Celecoxib, a Cyclooxygenase-2 Inhibitor, Reduces Neointimal Hyperplasia Through Inhibition of Akt Signaling

Han-Mo Yang, MD; Hyo-Soo Kim, MD; Kyung-Woo Park, MD; Hyun-Jeong You, BA; Soo-In Jeon, BA; Seock-Won Youn, MS; Sung-Hwan Kim, MD; Byung-Hee Oh, MD; Myoung-Mook Lee, MD; Young-Bae Park, MD; Kenneth Walsh, PhD

Background—Celecoxib has been shown to have antitumor effects that may be mediated through the cyclooxygenase-independent inhibition of Akt signaling. Here, we examined the effects of celecoxib on neointimal formation after balloon injury and its mechanism of action.

Methods and Results—In vitro experiments were performed to evaluate the effects of celecoxib on the Akt/GSK signaling axis and the viability of rat vascular smooth muscle cells (VSMCs). In vivo experiments examined the effects of celecoxib, aspirin, and vehicle on neointimal growth after denudation injury to rat carotid arteries. In vitro, celecoxib suppressed the phosphorylation of Akt and GSK in cultured VSMCs, leading to a reduction in viable cell number, which was reversed by transduction of constitutively active Akt. Such a reduction in cell number was mediated by inhibition of proliferation and induction of apoptosis. In vivo, celecoxib reduced injury-induced phosphorylation of Akt and GSK, reduced VSMC proliferation, and increased caspase-3 activation and VSMC apoptosis at 3 days after injury, whereas aspirin had no effect. At 2 weeks after injury, celecoxib reduced intima-to-media ratio, whereas aspirin had no effect. Adenovirus-mediated delivery of dominant negative Akt was as effective as celecoxib at inhibiting neointimal formation. Conversely, gene delivery of constitutively active Akt significantly reversed the inhibition of intimal hyperplasia by celecoxib, providing causal evidence that the modulation of Akt signaling by celecoxib is a physiologically relevant mechanism.

Conclusions—Celecoxib is a potential inhibitor of neointimal formation by blocking injury-induced Akt activation. These findings suggest a potential use for celecoxib in the prevention of restenosis after angioplasty. (Circulation. 2004;110:301-308.)

Key Words: Akt ■ cyclooxygenase inhibitors ■ restenosis ■ apoptosis ■ muscle, smooth

Celecoxib is a selective cyclooxygenase (COX)-2 inhibitor that has been used extensively to safely treat patients with arthritis. Moreover, treatment with celecoxib was found to reduce the number and size of colorectal polyps in patients with familial adenomatous polyposis. Selective inhibitors of COX-2 have also been demonstrated to exert antiproliferative effects and to induce apoptosis in colon, stomach, and prostate cancer cells, and clinical trials have recently shown that celecoxib has antitumor effects in lung, colon, and pancreatic cancer.

Recent evidence suggests that these antitumor and antiproliferative effects of celecoxib are exerted through COX-independent pathways. In particular, it has been demonstrated that celecoxib induces apoptosis by blocking Akt activation or by inhibiting 3-phosphoinositide-dependent kinase-1 activity in cancer cells. Akt regulates cellular processes including apoptosis, proliferation, migration, and survival, all of which are involved in the development of neointimal hyperplasia. Akt signaling mediates both proliferative and antiapoptotic signaling downstream of growth factors such as vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor. Akt has various downstream targets such as BAD, mTOR, and Forkhead family members. Akt also phosphorylates and inactivates GSK-3β. We and others have shown that the Akt-GSK axis is activated in the vessel wall after balloon injury and stent implantation. Furthermore, we recently reported that constitutively active (the dephosphorylated form) GSK-3β gene transfer inhibits the proliferation of vascular smooth muscle cells and reduces neointimal hyperplasia after balloon injury in rat carotid arteries. Thus, we reasoned that celecoxib-mediated suppression of Akt could inhibit SMC proliferation and neointimal hyperplasia in response to arterial injury after angioplasty or stent implantation.
To investigate the effects of celecoxib on neointimal formation after balloon injury and its mechanism of action, we examined whether celecoxib could reduce the proliferation of VSMCs by inhibiting PDGF-induced activation of Akt in vitro. We then examined whether celecoxib could suppress neointimal hyperplasia after balloon injury by blocking injury-induced activation of Akt in vivo. We also compared the effect of celecoxib and aspirin on neointima to investigate whether celecoxib exerts its effect through COX-dependent or COX-independent mechanisms. To test the hypothesis that the Akt inhibition by celecoxib is a component of a mechanism underlying the suppression of neointimal formation, we delivered adenoviral vectors expressing dominant negative (DN) or constitutively active Akt genes to the rat carotid artery after balloon injury.

