Endothelial Nitric Oxide Synthase Deficiency Enhanced Carotid Artery Ligation-Induced Remodeling by Promoting Vascular Inflammation

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ABSTRACT

Nitric oxide (NO) plays an important role in vascular protection. It has been reported that endothelial NO synthase (eNOS) deficiency exacerbated carotid artery ligation (CAL)-induced vascular remodeling, which, however, did not elucidate the role of inflammation. Overexpression of eNOS inhibited vascular inflammation and remodeling in a CAL model. However, there is no study that tested the hypothesis that eNOS deficiency can enhance the inflammatory response that plays a critical role in exacerbation of CAL-induced vascular remodeling. Thus, the present study used both eNOS knockout (eNOS-KO) mice and pharmacological blockade of nitric oxide synthase (NOS) to examine the temporal relationship between the inflammatory process and vascular remodeling by CAL and how elimination of NO production affects this.

The left common carotid artery was ligated in eNOS-KO, or wild type (WT) mice treated with or without an NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME). In WT mice, CAL induced vascular inflammation, characterized by neutrophil and macrophage infiltration into the vessel wall at 1 week. Although the inflammation diminished at 4 weeks, the ligated carotid artery developed prominent vascular

remodeling, manifested by smooth muscle cell rich neointimal formation, medial thickening, and adventitial proliferation with reduced luminal diameter. CAL also increased the expression of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1). VCAM-1 peaked at 1 week, and then slightly declined 4 weeks after CAL, while MCP-1 continued to increase. In eNOS-KO mice, the above changes were exacerbated. Treatment with L-NAME resulted in similar changes as in eNOS-KO mice. Thus, in complement to the previous finding that eNOS overexpression inhibited CAL-induced vascular remodeling, the present study further demonstrated that the absence of eNOS exacerbated vascular remodeling by promoting MCP-1 and VCAM-1 mediated vascular inflammation.

These data provided further in vivo evidence supporting the hypothesis that vasculo-protective effects of eNOS are mediated, at least in part, by inhibition of vascular inflammation and smooth muscle cell migration and proliferation induced by vascular injury and blood flow cessation.

INTRODUCTION

Revascularization of stenotic arteries by percutaneous transluminal angioplasty is a simple and low-risk procedure for the treatment of obstructing atherosclerotic lesions in arteries.[1] However, its long-term outcome has been blemished by the frequent occurrence of restenosis.[2] Recently a mouse model of vascular lesion formation was developed by blocking blood flow by ligating the common carotid artery just proximal to the bifurcation (carotid artery ligation [CAL]), creating a low-shear stress and turbulent blood flow environment.[3] This procedure results in highly reproducible neointimal formation and concentric arterial remodeling resembling the features of restenosis in human chronic total vascular occlusion.[4,5] Thus, the CAL model has been frequently used to study the mechanisms involved in injury-induced vascular remodeling.

Vascular inflammation plays a central role in the development of atherosclerosis and restenosis.[6,7] In this process, leukocyte adhesion to the endothelium and infiltration into the arterial wall is an early event mediated by adhesion molecules and chemokines, such as vascular cell adhesion molecule-1 (VCAM-1)[8] and monocyte chemoattractant protein-1 (MCP-1).[9] The initial interaction between leukocytes and endothelial cells subsequently enables the activation of more endothelial cells and attraction of more leukocytes to secrete large amounts of cytokines and growth factors, which can further result in smooth muscle cell (SMC) migration and proliferation, and ultimately neointimal formation, thus reducing vascular wall compliance and flow restriction.[6,7]

Endothelium-derived nitric oxide (NO) is an important modulator of vascular homeostasis, which is mainly synthesized by endothelial NO synthase (eNOS).[10,11] It has been shown that eNOS deficiency exacerbates both atherosclerosis[12,13] and CAL-induced vascular lesions.[14] However, the report of eNOS deficiency in a CAL model[14] did not elucidate the role of inflammation on vascular remodeling. By using eNOS transgenic mice, Kawashima and colleagues reported that eNOS overexpression inhibited leukocyte infiltration, neointimal formation, and expression of adhesion molecules induced by CAL, indicating that anti-inflammation was an important mechanism underlying the vasculo-protection.[15]

However, there is no study that tests the hypothesis that eNOS deficiency can enhance vascular inflammation, which plays a critical role in the exacerbation of CAL-induced vascular remodeling.

Table 1. Body Weight, Arterial Systolic Blood Pressure, and Heart Rate in Endothelial Nitric Oxide Synthase Knockout (eNOS-KO) and Wildtype (WT) Mice Treated With or Without NG-nitro-L-arginine methyl ester (L-NAME)

	WT	eNOS-KO	L-NAME
Body weight (g)	34.9 ± 0.7	33.7 ± 1.2	32.1 ± 0.4
Systolic blood pressure (mm Hg)	124 ± 2	144 ± 3*	140 ± 2*
Heart rate (beats/minute)	699 ± 12	587 ± 14*	596 ± 10*

Values are means ± SEM
*P<0.01, significantly different from WT. There was no significant difference between the eNOS-KO and L-NAME groups.

