Endothelial NO Synthase Deficiency Promotes Smooth Muscle Progenitor Cells in Association With Upregulation of Stromal Cell–Derived Factor-1α in a Mouse Model of Carotid Artery Ligation

Le-Ning Zhang, Dennis W. Wilson, Valdecia da Cunha, Mark E. Sullivan, Ronald Vergona, John C. Rutledge, Yi-Xin Wang

Background—Endothelial NO deficiency (endothelial NO synthase [eNOS]–knockout [KO]) enhanced smooth muscle cell (SMC)–rich neointimal lesion formation in a mouse model of carotid artery ligation (CAL). Recent evidence indicated that stromal cell–derived factor-1α (SDF-1α)–mediated recruitment of circulating SMC progenitor cells substantially contributed to the SMC-rich neointimal hyperplasia induced by vascular injury. The goal of this study was to investigate the effects of eNOS deficiency on the expression of SDF-1α and mobilization of circulating SMC progenitor cells in CAL model.

Methods and Results—Two- to 3-month-old C57BL/6J wild-type (WT) and eNOS-KO mice were evaluated 1, 2, or 4 weeks after CAL. CAL-induced expression of SDF-1α, as detected by immunohistochemical staining and further quantified by ELISA in the ligated carotid arteries, was moderate and transient with a peak at 1 week in WT mice. SDF-1α expression was significantly higher at 1 week and persisted through 2 weeks in eNOS-KO mice. CAL was associated with increased circulating stem cell antigen-1+ (Sca-1+)/c-Kit+/Lin− cells (interpreted as SMC progenitor cells), which peaked at 1 week in WT mice. This effect was also significantly greater and longer-lasting in eNOS-KO than WT mice. The number of circulating Sca-1+/c-Kit−/Lin− cells was positively correlated with the expression of SDF-1α but not vascular endothelial growth factor in the ligated carotid arteries. Furthermore, immunostaining showed abundant Sca-1+–positive cells in the adventitia of the 1-week ligated carotid arteries from eNOS-KO mice but not in WT mice. We also determined that eNOS deficiency enhanced CAL-induced intimal cell proliferation in the ligated arteries as detected by proliferating cell nuclear antigen staining but did not induce cell apoptosis as detected by staining for active caspase-3.

Conclusion—Our results indicate that eNOS deficiency exacerbates CAL-induced expression of SDF-1α and its receptor CXCR4. This is correlated with an increase in Sca-1+ cells in peripheral blood and adventitia, which may contribute to vascular remodeling and SMC-rich neointimal lesion formation. This suggests that constitutive eNOS inhibits SDF-1α expression and provides an important vasculoprotective mechanism for intact endothelium to limit SMC proliferation and recruitment in response to vascular injury. (Arterioscler Thromb Vasc Biol. 2006;26:765-772.)

Key Words: eNOS knockout mice ■ neointima ■ smooth muscle cell ■ progenitor cell ■ stromal cell–derived factor-1α
mobilization.11–16 Furthermore, Schober et al reported that SDF-1α blockade prevented recruitment of circulating SMC progenitor cells into neointimal lesions induced by vascular injury in apolipoprotein E–deficient mice.17

Synthesis of NO by endothelial NO synthase (eNOS) is an important homeostatic mechanism in the cardiovascular system. Our previous study showed that eNOS deficiency increased the recruitment of mononuclear cells, including monocytes and lymphocytes into the arterial wall, and enhanced SMC-rich neointimal lesion formation in a carotid artery ligation (CAL) model (L.N.Z., V.d.C., Martin-McNulty B., D.W.S., M.E.S., R.V., J.C.R., Y.X.W., unpublished data, 2005). In this model, remodeling of the vascular wall is thought to be driven by endothelial cell responses to altered flow and shear stress.18 These observations lead us to hypothesize that SDF-1α–mediated recruitment of circulating SMC progenitor cells into injured arteries contributes to enhanced neointimal SMC accumulation in eNOS-deficient mice. To test this hypothesis, the present study investigated the effects of eNOS deficiency on local expression of SDF-1α and mobilization of SMC progenitor cells in the CAL model.

