Direct Evidence for the Importance of Endothelium-derived Nitric Oxide in Vascular Remodeling

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Abstract

The vascular endothelium mediates the ability of blood vessels to alter their architecture in response to hemodynamic changes; however, the specific endothelial-derived factors that are responsible for vascular remodeling are poorly understood. Here we show that endothelial-derived nitric oxide (NO) is a major endothelial-derived mediator controlling vascular remodeling. In response to external carotid artery ligation, mice with targeted disruption of the endothelial nitric oxide synthase gene (eNOS) did not remodel their ipsilateral common carotid arteries whereas wild-type mice did. Rather, the eNOS mutant mice displayed a paradoxical increase in wall thickness accompanied by a hyperplastic response of the arterial wall. These findings demonstrate a critical role for endogenous NO as a negative regulator of vascular smooth muscle proliferation in response to a remodeling stimulus. Furthermore, our data suggests that a primary defect in the NOS/NO pathway can promote abnormal remodeling and may facilitate pathological changes in vessel wall morphology associated with complex diseases such as hypertension and atherosclerosis. (*J. Clin. Invest.* 1998. 101:731–736.) Key words: nitric oxide • eNOS knockout mice • proliferation • hyperplasty • carotid artery • atherosclerosis

Introduction

Chronic changes in blood flow cause alterations in blood vessel architecture to normalize wall shear stress. In normal conduit vessels, increases in blood flow are associated with an increase in lumen diameter, whereas decreases in flow are associated with a decrease in lumen diameter. The vascular endothelium, ideally situated at the interface between blood and underlying smooth muscle, senses the mechanical forces elicited by the pressure and flow of blood and coordinates these signals into biochemical events that regulate vascular tone and structure (1–5). This concept is supported by the pivotal observation that the decrease in vascular diameter normally accompanying a chronic flow reduction is dependent upon the vascular endothelium (6).

The mechanisms by which the endothelium controls vascular remodeling are not known, although several vasoactive molecules and growth factors have been implicated. Nitric oxide (NO)[1] produced by the endothelium has many of the attributes necessary to suggest its role as a mediator of vessel remodeling. Mechanical forces elicited by the flow of blood (shear stress) and pressure (cyclic strain) cause the acute release of NO, and after prolonged activation, induce endothelial nitric oxide synthase (eNOS) gene expression both in vitro and in vivo (7–11). NO is a potent vasodilator (12, 13) that inhibits both growth factor stimulated proliferation and migration of cultured vascular smooth muscle cells (14–16), neointima formation after vessel injury (17), endothelial cell locomotion and assembly into neovessels (18–20), and extracellular matrix turnover (21, 22). Collectively, these events are requisite for remodeling of the blood vessel wall. A recent study, in a high flow model of vessel remodeling demonstrated that treatment of rabbits with N^G-nitro-L-arginine methyl ester (L-NAME; a NOS inhibitor) reduced the flow-dependent increase in vessel diameter as well as basal remodeling of the nonsurgically manipulated vessel pointing toward a role for NO in remodeling (23). However, direct evidence for the role of endogenous NO or any other endothelial-derived mediators in vessel remodeling is lacking.

The purpose of this study was to develop a model of vascular remodeling in mice and to examine if eNOS-derived NO is important for remodeling using eNOS knockout mice. Here, we show that the typical decrease in lumen diameter seen in the common carotid artery after ligation of the ipsilateral external carotid artery is absent in eNOS knockout mice. Moreover, eNOS knockout mice exhibit an increase in vessel wall thickness, due to a hyperplastic response, only in the vessel sensing a remodeling stimulus. Thus, eNOS is the first gene directly implicated in normal vascular adaptation.

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1. *Abbreviations used in this paper:* BrdU, 5-Bromo-2′deoxyuridine; eNOS, endothelial nitric oxide synthase; LC, left carotid; L-NAME, N^G-nitro-L-arginine-methyl ester; NO, nitric oxide; RC, right carotid; −/−, eNOS knockout; +/+, wild-type.

Methods

Mice. C57BL/6J × 129/Ola +/− for the eNOS locus were intercrossed to generate litters of all three genotypes (+/+, +/−, −/−) identified by Southern blotting (24). 9–12 mo-old C57BL/6J × 129/Ola F2 generation hybrid littermate mice that were +/+ or −/− for the eNOS locus were used in this study on two separate occasions. eNOS protein was present in the endothelial cells lining the common carotid of +/+ mice and was absent in the −/− animals as previously described (24). Positive von Willebrand factor (vWf) staining (an endothelial marker) was found in the endothelium of both strains. Care of the mice was in accordance with institutional guidelines.