Methods

Materials and Adenoviral Vectors

Purified celecoxib was purchased from Pharmacia Korea and dissolved in dimethyl sulfoxide, and the final concentration in all cultures was 0.1%. To modulate Akt activity, we used 2 adenoviral constructs tagged with the hemagglutinin (HA) epitope, as described previously.17,18 The DN Akt construct contains 2 phosphorylation sites, serine 473 and threonine 308, both mutated to alanine, which results in an unphosphorylatable form of Akt. Myr-Akt is Akt that is myristoylated at its N-terminal end, resulting in its constitutively active form.

Cell Isolation and Culture

Rat aortic VSMCs were isolated from the thoracic aortas of Sprague-Dawley rats by enzymatic dispersion using a slight modification of a previously described method.19 Cells were cultured in DMEM (Gibco BRL) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 IU/mL streptomycin.

Cell Viability and Proliferation Assay

Cell viability and proliferation were measured using the WST-1 assay and incorporation of bromodeoxyuridine (BrdU) (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Cells were seeded in 96-well plates at 2 × 10³ cells per well in DMEM (100 μL). After serum starvation for 48 hours, various concentrations of celecoxib (0, 5, 10, and 25 μmol/L) were added and stimulated with either 10% FBS or PDGF-BB (R&D Systems). At the end of the incubation period, 10 μL of WST-1 or BrdU was added. For in vitro gene transfer experiments, VSMCs were infected with adenoviral vectors (50 MOI) overnight before celecoxib was added.

Flow Cytometry

Cell cycle status and apoptosis were evaluated by flow cytometry, as described previously.20 Cells were plated, allowed to attach overnight, and placed in DMEM plus 0.4% FBS for 48 hours. Celecoxib was then added in a serum-free medium composed of DMEM with 25 ng/mL PDGF-BB or 10% FBS 1 hour later. The cells were harvested and fixed at 20 hours for analysis of cell-cycle progression and at 48 hours for apoptosis. DNA content was analyzed by flow cytometry (Becton-Dickinson). Histograms of DNA contents were analyzed using ModFit LT software (Verity Software) to characterize population fractions in each phase of the cell cycle.

Western Blot Analysis

Confluent cells cultured in 6-cm dishes were incubated in serum-deprived medium for 24 hours. VSMCs were then stimulated using 25 ng/mL of PDGF-BB in the presence of different celecoxib concentrations (0, 10, and 25 mmol/L) for 2 hours. Western blot analysis was performed as described previously.14 The primary antibodies used were anti-phospho-Akt (Ser473) (1:500 dilution, Cell Signaling Technology), anti-total-Akt (1:500 dilution, Cell Signaling Technology), anti-phospho-GSK-3β (Ser9) (1:750 dilution, Cell Signaling Technology), and anti-α-tubulin (1:4000 dilution, Oncogene). For in vivo studies, 3 carotid arteries were pooled in each group.