Furthermore, the role of chemokines, such as MCP-1, is still not elucidated in the CAL model, which is an important mediator of macrophage accumulation and lesion formation.[16] Therefore, the present study used both eNOS deficient mice and a pharmacological blockade of nitric oxide synthase (NOS) by N^G-nitro-L-arginine methyl ester (L-NAME) to examine the temporal relationship between the inflammatory process and SMC proliferation induced by CAL and how elimination of NO production affects this. VCAM-1 and MCP-1 were selected as representative inflammatory mediators.

METHODS

Animal Preparation

All animal protocols were approved by the Institutional Animal Care and Use Committees at the University of California at Davis and Berlex Biosciences. Six-month-old male C57BL/6J wildtype (WT) control and eNOS knockout (eNOS-KO) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The eNOS-KO mice were generated with a C57BL/6J background strain. One week before CAL, some WT mice were provided water containing 0.5 mg/mL L-NAME to chronically inhibit NO synthase. Mice were anesthetized with inhaled 2% isoflurane. A midline neck incision was made and the left common carotid artery was dissected carefully under a dissecting microscope. The left common carotid artery was ligated with a 6-0 silk ligature just proximal to its bifurcation. The mice were allowed to recover and were maintained on standard chow diet for 1 or 4 weeks. Arterial systolic blood pressure and heart rate were measured noninvasively in conscious animals by the tail-cuff method as described by Wang et al.[17] One month after CAL, mice were killed and bilateral common carotid arteries were harvested for the following experiments.

Morphometric Analysis

For morphometric studies, mice were perfusion-fixed at a constant pressure (100 mm Hg) via cardiac puncture with 10% phosphate-buffered formalin for 10 minutes. The whole left and right carotid arteries (approximately 9 mm long) were excised and placed in 10% formalin for 24 hours to complete fixation. Specimens were embedded in paraffin. Cross sections at 2.5, 4.5, and 6.5 mm from ligation were cut, and stained with hematoxylin & eosin (H&E). Images of these sections were obtained with a digital camera (JVC TK-C1380, Japan) and morphometrically analyzed in a blind fashion by using the computer assisted stereological toolbox (C.A.S.T.) Grid system (Olympus Danmark, Denmark). A total of 3 sections for each carotid artery were measured, and the data were averaged. The circumferences of the lumen, internal elastic lamina (IEL),

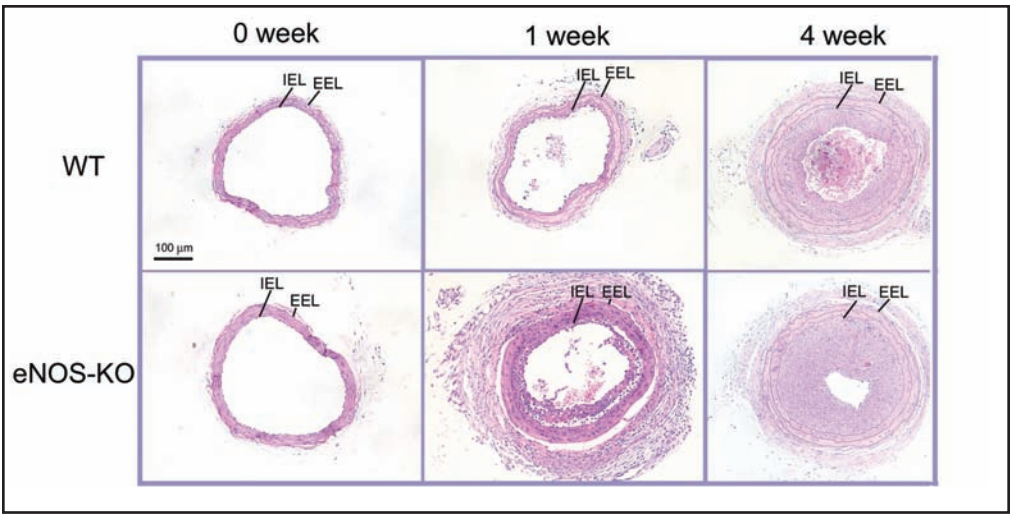

Figure 1. Representative hematoxylin and eosin-stained (H&E) sections of the control nonligated (0-week), 1-week, and 4-week ligated carotid arteries from wildtype (WT) and endothelial nitric oxide synthase knockout (eNOS-KO) mice. IEL, internal elastic lamina; EEL, external elastic lamina. Original magnifications X 200.

external elastic lamina (EEL), and the outer edge between tightly packed and surrounding loose tissue of the carotid artery were measured by tracing the contours on digitized images. Under the assumption that the structures were circular, the radius of the individual layer of the vessel was calculated from the circumference as the circumference divided by 2π. Intimal thickness (distance between lumen and IEL) was calculated by subtracting the radius defined by the lumen from the radius defined by the IEL. Similarly, medial (distance between IEL and EEL), and adventitial thicknesses (distance between EEL and the outer edge between tightly packed and surrounding loose tissue) were calculated.

The extent of inflammatory responses was semi-quantitatively evaluated by scoring as follows: grade 0, no inflammation in arterial wall; grade 1, small number of inflammatory cells only attaching to endothelium but without infiltrating into vascular wall; grade 2, moderate number of individual inflammatory cells infiltrating into the intima, media, and adventitia; grade 3, large aggregates of inflammatory cells infiltrating into the intima, media, and adventitia.