Methods

Experimental Design and Surgical Procedures

All protocols were approved by the institutional animal care and use committees at the University of California at Davis and Berlex Biosciences. Two- to 3-month-old male C57BL/6J wild-type (WT) controls and eNOS-knockout (KO) mice with C57BL/6J background (Jackson Laboratories; Bar Harbor, Me) were anesthetized with inhalation of 2% isoflurane. A midline neck incision was made and the left common carotid artery carefully dissected under a dissecting microscope. The left common carotid artery was ligated with a 6-0 silk ligature just proximal to its bifurcation, and the right carotid artery was used as sham-operated control by passing a suture underneath without tightening. After closing the wound, the mice were allowed to recover and were maintained on standard chow diet for 1, 2, or 4 weeks. Then mice were euthanized, and both common carotid arteries were removed for the following experiments. Each experimental group included 5 to 7 animals.

Morphometric Analysis

For morphometric studies, mice were perfusion-fixed at a constant pressure (100 mm Hg) via cardiac puncture with 10% PBS formalin for 10 minutes. The whole left and right carotid arteries (~9 mm long) were excised and placed in 10% formalin for 24 hours to complete the fixation. Cross-sections at 2.5, 4.5, and 6.5 mm from the ligation were cut, embedded in paraffin, and stained with hematoxylin and eosin. Images of these sections were obtained with a Zeiss Microscope mounted with a digital camera (JVC TK-C1380) and morphometrically analyzed by using the cast grid system (Olympus). Three sections for each carotid artery were measured, and the data were averaged. The circumferences of the lumen, internal elastic lamina (IEL), external elastic lamina (EEL), and the outer edge between tightly packed and surrounding loose tissue of the carotid artery were obtained by tracing their contours on digitized images. Adventitial, medial, and intimal areas were calculated from these parameters. The adventitial area is the area between EEL and IEL, and the intimal lesion area was calculated by subtracting lumen area from the IEL area.

Immunohistochemistry

Immunostaining for SDF-1α was performed with a mouse anti-m/h SDF-1α antibody (clone 79018.111 R&D systems). The sections (5 μm) were deparaffinized and rehydrated. Antigen retrieval was performed by boiling the sections in 0.01 mol/L citrate buffer, pH 6.0, 4 times for 5 minutes at 700 watts in a microwave, followed immediately by a 20-minute cooling period. The sections were then treated with 3% H2O2 in methanol for 10 minutes to abolish endogenous peroxidase activity. To block nonspecific antibody binding, the Histomouse Max Kit (Zymed Laboratories Inc.) was used according to manufacturer instructions. Subsequently, sections were incubated overnight at 4°C with primary SDF-1α antibody (1:200 dilution). For negative controls, the primary antibody was replaced with isotype control mouse monoclonal IgG. On the second day, after several washes with PBS, the sections were incubated with biotinylated secondary antibody and avidin-biotin horseradish peroxidase for 30 minutes, respectively, visualized with 3,3′-diaminobenzidine followed by counterstaining with 10% Mayer’s hematoxylin, and finally, mounted in Permount and examined by light microscopy. The extent of SDF-1α expression was semiquantitatively evaluated by scoring as follows: grade 0, negative stain; grade 1, variable or weak stain; and grade 2, moderately or strongly positive stain.

Immunohistochemical staining for stem cell antigen-1 (Sca-1) with a rat anti-mouse monoclonal antibody 553333 (1:100; Pharmingen), proliferating cell nuclear antigen (PCNA) with a rabbit polyclonal antibody sc-7907 (1:500 dilution; Santa Cruz Biotechnology), and active caspase-3 with a rabbit polyclonal antibody G748A (1:500 dilution; Promega) for apoptosis in the arterial wall were performed with a similar procedure as described above.