Rat Carotid Artery Balloon Injury Model, Drug Treatment, and Adenoviral Vector–Mediated Gene Delivery

Male Sprague-Dawley rats, 13 weeks old, weighing ~400 g (Daehan Biolink Co) were fed standard pellet feed and given water ad libitum. The experimental protocol was designed in accordance with the Guide for Experimental Animal Research issued by the Laboratory for Experimental Animal Research, Clinical Research Institute, Seoul National University Hospital. Animals were anesthetized with ketamine hydrochloride (50 mg/kg, Yuhan Corp, Bayer Korea) and xylazine (7 mg/kg, Yuhan Corp, Bayer Korea). The left carotid artery was exposed, and a 2F Fogarty balloon embolectomy catheter (Baxter) was inserted via an external carotid arteriotomy incision.21 The catheter was advanced to the common carotid artery, inflated with 0.2 mL of saline, and withdrawn 3 times with rotation. In the first in vivo study, rats were divided into 3 groups (n=10/group): celecoxib (50 mg · kg⁻¹ · d⁻¹), aspirin (50 mg · kg⁻¹ · d⁻¹), and vehicle (0.5% methylcellulose, 0.025% Tween 20; Sigma Chemical Co). Drugs were administered daily by oral gavage, in a 0.5-mL suspension of vehicle, for 3 days before the balloon injury and were continued for 2 weeks after injury. In the second in vivo study, rats that underwent balloon denudation injury of the carotid artery were divided into 4 groups (n=10/group): adeno-green fluorescent protein (GFP), adeno-DN-Akt, celecoxib+adeno-GFP, and celecoxib+constitutively active (myr)-Akt. After balloon injury, 5 × 10⁷ pfu of adenovirus diluted in a total volume of 20 μL was delivered to injured segments, which were then incubated for 20 minutes with vascular clamp. At 3 days or 2 weeks after injury, rats were euthanized by pentobarbital overdose and perfused with 10% formaldehyde. Carotid arteries were removed and placed in the same fixative. Tissues were then embedded in paraffin, and 4 to 5 sections (4 μm) were cut at multiple levels. These were stained with hematoxylin-eosin or elastic-van Gieson stain. Sections were then examined, and the cross-sectional areas of the lumen, neointima (from the internal elastic lamina to the lumen), and media were determined using an Image Pro Plus Analyzer Version 4.5 (Media Cybernetics Inc). The intima-to-media (I/M) ratio was then calculated from the determined means.

Immunohistochemical Staining and TUNEL Assay

Immunohistochemistry (IHC) was performed as previously described.14 The primary antibodies used were anti-PC10, for proliferating cell nuclear antigen (PCNA 1:200, Dako), anti-α-HA (1:200, Santa Cruz), anti-cleaved caspase-3 (1:200, Cell Signaling Technology), and anti-ED1 for macrophages (1:200, Serotec). The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) assay was performed with minor modification to a previously described method,14 using an Apoptag kit (Intergen Co). DAB was used as a chromogen. Sections were counterstained with methyl green or Mayer’s hematoxylin. The percentages of PCNA-positive, TUNEL-positive, and ED1-positive cells versus total nucleated cells were quantified in 3 different sectors per tissue section. All samples were coded so that analysis was performed without knowledge of which treatment each individual vessel had received.

Statistical Analysis

Data are presented as mean±SEM. Analysis was performed with 1-way ANOVA with the Bonferroni test for multiple comparisons and with the Student t test using SPSS 11.0 software. A probability value of P<0.05 was considered significant.
Results

Celecoxib Reduces VSMC Viability Through Inhibition of Akt Pathway

On microscopic examination, VSMCs treated with 10 or 25 μmol/L of celecoxib became rounded and detached from the culture dish (Figure 1A). The loss of cell viability was associated with a significant attenuation of both phosphorylated Akt and phosphorylated GSK-3β, a downstream substrate of Akt. Western blot analysis showed that celecoxib markedly reduced the basal level of phospho-Akt and phospho-GSK-3β in nonstimulated conditions (Figure 1B). The enhanced Akt phosphorylation and paralleled increase in the phospho-GSK-3β after PDGF stimulation was also reduced in a dose-dependent manner with celecoxib treatment (Figure 1B).