Immunohistochemistry

Immunostaining of paraffin sections for Mac-3 was performed to confirm the presence of macrophages in the inflammatory infiltrate. Paraffin sections (5 µm) were deparaffinized and rehydrated. Antigen retrieval was performed by boiling the sections in 0.01 M citrate buffer (pH 6.0) 4 times for 5 minutes at 700 watts in a microwave, followed immediately by a 20-minute cool-down period. The sections were then treated with 0.3% H_2O_2 in methanol for 30 minutes to abolish endogenous peroxidase activity and with 2% rabbit serum for 30 minutes to block nonspecific antibody binding. Subsequently, sections were incubated overnight at 4˚C with a rat anti-mouse Mac-3 monoclonal primary antibody (1:25 dilution, 550292, BD Bioscience Pharmingen, San Diego, CA). For negative controls, the primary antibody was replaced with nonimmune rat serum (Sigma Chemical, St. Louis, MO). On the second day, after several

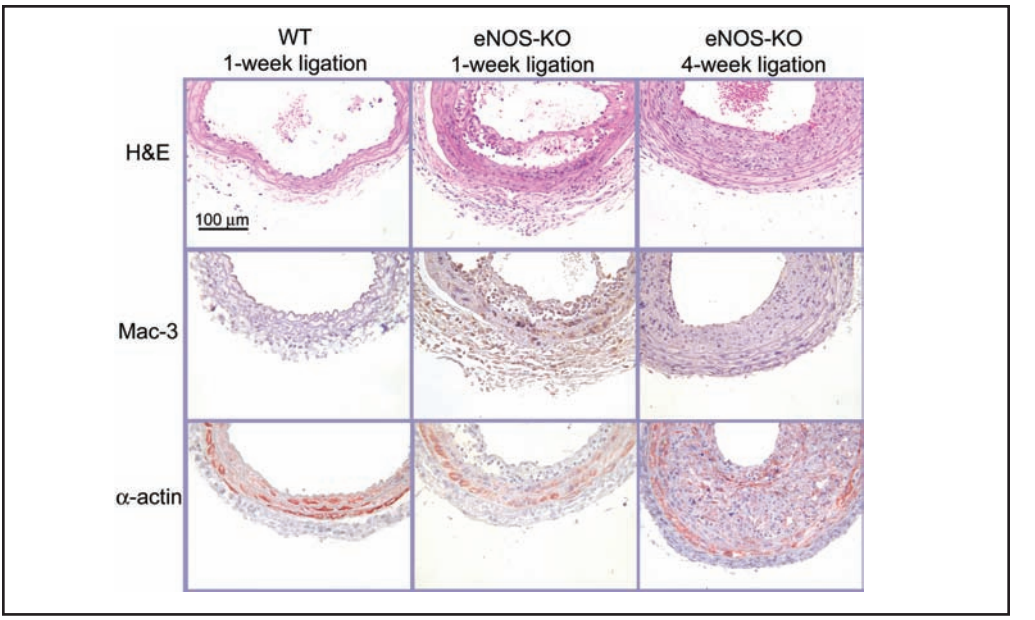

Figure 2. Representative hematoxylin and eosin (H&E), Mac-3, and α-actin stained sections of the 1-week ligated carotid artery from wildtype (WT) and the 1-week and 4-week ligated carotid arteries from endothelial nitric oxide synthase knockout (eNOS-KO) mice. Original magnifications X 400.

washes with phosphate buffered saline, the sections were incubated with biotinylated rabbit anti-rat secondary antibody and avidin-biotin horseradish peroxidase (Vector Elite Avidin Biotin Complex system, Vector Laboratories, Burlingame, CA) for 30 minutes, respectively, visualized with 3,3'-diaminobenzidine (DAB kit, Vector Laboratories) followed by counterstaining with 10% Mayer's hematoxylin, and finally, mounted in Permamount (Vector Laboratories, Burlingame, CA), and examined by light microscopy.

To identify SMCs, a commercially available immunohistochemistry kit for α-actin (Sigma Chemical) was used similarly to the aforementioned procedure with minimal modification according to the manufacturer's instructions.

Ex Vivo Carotid Artery Culture and Quantification of Soluble VCAM-1 and MCP-1

Left and right carotid arteries were dissected aseptically and immediately place in 300 µL Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Gaithersburg, MD), then incubated for 24 hours at 37°C, 5% CO_2 atmosphere. Soluble VCAM-1 and MCP-1 levels in tissue culture supernatants were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN) and assays were performed according to the manufacturer's instructions. Both VCAM-1 and MCP-1 values were normalized to the weight of the carotid artery segment and expressed as ng/mL per mg tissue.

Statistics

All results are presented as the mean ± standard error of the means with the number of animals indicated as N. An unpaired Student's *t*-test or Mann-Whitney *U* test was used to detect significant differences when 2 groups were compared. Multiple comparisons of