Ex Vivo Carotid Artery Culture and Quantification of Soluble SDF-1α, Vascular Endothelial Growth Factor, and Active Matrix Metalloproteinase-2

Left and right carotid arteries were dissected aseptically and immediately placed in 300 μL DMEM (GIBCO-BRL), then incubated for 24 hours at 37°C, 5% CO2 atmosphere. Soluble SDF-1α and vascular endothelial growth factor (VEGF) levels in tissue culture supernatants were determined by using commercially available ELISA kits (R & D Systems). By using the matrix metalloproteinase-2 (MMP-2) Biotrack Activity Assay System (Amersham Biosciences), active MMP-2 level in tissue culture supernatants was also measured. SDF-1α, VEGF, and MMP-2 values were normalized to the weight of the carotid artery segment and expressed as nanograms per milliliter per milligram of tissue.

Flow Cytometry

To enumerate circulating SMC progenitor cells, anticoagulated blood was collected by cardiac puncture. After removal of red blood cells by erythrocyte lysis, peripheral blood cells in staining buffer (Mg2+/Ca2+ free PBS with 1% FCS and 0.1% sodium azide, pH 7.4) were first incubated at 4°C with rat anti-mouse CD16/CD32 monoclonal antibody (mAb) to block nonspecific binding for 5 minutes, then with biotin-conjugated mAbs against allophycocyanin (activ-ated protein C [APC])–conjugated lineage markers (Lin; CD3, CD11b, CD45R/B220, Ly-6C/G, TER119), fluorescein isothiocyanate–FITC–conjugated Sca-1, and phycoerythrin (PE)–conjugated c-Kit (CD117; all Pharmingen) for 40 minutes in the dark. In addition, for baseline setting, cells were incubated with an APC mouse lineage isotype control cocktail, FITC-rat IgG2a, and PE-rat IgG2b as negative controls, and the BD CompBeads Compensation antibodies (PharMingen) were incubated with APC lineage markers, FITC–Sca-1–, and PE–c-Kit as positive controls. At the end, the Ab/PharMingen cocktail was added, and the cells were counted with a FACScalibur system (Becton Dickinson).

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from carotid artery samples (5 carotid arteries pooled as 1 sample) using the Trizol Reagent (Invitrogen) and purified by RNasy Mini Kit (Qiagen) according to manufacturer instructions. cDNA was synthesized using 10 μg of total RNA with High Capacity cDNA Archive Kit (Applied Biosystems). For amplification of cDNA, primers of mouse CXCR4 (forward, 5′-TCAG-
TGGCTGACCTCCTCTT-3'; backward, 5'-TTTCAGCCAGCA-GTTTCCCT-3', 219 bp) and GAPDH (forward, 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; backward, 5'-AGCCTTCTCCAGGTGGTGAAAGAC-3', 307 bp) were used. All polymerase chain reactions (PCRs) were performed in 100-μL reaction mixtures on a GeneAmp PCR System 2400 (Perkin-Elmer) for 35 cycles in which each cycle consisted of 15 s at 92°C, 30 s at 51.3°C, with a final extension step of 2 minutes at 68°C. Equal amounts of PCR products of the sample were analyzed on 3% agarose gels and stained with ethidium bromide.

Statistics

All results are presented as the mean±SEM with the number of animals indicated. An unpaired Student t test was used to detect significant differences when 2 groups were compared. Multiple comparisons of mean values were performed by ANOVA followed by a subsequent Student-Newman–Keuls test for repeated measures. The correlation between Sca-1/c-Kit/Lin− cells and expression of SDF-1α and VEGF was assessed by Spearman rank correlation coefficient. Differences were considered statistically significant when the P value was <0.05.