The WST-1 assay was used to quantify the effect of celecoxib on the decrease in cell number and to examine whether its effect is mediated through the inhibition of the Akt pathway. This assay showed that celecoxib decreased the number of viable VSMCs in the presence of either PDGF or 10% FBS in a dose-dependent manner (Figure 1C). The inhibitory effect of celecoxib on viable cell number was more potent with PDGF than with 10% FBS (viable cells, 41.2±6.7% at celecoxib 10 μmol/L; 27.3±4.4% at 25 μmol/L compared with 100% at vehicle under PDGF stimulation; 76.8±7.0% at celecoxib 10 μmol/L; 63.6±8.6% at 25 μmol/L compared with 100% at vehicle under 10% FBS stimulation; both P<0.01). Transduction of Akt with constitutively active myr-Akt reversed the action of celecoxib, resulting in a significant increase in viable cells (27.3±4.4% with adeno-GFP, 91.3±13.2% with adeno-myrAkt under PDGF; 63.6±8.6% with adeno-GFP, 84.8±8.5% with adeno-myrAkt under 10% FBS at celecoxib 25 μmol/L; both P<0.01) (Figure 1C).

To assess the contributions of apoptosis and cell cycle arrest to the reduction in VSMC number, we performed BrdU incorporation assay and flow cytometry for DNA content. Celecoxib inhibited DNA synthesis in a dose-dependent
manner (Figure 2A). This treatment arrested the cell cycle at G₁ phase, resulting in a decrease in the fraction of cells in the S phase (Figure 2B). Celecoxib significantly increased the subdiploid fraction of DNA by FACS analysis, which is indicative of DNA fragmentation during apoptosis (Figure 2C). The effects of celecoxib on BrdU incorporation, S phase fraction, and apoptosis were greater with PDGF culture conditions compared with 10% FBS (data not shown).

**Figure 2.** Effect of celecoxib on DNA synthesis, cell cycle, and apoptosis in PDGF culture condition. A, BrdU incorporation to measure DNA synthesis. DNA synthesis was reduced in a dose-dependent manner after celecoxib treatment. B, Cell cycle analysis. Celecoxib treatment resulted in G₁ cell-cycle arrest and inhibited transition to S phase. C, FACS analysis of proportion of subdiploid cells. Celecoxib significantly increased apoptosis of VSMCs. Data are representative of 4 separate experiments. All experiments were performed under PDGF stimulation. *P<0.05 vs vehicle (CXB 0 μmol/L) or only PDGF treatment. CXB, celecoxib.

**Celecoxib Inhibits Neointimal Hyperplasia In Vivo**

At 2 weeks after injury, the vehicle-treated group showed abundant neointimal hyperplasia. The celecoxib-treated group showed significant suppression of neointimal hyperplasia and reduction of the I/M ratio (I/M ratio, vehicle versus celecoxib, 0.98±0.04 versus 0.48±0.05, P<0.001). In contrast, the aspirin-treated group showed no significant reduction of neointimal hyperplasia (I/M ratio, vehicle versus aspirin, 0.98±0.04 versus 0.89±0.05, P=0.25) (Figure 3, A and B). At 3 days after injury, no intimal hyperplasia could be detected in the 3 groups (data not shown).

**Celecoxib Inhibits VSMC Proliferation and Induces Apoptosis In Vivo**

To examine the effects of celecoxib on VSMC proliferation in vivo, IHC for PCNA was performed. At 3 days, 29.8±1.7% of the nuclei in the vehicle-treated group were PCNA-positive, whereas only 17.1±1.5% in the celecoxib-treated group were PCNA-positive (P<0.001) (Figure 4, A and B). At 2 weeks, the PCNA-positive percentage in the vehicle-treated group was 9.7±0.8%, whereas in the celecoxib-treated group, it was 5.9±0.8%. However, no inhibition of proliferation was found in the aspirin-treated group (3 days and 2 weeks, 25.6±1.9% and 8.5±0.7%).

To examine the effects of celecoxib on VSMC apoptosis in vivo, we performed TUNEL assay and IHC for activated caspase-3. At 3 days after injury, the celecoxib-treated group showed higher levels of apoptosis than either the vehicle- or aspirin-treated groups (celecoxib versus vehicle versus aspirin, 19.4±2.0% versus 12.3±1.3% versus 11.8±1.2%). At 2 weeks, this tendency was also present, but to a lesser extent (celecoxib versus vehicle versus aspirin, 8.1±0.9% versus...
4.6±0.7% versus 5.3±0.8%). Consistent with these data, IHC for activated caspase-3 showed more staining in the celecoxib-treated group (Figure 4, B and C).