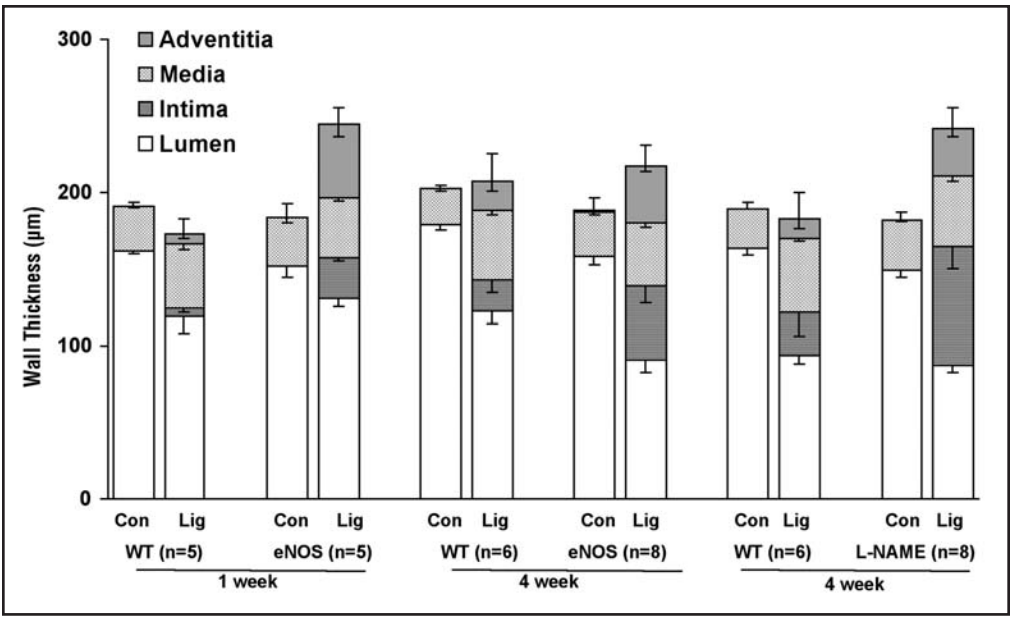

Figure 3. Quantitative analysis of vascular remodeling in response to carotid artery ligation in wildtype (WT), endothelial nitric oxide synthase (eNOS) deficient, and N^G-nitro-L-arginine methyl ester (L-NAME)–treated WT mice. Con = control; Lig = ligated

mean values were performed by analysis of variance (ANOVA) followed by a subsequent Student-Newman-Keuls test for repeated measures. Differences were considered statistically significant when $P<0.05$.

RESULTS

Arterial systolic blood pressure was significantly higher, whereas heart rate was lower, in eNOS-KO mice compared with WT controls (Table 1). Long-term treatment with L-NAME in WT mice resulted in similar changes to those observed in eNOS-KO mice.

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

In both WT and eNOS-KO mice, the nonligated carotid arteries (Time 0) appeared normal with the intima limited to endothelial cells overlying the IEL, a modest, concentrically oriented media, and a sparse, primarily acellular adventitia (Figures 1 and 2). At 1 week after ligation, there was endothelial cell hypertrophy and adherence of neutrophils and monocytes to the luminal endothelial surface. Individual neutrophils were present between the endothelium and the IEL. The media appeared moderately thickened, but not more than could be expected subsequent to the slightly reduced lumen diameter. The adventitia was markedly cellular and expanded by increased amounts of extracellular matrix. Adventitial cellularity consisted primarily of mononuclear cells with elongate nuclei concentrically arranged around the media and embedded in the fibrillary matrix. Mac-3 staining showed no positive staining cells in the intima and few positive cells in adventitia at 4 weeks after ligation.

The most prominent change was organization and thickening of the intima by smooth muscle actin positive cells forming a circumferential sheet of irregularly oriented cellular bundles. The media at 4 weeks was not markedly dif-

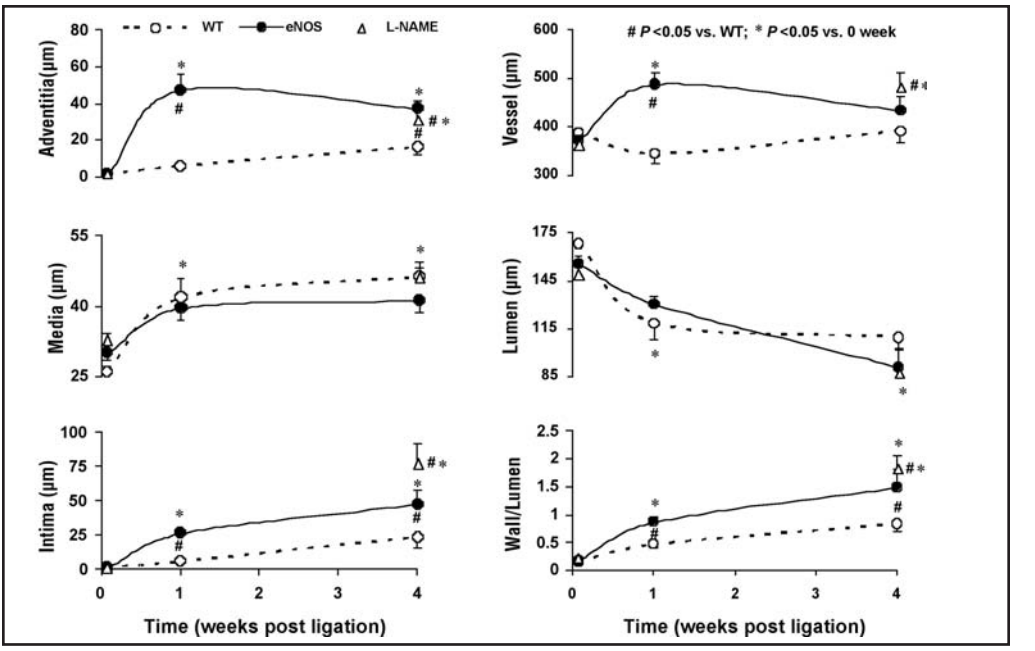

Figure 4. Morphometric changes of each individual component of the carotid arteries from wildtype (WT), endothelial nitric oxide synthase (eNOS) deficient, and N^G-nitro-L-arginine methyl ester (L-NAME)–treated WT mice in response to carotid artery ligation.