Results

eNOS Deficiency Exacerbated CAL-Induced Arterial Remodeling: Neointimal Formation and Adventitial Proliferation

Morphometric analysis confirmed our previous report (L.N.Z., V.d.C., Martin-McNulty B., D.W.S., N.E.S., R.V., J.C.R., Y.X.W., unpublished data, 2005) that CAL gradually increased the area of all 3 layers of the vessel wall over time, including intima, media, and adventitia (supplemental Figure I, available online at http://atvb.ahajournals.org). CAL-induced adventitial proliferation and neointimal formation were significantly greater in eNOS-KO than WT mice. There was no significant difference in medial thickening between WT and eNOS-KO mice.

eNOS Deficiency Enhanced CAL-Induced SDF-1α and VEGF Expression and MMP-2 Activity

The temporal expression of SDF-1α in the carotid arteries after CAL was evaluated by immunohistochemistry staining and further quantified by ELISA. As shown in Figure 1A, SDF-1α staining was barely detectable in WT mice but was extensively expressed in eNOS-KO mice, especially at 2 weeks after CAL, when it was prominently evident throughout the arterial wall from adventitia to media and neointima. Semiquantitative evaluation of SDF-1α staining revealed that the expression was significantly higher in the carotid artery from eNOS-KO than WT mice (Figure 1B). Quantification of SDF-1α expression in the carotid artery by ELISA similarly demonstrated that CAL only induced transient and low-level expression of SDF-1α in WT mice, whereas the response was significantly greater and longer lasting in eNOS-KO mice (Figure 2, top). To evaluate other factors also potentially involved in the homing and retention of progenitor cells in the neointimal lesions, additional ELISA results showed that CAL-induced expression of VEGF in the carotid artery occurred mainly at 1 and 2 weeks, which was significantly greater in eNOS-KO than WT mice (Figure 2, middle). MMP-2 activity was elevated at 1 week and returned to baseline 2 weeks after CAL, with the responses also being

Figure 1. Immunostaining (A) and semiquantitative evaluation (B) of SDF-1α expression in the carotid arteries from WT and eNOS-deficient (eNOS-KO) mice at 1, 2, and 4 weeks after CAL. *P<0.05 vs WT. Original magnifications ×200.
ligation. Compared with eNOS-KO mice, Sca-1 was ex-
pressed in a few adventitial cells at 1 week and absent at later
time points in WT mice. Semiquantitative assessment showed
that the Sca-1 expression was significantly higher in the
carotid artery from eNOS-KO than WT mice at 1 week after
CAL (supplemental Figure II). Furthermore, immunostaining
showed no c-Kit$^+$ in the ligated carotid arteries (data not
shown).

eNOS Deficiency Increases CXCR4 Gene
Expression in Ligated Carotid Arteries

Because it is reported that recruitment of circulating SMC
progenitors to sites of vascular injury is CXCR4 dependent
and that accumulating SMCs express CXCR4 receptor,21 we
asked whether the accumulations of SMCs in eNOS-KO mice
transcribed the CXCR4 gene. Indeed, semiquantitative RT-
PCR revealed a significantly greater upregulation of CXCR4
mRNA in eNOS-KO than WT mice at 2 weeks (supplemental
Figure III).

eNOS Deficiency Increased CAL-Induced
Cell Proliferation

Increased intimal cell populations could result from a com-
bination of emigration, local proliferation, or a decreased rate
of apoptosis. A recent report suggests SDF-1α expression is
preceded by apoptosis.21 We evaluated immunohistochemical
staining for PCNA (indicating cell proliferation) and active
caspase-3 (indication of apoptosis) in the arterial wall (sup-

tamental Figure IV). PCNA$^+$ cellular proliferation was
prominent at 1 week and dramatically declined at 2 and 4
weeks after CAL. This response was also significantly greater
in eNOS-KO than WT mice (supplemental Figure IVA and
IVB). Caspase-3 immunostaining demonstrated only rare
positive cells and did not reveal differences between the WT
and eNOS-KO mice (supplemental Figure IVC).