In tissues harvested 2 weeks after injury, the location of macrophages in the neointima was not periluminal but rather near the internal elastic lamina (data not shown). In contrast to the vehicle-treated group, the aspirin- and celecoxib-treated groups showed a small reduction in macrophage infiltration (macrophage percent, control versus aspirin versus celecoxib, 7.1±0.9% versus 5.1±0.6% versus 5.4±0.5%).

Celecoxib Inhibits Neointimal Hyperplasia by Blocking Injury-Induced Akt Activation

To examine the time course of Akt activation after injury in vivo, Western blotting for phospho-Akt and phospho-GSK-3β was performed with the tissues taken before injury and 3 days and 2 weeks after injury. Akt activity at 3 days after injury was significantly elevated, as was the phosphorylation of GSK-3β (Figure 5A). At 2 weeks after injury, activated Akt levels decreased dramatically. To investigate the mechanism of celecoxib action after vascular injury, Western blot analysis for Akt and GSK-3β.
was performed in all 3 groups at 3 days after balloon injury. At this time point, the levels of phosphorylated Akt and GSK-3β were significantly suppressed in the celecoxib-treated group compared with the vehicle- and aspirin-treated groups (Figure 5B).

Effects of Celecoxib on Neointimal Hyperplasia Are Mediated by Akt Signaling

To test whether the inhibition of Akt activation by celecoxib contributes to the suppression of neointimal hyperplasia, we evaluated the effect of DN-Akt gene transfer in the balloon-injury model. Expression of the HA-tagged transgene was detected by IHC at 2 weeks after delivery. HA-tagged transgene expression was also detected by Western immunoblot analysis (data not shown).

At 14 days after injury, DN-Akt gene transfer led to a significant reduction in intimal hyperplasia (I/M ratio, control versus DN-Akt, 0.86±0.05 versus 0.51±0.04, P<0.001) (Figure 6A). A significant decrease in the percentage of PCNA-positive VSMCs was also observed in the neointima and media of the DN-Akt group at 3 days and 2 weeks (control versus DN-Akt, 3 days, 25.4±1.7% versus 15.7±1.8%; 2 weeks, 7.6±0.9% versus 4.7±0.6%, P<0.05). At 3 days after injury, the DN-Akt–treated group showed more apoptosis by TUNEL assay (control versus DN-Akt, 13.8±1.6% versus 19.7±2.1%, P<0.05), and at 2 weeks after injury, although the absolute value decreased, the tendency was sustained (control versus DN-Akt, 3.3±0.5% versus 5.3±0.7%, P<0.05) (Figure 6B).

To test the hypothesis that modulation of Akt signaling by celecoxib is a physiologically relevant mechanism, we delivered adenoviral vectors expressing constitutively active Akt genes to the rat carotid artery after balloon injury. At 2 weeks after injury, myr-Akt gene transfer reversed the inhibition of intimal hyperplasia by celecoxib (I/M ratio, adeno-GFP versus celecoxib 0.86±0.05 versus 0.45±0.04 versus 0.78±0.05) (Figure 6A). In IHC for PCNA, myr-Akt gene transfer reversed the inhibition of VSMC proliferation by celecoxib (celecoxib+adeno-GFP versus celecoxib+myr-Akt, 3 days: 16.9±1.3% versus 20.7±1.3%; 2 weeks: 5.1±0.5% versus 6.9±0.7%, P<0.01 vs control group. n=10 in each group. B, Immunohistochemical staining for PCNA and TUNEL assay. DN-Akt gene transfer reversed favorable effects observed with celecoxib treatment. I/M ratio, intima-to-media ratio; CXB, celecoxib; Myr-Akt, constitutively active Akt; DN-Akt, dominant-negative Akt; Con, control. *P<0.01 vs control group.
after the finding that DN-Akt gene transfer results in a significant reduction of VSMC proliferation and neointimal growth and that constitutively active myr-Akt gene transfer reverses the favorable effects observed with the administration of celecoxib.