ferent from the 1-week specimens. The adventitia remained thickened but had more circumferentially oriented fibrillar extracellular matrix investing fewer cells with smaller, lighter staining nuclei than those from the 1-week specimens. Changes in both eNOS-KO– and L-NAME–treated mice were more marked. At 1 week, most sections had partial to circumferential separation of the endothelium from the IEL. The endothelium was reactive, characterized by enlarged rounded nuclei and dark staining cytoplasm. Underlying the endothelium in many regions was a layer of randomly oriented Mac-3 positive mononuclear cells with indistinct cytoplasm. The subendothelial space contained inflammatory infiltrates, predominantly neutrophils that were often multilayered and present in loose aggregates. Many subendothelial spaces also contained red blood cells and fibrin. Infiltration of the media by neutrophils was common, especially in the inner layer and was often associated with necrotic SMCs containing granular cytoplasm and fragmented nuclei. Adventitial thickening was also more prominent and highly cellular in eNOS-KO mice compared with WT controls. The principal difference in eNOS-KO– and L-NAME–treated mice from WT controls at 4 weeks was a marked increase in intimal thickening by α-actin positive cells. Intimal remodeling was not only more extensive, but α-actin positive cells were also more haphazardly arranged in the ligated carotid arteries from eNOS-KO mice than those from WT controls.

Figure 3 demonstrates morphological changes in both the ligated and nonligated (control) carotid arteries. Figure 4 is the comparison of the changes of each individual component of the carotid arteries between WT and eNOS-KO mice over the time course after CAL. CAL gradually increased all 3 layers of the vessel wall, including intima, media, and adventitia, over time. In WT mice, the increase in the wall thickness

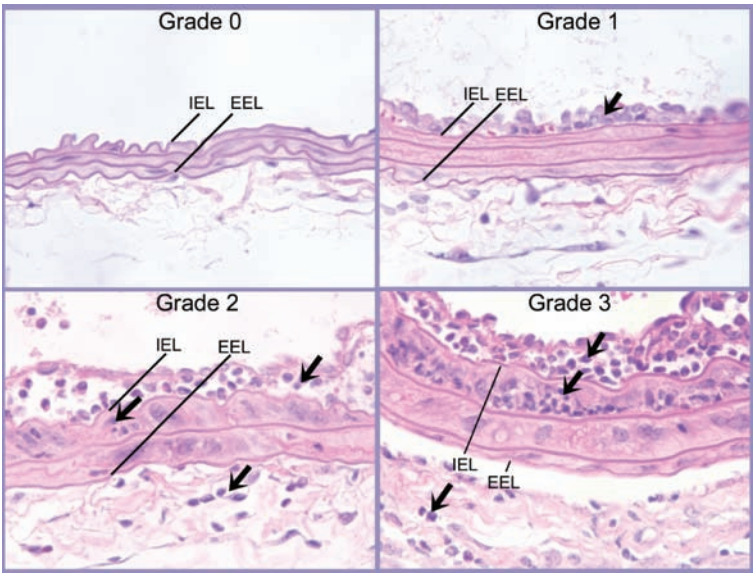

Figure 5. Representative hematoxylin and eosin-stained (H&E) sections for inflammatory response scoring. IEL, internal elastic lamina; EEL, external elastic lamina. Arrows indicate inflammatory cells. Original magnification X 400.

was mainly concentric because the lumen narrowed without significant vessel expansion. Thus, the ratio of wall to lumen significantly increased. The previously mentioned CAL-induced changes were dramatically enhanced in eNOS-KO mice. Specifically, CAL induced a significantly greater adventitial proliferation and neointimal formation in eNOS-KO than in WT mice. However, the time course of the 2 changes was different. Adventitial proliferation peaked at 1 week and slightly declined at 4 weeks, while neointimal formation further increased at 4 weeks after CAL. There was no significant difference in medial thickening and luminal narrowing between WT and eNOS-KO mice. In contrast to WT mice, CAL-induced remodeling in eNOS-KO mice was both expansive and concentric, ie, the total vessel size increased and lumen narrowed, thus, the ratio of wall to lumen gradually increased over time and was greater than that in WT mice.

Inhibition of NO production by blockade of NOS with L-NAME resulted in similar changes on vessel remodeling as observed in eNOS-KO mice after CAL at 4 weeks. The treatment with L-NAME greatly enhanced adventitial proliferation, neointimal formation, vessel expansion, and increment of the wall over lumen ratio lumen without significantly affecting the magnitude of medial thickening and luminal narrowing compared with those in the control WT mice.

eNOS Deficiency Enhanced CAL-Induced Inflammatory Responses: Monocyte/Macrophage Infiltration and SMC Migration

CAL induced massive inflammatory cell recruitment in the vessel wall in eNOS-KO mice, although the response in WT mice was very mild (Figures 1 and 2). To further evaluate the time course and extent of the inflammatory responses, a semi-quantitative scoring method was

