lesion formation in mice.

In addition to expression of chemokines, we found that OxPAPC induced expression of several other atherosclerosis-related genes. We demonstrate that oxidized phospholipids induce IL-6 transcripts in a native murine artery in vivo. Circulating levels of IL-6 predict future myocardial infarction in apparently healthy men. Because IL-6 was shown to be expressed in atherosclerotic lesions in humans and ApoE⁻/⁻ mice, atherosclerotic sites themselves likely contribute to elevated circulating IL-6 levels. Thus, our data suggest oxidized phospholipids contributing to IL-6 production in atherosclerosis. Moreover, we demonstrate induction of TF by OxPAPC. Enhanced TF expression has been demonstrated in atherosclerotic plaques, a process that may account for thrombotic events associated with early and advanced atherosclerosis. A transcription factor capable of binding to the TF promoter is EGR-1, which is critically involved in TF gene regulation. We have previously shown that OxPAPC increases EGR-1 as well as TF expression in cultured endothelial cells, and that TF induction by OxPAPC is dependent on EGR-1. Our data suggest that oxidized phospholipids contribute to EGR-1 expression in atherosclerosis, thereby enhancing expression of TF and possibly other EGR-1–inducible genes. Finally, we found induction of HO-1 by OxPAPC in murine arteries. HO-1 is expressed in experimental as well as human atherosclerotic lesions, where it is thought to counteract continuous oxidative stress by its antioxidant and anti-inflammatory properties. Thus, induction of HO-1 by oxidized phospholipids in the artery wall may constitute an adaptive response to limit the inflammatory reaction. It was demonstrated that formation of atherosclerotic lesions in ApoE⁻/⁻ mice was accompanied by decreased expression of various antioxidant enzymes, whereas HO-1 mRNA levels remained high during the course of atherogenesis, indicating continuous stimulation likely to be caused by oxidized phospholipids.

Finally, we show that OxPAPC-induced expression of inflammatory genes in carotid arteries differs from that induced by LPS. Although LPS-induced or tumor necrosis factor-induced inflammation would result in adhesion and accumulation of neutrophils and monocytes, the gene expression pattern elicited by “lipid-induced inflammation” may determine monocyte specificity.

In conclusion, we have shown that oxidized phospholipids, known to accumulate in atherosclerotic lesions, induce expression of atherogenic chemokines and other inflammation-related genes in the arterial wall in vivo. Furthermore, we demonstrate a major role for KC in mediating oxidized phospholipid-induced monocyte adhesion to murine arteries. Thus, oxidized phospholipids can be considered as triggers of the inflammatory process in the vascular wall and therefore represent promising molecular targets to combat atherosclerosis and its clinical consequences.

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References


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Surgical procedure and tissue harvesting

All procedures were approved by the Animal Care and Use Committee of the Vienna University. C57BL/6J mice were used at 12-15 weeks. Mice were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylacine (4 mg/kg). Using sterile techniques, a left anterior neck incision was set and the left common carotid artery was freed from surrounding tissue. Subsequently, 60 µl of F-127 with or without 50 µg OxPAPC, 50 µg PAPC or 6 µg lipopolysaccharide (E. coli serotype 055:B5, Sigma-Aldrich) was applied to the vessel. Upon gelling of the solution, the skin wound was closed by interrupted sutures. At indicated time points animals were sacrificed, perfused for 5 minutes with PBS via the left ventricle, and the treated part of the common carotid artery was removed. For comparison of gene expression levels between ApoE⁻/⁻ and wildtype animals, mice were used at 12 months of age, at which time common carotid arteries of ApoE⁻/⁻ mice showed typical macrophage-rich lesions at the proximal and distal bifurcation. Common carotid arteries were harvested as described for lipid-treated animals.

RNA isolation and quantitative RT-PCR

Freshly harvested arterial tissue was immediately immersed into ice-cold RNAlater (Ambion, Austin, Texas) and stored at -70°C upon analysis. For each determination, 3 arteries were pooled and 100 ng of total RNA isolated with TRIzol reagent (Invitrogen, Carlsbad, California) was reverse transcribed to cDNA using GeneAmp RNA PCR core kit (Applied Biosystems, Foster City, California). mRNA sequences of the investigated genes were obtained from GenBank. PCR Primers were designed using PRIMER3
software from the Whitehead Institute for Biomedical Research (Cambridge, Massachusetts) (Table I). Amplified cDNA regions were chosen to span one or more large introns in the genomic sequence to avoid coamplification of genomic DNA. Melting point analysis, agarose gel electrophoresis and DNA sequencing of the PCR product confirmed primer specificity. Quantitative real-time PCR was performed by LightCycler technology using FastStart SYBR Green I (Roche Diagnostics, Basel, Switzerland) as recommended by the manufacturer. The housekeeping gene porphobilinogen deaminase (PBGD) was used as endogenous control. PCR efficiency was determined for each primer pair from dilution series of a typical sample cDNA.

**Immunohistochemistry**

Immunostaining for MCP-1 and KC was performed on 5 µm cryosections of acetone-fixed carotid arteries. Slides were incubated with 10% normal rabbit serum for 20 minutes, followed by 15 minutes of avidin and biotin blocking reagent, respectively (Vector Laboratories, Burlingame, California). Polyclonal goat anti-mouse MCP-1 or KC primary antibodies (Santa Cruz Biotechnology, Santa Cruz, California) were used at 2 µg/ml in the presence of 10% normal rabbit serum for 16 hours at 4°C. Slides were developed with Vectastain Elite ABC kit and 3,3′-diaminobenzidine (Vector Laboratories), and counterstained with hematoxylin. Detection of VCAM-1 was performed by incubating 5 µm cryosections of carotid arteries with 5 µg/ml monoclonal anti-mouse VCAM-1 primary antibodies (clone M/K2, Southern Biotechnology Associates, Birmingham, Alabama) for 16 hours at 4°C, followed by AlexaFluo-488-
labeled secondary antibodies (Molecular Probes, Eugene, Oregon). Secondary antibodies were visualized by epifluorescence microscopy (Olympus).

**Table I.** Real-time PCR primers

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