In the present study, celecoxib resulted in a significant attenuation of Akt phosphorylation that was dose-dependently associated with decreased VSMC viability in vitro. The attenuation of Akt phosphorylation was paralleled by a decrease in GSK-3β phosphorylation. The activation of the Akt/GSK axis by PDGF was also blocked by celecoxib. In addition, myr-Akt transfection reversed the reduction in cell viability, demonstrating that the inhibition of Akt activation is a primary mechanism of the inhibitory effect of celecoxib on VSMC viability. We found that BrdU incorporation was significantly reduced by celecoxib, whereas the fraction of apoptotic cells increased. Furthermore, cell-cycle analysis showed G1 arrest and a decreased fraction of cells in the S-phase. Taken together, we believe that the decrease in cell number after celecoxib treatment results from both decreased VSMC proliferation and enhanced apoptosis. We also noticed that the decrease in viable cell number, decrease in BrdU incorporation, and increase in apoptosis fraction were seen to a greater degree with PDGF than 10% FBS conditions. This finding appears to support our hypothesis that the antiproliferative and proapoptotic effects of celecoxib are mediated through the Akt pathway, because PDGF acts more directly through the Akt pathway than 10% FBS, which contains multiple cytokines and growth factors.

The administration of celecoxib in vivo led to a significant reduction of neointimal growth 2 weeks after injury. Western blotting of tissues harvested 3 days after injury showed that celecoxib attenuated Akt/GSK signaling. Celecoxib significantly reduced proliferation and increased apoptosis of VSMCs at both 3 days and 2 weeks after injury as measured by IHC. In contrast, aspirin, a COX inhibitor, had no effect on the Akt/GSK axis and showed no effect on neointima formation compared with controls. Collectively, these data suggest that the effect of celecoxib is independent of COX inhibition.

A potential concern with some COX-2 inhibitors is its thrombogenic potential. Previous animal studies showed that selective COX-2 inhibitors block the formation of prostaglandin I2, without inhibiting thromboxane A2, which increases the possibility of thrombosis. In the VIGOR (Vioxx Gastrointestinal Outcome Research) trial, the rate of myocardial infarction was higher in the rofecoxib group than in the naproxen group. However, in the CLASS (Celecoxib Long-Term Arthritis Safety Study) trial, the celecoxib group showed no increase in cardiovascular events versus other NSAID-treated groups. Moreover, White et al demonstrated, in a large arthritis clinical trial database for celecoxib, that there was no evidence of an increased risk of cardiovascular thrombotic events in a celecoxib group (18942 patients) versus those treated with either conventional NSAIDs (11143 patients) or a placebo (1794 subjects). In our study, we observed no thrombus inside rat carotid arteries at harvest in the celecoxib-treated group.

In summary, this is the first study to demonstrate that celecoxib inhibits neointimal hyperplasia after angioplasty. Our results suggest that celecoxib affects the Akt/GSK signaling axis, leading to an inhibition of VSMC proliferation and an increase in VSMC apoptosis. In this regard, it may be of interest to investigate the feasibility of celecoxib administration, either orally or as an agent for drug-eluting stents, for anti-restenosis therapy.

Acknowledgments
This study was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (02-PJ10-PG8-EC01-0026). Dr Hyo-Soo Kim is the investigator of the Aging and Apoptosis Research Center at Seoul National University sponsored by KOSEF and is the recipient of the Seoul National University Hospital Research Fund (03-2004-005). Dr Young-Bae Park is the investigator of the Stem Cell Research Center, Republic of Korea (SC 13122).

References


Celecoxib, a Cyclooxygenase-2 Inhibitor, Reduces Neointimal Hyperplasia Through Inhibition of Akt Signaling
Han-Mo Yang, Hyo-Soo Kim, Kyung-Woo Park, Hyun-Jeong You, Soo-In Jeon, Seock-Won Youn, Sung-Hwan Kim, Byung-Hee Oh, Myoung-Mook Lee, Young-Bae Park and Kenneth Walsh

Circulation. 2004;110:301-308; originally published online July 6, 2004;
doi: 10.1161/01.CIR.0000135467.43430.16
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/3/301

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation 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 is online at:
http://circ.ahajournals.org//subscriptions/