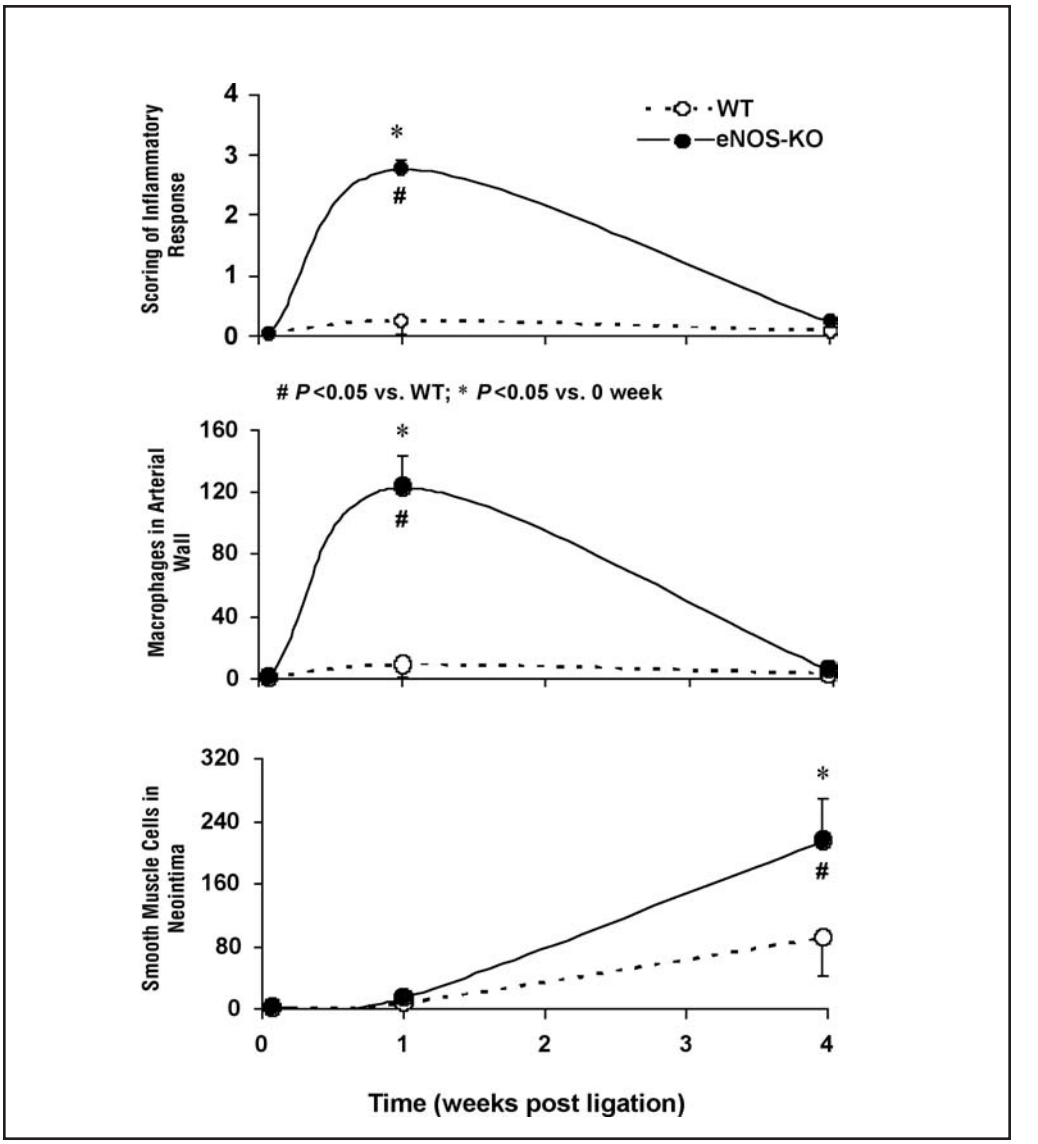

Figure 6. Time course of inflammatory scoring and quantitative analysis of macrophage infiltration in vascular wall and small muscle cell proliferation in the neointima after carotid artery ligation. WT, wildtype, eNOS-KO, endothelial nitric oxide synthase knockout.

used (Figure 5). CAL resulted in a significantly stronger inflammatory cell infiltration in the carotid arteries from eNOS-KO than WT mice at 1 week (Figure 6, top). The inflammatory response was minimal at 4 weeks. In addition to large numbers of neutrophils, immunohistochemical staining showed that a large number of infiltrated inflammatory cells were Mac-3 positive macrophages (Figure 2, middle) in the 1-week ligated carotid artery from eNOS-KO mice. Again, the number of macrophages in the ligated carotid artery were significantly increased only in eNOS-KO mice at 1 week and returned to basal level at 4 weeks after CAL (Figure 6, middle). CAL also resulted in α-actin positive SMC migration into neointima at 4 weeks (Figure 2, bottom).