**Discussion**

Proliferation and migration of SMCs during the development
of intimal hyperplasia induced by arterial injury is a critical
component of restenosis after angioplasty of human coronary
arteries and an important feature of atherosclerotic lesions.
The origin of SMCs in the neointimal lesions of vascular
disease is not yet fully understood. Medial SMCs are com-
monly proposed as an important source that can undergo
phenotypic modification and migrate into the intima, where
they proliferate and secrete extracellular matrix compo-
nents.7,8 Recently, several groups developed persuasive
evidence showing that in models of postangioplasty resteno-
sis, graft vasculopathy, and hyperlipidemia-induced ather-
sclerosis, bone marrow–derived progenitor cells were re-
cruited into the arterial wall and differentiated into SMC-like
cells, contributing to the in situ formation of the neointima.9,22

Moreover, SDF-1α/CXCR4 interactions and signaling have
been implicated as a principal axis regulating mobilization,
migration, and retention of hematopoietic progenitor cells
during steady-state homeostasis and injury.11,23 SDF-1α is a
unique chemokine that is highly conserved in mammals and
is necessary for embryonic survival. SDF-1α binds to and

![Graph](http://atvb.ahajournals.org/)

**Figure 2.** Evaluation of SDF-1α (top) and VEGF (middle) level by
ELISA or activity of active MMP-2 by activity assay (bottom) in
the carotid arteries from WT and eNOS-deficient (eNOS-KO)
mice at 0 (no ligation), 1, 2, and 4 weeks after CAL. *P<0.05
vs WT.
signals exclusively through CXCR424 and was found to be highly expressed in human atherosclerotic plaques.25 Recent studies demonstrated that the SDF-1\textsuperscript{α}/CXCR4 axis plays a crucial role in neointima formation in a wire-induced vascular injury model\textsuperscript{17,21} and transplant arteriosclerosis.26 In these studies, SDF-1\textsuperscript{α} was the key molecule recruiting CXCR4\textsuperscript{+} circulating progenitor SMCs into the arterial wall, where they differentiate into SMCs in the neointima.

Results of our studies confirm that eNOS deficiency augments the accumulation of smooth muscle actin\textsuperscript{+} cells in the neointima and proliferation of adventitia in the CAL model of vascular remodeling. Neointima formation involved local proliferation of cells based on PCNA staining but was apparently not related to altered rates of apoptosis as evaluated by caspase-3 activation. We also demonstrate upregulation of SDF-1\textsuperscript{α}, VEGF and MMP-2 protein, and CXCR4 message in the remodeling vascular wall. CAL resulted in an increase in circulating vascular smooth muscle progenitor cells that was also augmented in eNOS-deficient mice. Furthermore, the number of circulating progenitor cells was correlated with levels of SDF-1\textsuperscript{α} but not VEGF, and Sca-1\textsuperscript{+} cells were abundant in the arterial wall of eNOS-KO but not WT mice. Our data suggest a hypothesis that SDF-1\textsuperscript{α}-mediated recruitment of progenitor SMCs into the arterial wall is an important source of SMC-like cells in the vascular remodeling and neointimal lesion induced by CAL in eNOS-

Figure 3. A, Representative flow-cytoMetric charts from WT and eNOS-deficient (eNOS-KO) mice. FL indicates fluorescence. B, Flow cytometric analysis of progenitor cells in peripheral blood in WT and eNOS-KO mice at 0 (no ligation), 1, 2, and 4 weeks after CAL. The Sca-1\textsuperscript{+}/c-Kit\textsuperscript{+}/Lin\textsuperscript{−} (top) and Sca-1\textsuperscript{+}/c-Kit\textsuperscript{+}/Lin\textsuperscript{−} (bottom) cells were counted. *P<0.05 vs WT.
deficient mice. This also suggests that the persistence of endothelium in the remodeling ligated artery is an important modulator of neointimal formation.