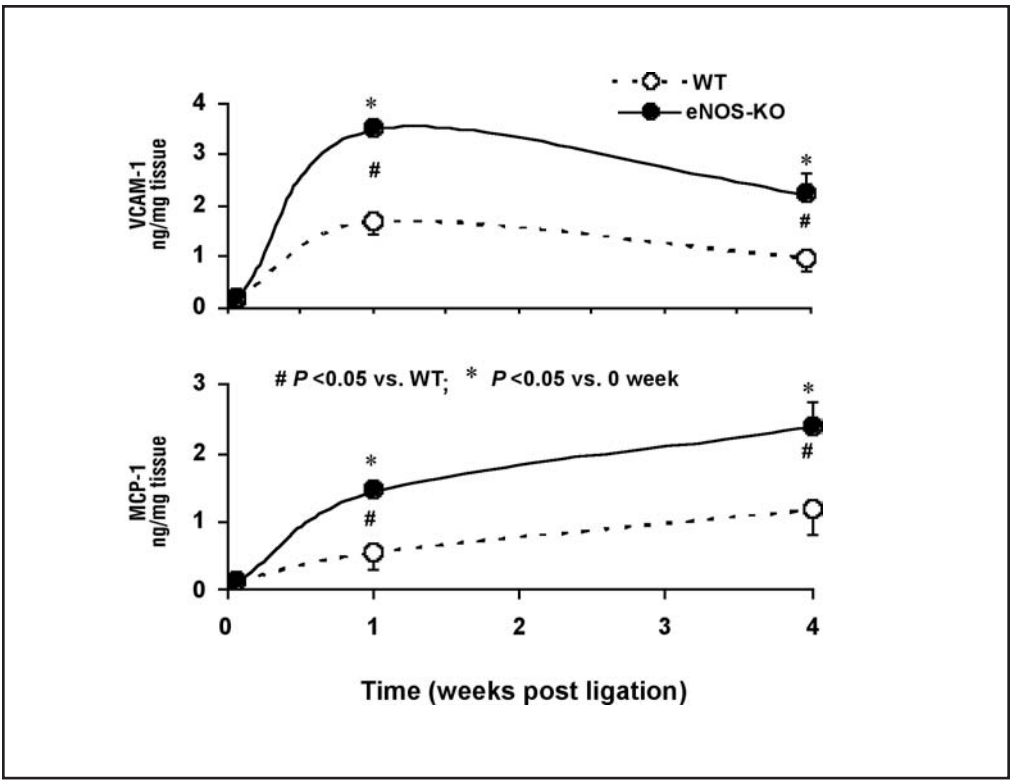

Figure 7. Ex vivo vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) secretion by the nonligated (Time 0) and ligated carotid arteries isolated from wildtype (WT) and endothelial NO synthase knockout (eNOS-KO) mice.

This response was significantly increased in eNOS-KO, compared with WT mice at 4 weeks after CAL (Figure 6, bottom).

eNOS Deficiency Increased CAL-Induced Production of Endothelial Cell Adhesion Molecules and Chemokines

To determine the expression of VCAM-1 and MCP-1, production of these 2 proteins was measured by ELISA after 24-hour incubation of the freshly isolated carotid arteries. The production of VCAM-1 and MCP-1 significantly increased in the ligated carotid arteries (Figure 7). VCAM-1 peaked at 1 week, then slightly declined at 4 weeks, whereas MCP-1 continued to increase at 4 weeks after CAL. Compared with WT mice, CAL-induced increase in VCAM-1 and MCP-1 was significantly higher in eNOS-KO mice (Figure 7).

DISCUSSION

The present study showed that CAL-induced vascular remodeling was greatly enhanced in both eNOS-KO mice and in WT mice treated with L-NAME compared with WT controls. Significant changes included adventitial proliferation, neointimal formation, and vessel expansion, but with no significant effects on medial thickening and luminal narrowing. The arterial remodeling was accompanied by a significant increase in inflammatory cell infiltrate, including neutrophil and macrophage localization in the artery wall and SMC proliferation in the neointima, as well as elevated production of pro-inflammatory mediators, VCAM-1 and MCP-1.

The principal pathological changes in restenosis are inflammatory cell infiltration, neointimal formation, and SMC

proliferation and migration into the neointima, resulting in lumen narrowing.[4,5] Although the interruption of blood flow is an initial stimulus for the vascular remodeling in this model, and thus differs from the vascular injury resulting from restenosis after angioplasty in humans, the resulting inflammatory response and vascular remodeling are strikingly similar. The pathological changes observed in the present study are also consistent with previous studies[3,18,19] except that the extent of the remodeling was very mild in WT mice in the present study. This discrepancy could be attributed to the older mice used in the study (6 months old) compared with those used by other groups (2-3 months old).[3,14,15] It has been reported that vascular remodeling in response to changes in blood flow is highly dependent on age, ie, older animals exhibit less remodeling.[20,21] Neutrophil infiltration into the artery wall, in addition to macrophage infiltration, at 1 week after CAL was a novel finding in this study. Although neutrophils have long been observed in the experimental models of arterial injury, their pathological role in vascular diseases is not well defined.[22-26] Their effect on stimulating SMC proliferation might play an important role in SMC-rich neointimal formation in this CAL model.[27]

eNOS serves important regulatory functions in the cardiovascular system. In response to stimuli such as shear stress or acetylcholine, eNOS catalyzes the production of NO from L-arginine. NO diffuses across the endothelial cell membrane into neighboring SMCs and induces vasodilation.[10,11] Numerous data suggest that eNOS derived NO is an important endogenous anti-atherosclerotic factor in addition to a potent vasodilator. For example, oral administration of L-arginine, the substrate of NOS, inhibited atherosclerosis in rabbits[28] and mice,[29] while blockade of NO production with NOS inhibitors promoted atherosclerosis.[30] Furthermore, atherosclerosis in apolipoprotein E-deficient (apoE-KO) mice was increased when eNOS was also deficient,[12,13] and was reduced when eNOS was overexpressed.[31] Regarding the role of eNOS/NO in the vascular remodeling after vascular injury, it has been reported that neointimal formation induced by cuff placement around the femoral artery[32] or by CAL[14] was exacerbated in eNOS-KO mice, and attenuated in eNOS-transgenic mice.[15] The present data are consistent with these findings, demonstrating that the eNOS/NO system plays a vasculo-protective role against injury-induced abnormal vessel remodeling.

Chronic vascular wall inflammation has been proposed to play an important role in pathogenesis of both atherosclerosis and restenosis.[6,7] Although the exact mechanism by which eNOS/NO plays anti-atherogenetic and vasculoprotective roles is not clearly understood, its anti-inflammatory actions might be an important mechanism. This hypothesis was supported mainly by in vitro studies. For example, NO has been reported to attenuate the expression of proinflammatory mediators such as VCAM-1 and MCP-1,[33,34] inhibit monocyte adhesion to the endothelium,[33] limit SMC proliferation and migration[35,36] and platelet aggregation,[37] and induce macrophage and SMC apoptosis.[38] These reports suggest that the eNOS/NO system plays a beneficial role in multiple pathways causing atherogenesis and abnormal vascular remodeling. The present study shows that eNOS deficiency exacerbated CAL-induced initiation of inflammatory cell filtration into the vascular wall, increased the expression of proinflammatory mediators, such as VCAM-1 and MCP-1, and exacerbated vascular remodeling. These results are consistent with a previous study that

eNOS overexpression in the endothelium reduced expression of adhesion molecules and leukocyte infiltration, and inhibited CAL-induced vascular remodeling.[15] These findings provide direct in vivo evidence that the vasculo-protective effects of eNOS/NO can be mediated by inhibiting vascular injury-induced initiation of vascular inflammation. The results of this study further indicate that the underlying molecular mechanism could be attributed to inhibiting the expression of VCAM-1 and MCP-1.

Another novel observation in the present study is that the time courses of the expression of VCAM-1 and MCP-1 were different, which may indicate different roles of these 2 proinflammatory molecules in the process of CAL-induced neointimal formation. The results of this study showed that VCAM-1 levels peaked at 1 week, whereas MCP-1 continued to increase at 4 weeks after CAL (Figure 7). It is possible that CAL first activates endothelial cells to express VCAM-1, which allows leukocytes to attach to endothelial cells, then penetrate into the arterial wall under the attraction of MCP-1. VCAM-1 is usually generated in activated endothelial cells, while MCP-1 is widely expressed by endothelial cells, macrophages, and SMC.[39] The data from the present study showed morphologic evidence that endothelial cell activation and vascular inflammation was prominent at 1 week and diminished at 4 weeks after CAL, which was coincident with the changes of VCAM-1, while SMCs were predominant in the neointima at 4 weeks when MCP-1 was at the highest level. Thus, the expression of VCAM-1 appears to reflect vascular inflammation, while a key source of MCP-1 may be from SMCs in the neointima.

Sustained expression of MCP-1 may be more important than VCAM-1 in the process of neointimal hyperplasia featured by predominant SMC in the CAL mouse model since MCP-1 may both trigger the initial influx of leukocytes into the vessel wall and help to perpetuate the development of the intimal lesion by stimulating the proliferation and migration of SMC in intima.[9,40] Thus, inhibiting MCP-1–induced leukocyte infiltration and SMC proliferation and migration might be an important molecular mechanism by which eNOS/NO attenuates CAL-induced neointimal lesions. Because stimulation of SMC proliferation may play an important role in SMC-rich neointimal formation in the CAL model, the results of the present study suggest that inhibition of MCP-1 could be a promising therapeutic strategy for restenosis after percutaneous transluminal angioplasty.

Furthermore, prominent fibrin deposition in subendothelial space in the carotid arteries from eNOS-KO mice at 1 week after ligation was observed. Fibrin deposition was the result of activation of the coagulation system. It could play an important role in neointimal formation by several mechanisms: serving as a provisional matrix that can be invaded by SMC, directly stimulating SMC proliferation, and finally, indirectly stimulating SMC proliferation by binding and enhancing the function of vascular SMC growth factors.[41]

In the present study, pharmacologically inhibiting NO production by blockade of NOS with L-NAME in WT mice resulted in a similar enhancement of vascular remodeling as in eNOS-KO mice at 4 weeks after CAL, ie, neointimal formation, medial thickening, and adventitial proliferation. This result reinforces the concept that NO, especially eNOS derived NO, is vasculo-protective in development of restenosis.

In summary, the present study showed that eNOS deficiency or long-term pharmacological inhibition of NOS with L-NAME exacerbated CAL-

induced vascular remodeling, which was associated with aggravated vascular inflammation. Increased VCAM-1 may be associated with initiation of vascular inflammation, although increased MCP-1 may be an important molecular mechanism of SMC-rich neointimal formation in the CAL model. These data provided further in vivo evidence supporting the hypothesis that vasculo-protective effects of eNOS are mediated, at least in part, by inhibition of vascular inflammation and SMC migration and proliferation induced by vascular injury and blood flow cessation.

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