Recent work by Sata’s group found that circulating progenitor cells were not a major source of SMC-like cells in the neointima lesion in the CAL model. In their study, 3 distinct types of mechanical injuries, including wire injury of the intima, external cuff restriction, and CAL were compared in C57BL/6J mice. The results showed that in the wire injury model, a significant number of neointimal and medial cells were derived from bone marrow, but there were almost no bone marrow cells detected in the lesion induced by perivascular cuff replacement and only a few bone marrow–derived cells in the neointima after CAL. Our data show that in WT mice, the upregulation of SDF-1α and circulating smooth muscle progenitor cells induced by CAL were transient and at a low level. This finding is consistent with the limited number of bone marrow–derived neointimal cells in the work of Sata’s group. However, in eNOS-KO mice, we found that CAL-induced expression of SDF-1α and the increase in circulating smooth muscle progenitor cells was significantly higher and lasted longer. Furthermore, immunostaining and RT-PCR data revealed that Sca-1+ cells and vascular wall expression of the CXCR4 gene were more prominent in eNOS-KO than WT mice. The presence of SDF-1α and Sca-1+ cells as well as transcription of its receptor CXCR4 in the vessel is supportive of our hypothesis that the SDF-1α/CXCR4 axis modulated mobilization of progenitor cells induced by CAL in eNOS-KO mice. These results may provide a molecular mechanism explaining the finding of Sata’s group, in which circulating progenitor cells contributed to neointima formation in wire injury model that is characterized by severe endothelium damage but not in the perivascular cuff replacement and ligation models in which endothelium remains intact.

It is becoming well accepted that mobilization of bone marrow precursors is an important component of vascular repair and remodeling. Although identification of circulating vascular progenitors as endothelial precursors with specific markers such as CD34 and CD133 seems relatively specific, markers for SMC precursors are less well defined. However, the contribution of circulating SMC precursors to neointimal formation in arterial disease is increasingly evident, and recent work demonstrates a key role for the SDF-1α/CXCR4 axis in recruitment to sites of vascular injury. Sca-1+/c-Kit+/Lin− cells in peripheral blood have been regarded as the principle population of circulating vascular progenitor cells. In contrast, Zernecke et al recently found that the subpopulation of circulating progenitor cells that was mobilized by SDF-1α in neointimal hyperplasia were Sca-1+/c-Kit+/Lin− cells. In agreement with their findings, our study showed that CAL increased the number of Sca-1+/c-Kit−/Lin− cells rather than Sca-1+/c-Kit+/Lin− cells, further supporting the concept that SDF-1α mobilizes c-Kit+ cells in peripheral blood more readily than c-Kit+ cells. Although it has been shown that VEGF plays an important role in the mobilization of bone marrow progenitor cells, especially endothelial progenitor cells, our finding that CAL-induced expression of

Figure 4. Correlation of Sca-1+/c-Kit+/Lin− cells in peripheral blood with the levels of SDF-1α or VEGF in the ligated carotid arteries.

Figure 5. Immunostaining of Sca-1 expression in the carotid arteries from WT and eNOS-deficient (eNOS-KO) mice at 1, 2, and 4 weeks after CAL. Original magnifications ×400.
VEGF was not correlated with an increase in circulating SMC progenitors suggests that VEGF is not a primary mediator for the mobilization and homing of bone marrow progenitor cells into the arterial wall in the CAL model. Although the role of VEGF in the CAL model remains to be determined, its proinflammatory action may be important because our previous study showed that inflammation was an significant early response in vascular remodeling after CAL (L.N.Z., V.d.C., Martin-McNulty B., D.W.S., M.E.S., R.V., J.C.R., Y.X.W., unpublished data, 2005).

Although we did not detect Sca-1⁺ SMCs in the neointima, abundant Sca-1⁺ cells were observed in the adventitia of the ligated arteries from eNOS-KO mice. These data suggest that SMC progenitor cells may first home to the adventitia and then migrate to neointima. In the process of differentiation, the precursor cells migrating from the adventitia may lose progenitor markers. In support of this hypothesis, bone marrow, blood circulation, and local adventitia have been shown as sources for neointimal SMCs. Although the exact route of entry and process of mobilization and migration of vascular progenitor cells into the arterial wall is still not clear, the role of adventitia is being actively explored. The present data cannot specify the exact origin of Sca-1⁺ cells in adventitia, and further investigations are needed to determine the origin of these cells.

In the present study, we also found that CAL upregulated the level of active MMP-2 in the ligated arteries, and that this was significantly enhanced in eNOS-KO mice. Active MMP-2 is a critical enzyme in extracellular matrix degradation, a key process in pathological vascular remodeling in cardiovascular diseases. Moreover, active MMP-2 may degrade collagen type IV and fibronectin in the basement membrane, thus creating a favorable environment for stem cell migration, homing, and retention in the tissues. Shi and Kuzuya et al further reported that MMP-2 is key factor promoting cell migration from adventitia or media to neointima. Hence, it seems plausible that upregulation of MMP-2 activity may be a mechanism facilitating SDF-1α-mediated emigration of SMC progenitor cells to the intima in eNOS-KO mice.

Cardiovascular protective roles of endothelial-derived NO include regulation of blood pressure and vascular tone, inhibition of platelet aggregation and leukocyte adhesion, and prevention of SMC proliferation. Although the roles of eNOS/NO in cardiovascular systems have been studied extensively, understanding the influence of eNOS/NO system on the regulation of progenitor cell function in the cardiovascular system is currently only beginning to be explored. The increase in neointima appears to be a combination of cellular infiltration and local proliferation. An increased cell mass at the site of injury could also depend on the balance between apoptosis and proliferation. Our result shows that eNOS deficiency enhances cell proliferation induced by CAL, but that apoptosis does not seem to be important in its progression. Recently, Aicher et al reported that eNOS was essential for mobilization of endothelial progenitor cells from bone marrow to tissues, and that this improved neovascularization and repair of ischemic tissue in myocardial infarction or limb ischemia. Our current findings demonstrate that eNOS deficiency is not sufficient to prevent mobilization of circulating vascular progenor cells and suggest that endothelial cell-derived NO is an important regulator of local SDF-1α upregulation in mediating neointima formation. This effect would be a novel aspect of the vasculoprotective action of the eNOS/NO system and should be considered in interventions directed at SMC accumulation in the neointimal lesion that is a major component in the chain of events leading to intimal hyperplasia after percutaneous transluminal coronary angioplasty. An alternative explanation for exacerbated remodeling in eNOS-KO mice is as a consequence of higher blood pressure. Elevated blood pressure has been observed in eNOS-KO mice, and hypertension may certainly play a role in vascular remodeling and the development of atherosclerosis. However, arguing against this is the demonstration that hypertension does not account for the accelerated atherosclerosis in eNOS-KO mice. Furthermore, Yogo et al reported that antihypertensive treatment with bunazosin failed to suppress the neointimal formation in the ligated carotid artery from eNOS-KO mice.

In summary, this is the first study demonstrating that eNOS deficiency enhanced CAL-induced upregulation of SDF-1α protein, recruitment of Sca-1⁺ cells, and CXCR4 gene expression in injured carotid arteries in association with an increase in Sca-1⁺ progenitor cells in peripheral blood. These results suggest that SDF-1α–mediated recruitment of Sca-1⁺ CXCR4⁺ SMC progenitor cells into an injured artery might significantly contribute to SMC-rich neointimal hyperplasia. An intact eNOS/NO system may inhibit the SDF-1α/CXCR4 system, thereby protecting vessels from pathologic remodeling during injury.

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Figure I

![Graph showing the comparison of Adventitia Area, Media Area, and Intima Area between WT and eNOS-KO over time (weeks after ligition). The graph indicates a significant difference (*) and a very significant difference (**) at certain time points.]
Figure II

Scoring of Immunostaining for Sca-1

- Open circle: WT
- Solid circle: eNOS-KO

** p < 0.01

Time (weeks after ligation)
Figure III

WT  eNOS-KO

CXCR4

GAPDH

CXCR4/GAPDH

0  0.2  0.4  0.6  0.8  1

0  0.2  0.4  0.6  0.8  1
Figure IV

A

WT

100 μm

IEL

IEL

eNOS-KO

B

Positive PCNA In Adventitia (%)

WT

- eNOS-KO

Positive PCNA In Media (%)

C

Positive PCNA In Intima (%)

Positive Caspase-3 In Intima (%)

Positive Caspase-3 In Media (%)

Positive Caspase-3 In Adventitia (%)

Time (weeks after ligation)

WT

- eNOS-KO