External carotid artery ligation. Mice were anesthetized with an intramuscular injection of ketamine (Ketaset; Fort Dodge Laboratories, Inc., Fort Dodge, IA), and xylazine (Rompun; Bayer Corporation, Shawnee Mission, KS) mix and a neckline incision made. The distal left common carotid artery and its bifurcation into the external and internal carotid were exposed using minimal dissection. 9-0 nylon sutures (USSC Sutures, Norwalk, CT) were then used to ligate the external carotid at its origin and incisions were closed (7-0 polypropylene). After 2 wk, 5-Bromo-2′deoxyuridine (BrdU) was injected subcutaneously (100 mg/kg) and intraperitoneally (30 mg/kg) at 18 h, and again intraperitoneally (30 mg/kg) 12 h before death.

Tissue harvest and histology. At death, mice were perfused at a constant pressure (60–80 mmHg) via the left ventricle with phosphate buffered saline (PBS, pH 7.4) containing 0.1 mM adenosine (Sigma Chemical Co., St. Louis, MO), 0.3 mM papaverine (Sigma Chemical Co.) and heparin sodium (50 U/ml), pH 7.4, at 37°C, followed by perfusion fixation with freshly depolymerized, 4% paraformaldehyde in PBS (pH 7.4; reference 25). In all experiments, perfusion pressures were monitored via a mercury manometer. In one set of experiments, systemic arterial perfusion pressures were measured and maintained at 80 mmHg via a catheter inserted into the abdominal aorta. After perfusion, both common carotid arteries were carefully excised and postfixed overnight at 4°C. At the midpoint of the vessels, parallel blocks of the left common carotid artery (LC) and right common carotid artery (RC) were excised, embedded in O.C.T. (Tissue-Tek Elkhart, IN), and cross sections (3–5 μm thickness) were obtained for hematoxylin/eosin staining, morphometry and BrdU immunohistochemistry.

Morphometry. Morphometric analysis of carotid arteries was performed using video microscopy at a final magnification of 450 (25). The image was captured and displayed on a computer monitor and vessel perimeter measured using the imageSCION program. Perimeter of the vessel lumen was taken as the circumference (C) of a circle and lumen diameter (D) determined from the equation $D = C/\pi$, assuming that the vessel cross sections were circular in vivo. Eight serial cross sections spanning a total length of 150 μm were measured and averaged from each vessel segment. The coefficient of variation (SEM/mean) between sections was < 2%. Medial or wall thickness was measured as the linear distance from the inner elastic lamina to the external elastic lamina measured at a magnification of 630 (Carl Zeiss, Inc., Thornwood, NY; ×63 objective) using a calibrated eyepiece reticle. In each vessel section, four locations 90° apart were measured in two serial sections and the values were averaged. The coefficient of variation at each point measured was < 10%.

Nuclei number. Frozen sections from RC and LC of mice +/+ and −/− eNOS genotypes were stained with 10% Harris' hematoxylin. In four to six serial cross sections spanning 100 μm of vessel length, hematoxylin positive nuclei including endothelial and smooth muscle cells, were counted under oil immersion and then averaged.

Immunohistochemistry for proliferating cells. BrdU immunohistochemistry was performed using a mouse anti-BrdU antibody (Becton Dickinson) essentially as described. In brief, O.C.T embedded sections were immersed in 70% ethanol for 30 min, washed in distilled water for 5 min, and then endogenous peroxidases were blocked with 0.3% hydrogen peroxide in methanol for 30 min. Slides were washed again in distilled water and then boiled for 5 min by microwave in 0.01 M citrate buffer (pH 6) two times with an intermediate cool-down period. Nonspecific sites were blocked with horse serum for 30 min, followed by an overnight incubation at 4°C with mouse anti-BrdU. The next day slides were washed in PBS and then incubated at room temperature with secondary antibody. Slides were washed, incubated with the ABC complex for 1 h at room temperature. Slides were developed using diaminobenzidine and counterstained with 10% Harris' hematoxylin. Immunoperoxidase staining was performed using commercially available reagents (Vector Elite Avidin Biotin Complex kit [ABC]; Vector Laboratories, Inc., Burlingame, CA). In four to six serial cross sections spanning 100 μm of vessel length, BrdU and hematoxylin positive cells were counted under oil immersion and then averaged. BrdU labeling index was the ratio of BrdU positive cells to total nuclei multiplied by 100.

Calculations and statistics. All values are expressed as mean± SEM. Comparisons of left versus right common carotid diameter, wall thickness, BrdU positive cells, and total cells were made by paired Student's *t* test in mice of the same genotype. Comparisons between wild-type and knockout mice were made by unpaired Student's *t* test. For all statistical analysis, a P value < 0.05 was considered significant.

Results

To take advantage of genetic models that manipulate genes relevant for vascular disease in mice, we applied a model of vascular remodeling previously used in rats and rabbits to mice. In rats and rabbits, ligation of the left external carotid artery has been shown to increase upstream resistance in the vascular bed supplied by the left internal carotid artery thereby reducing flow and lumen diameter in the LC by 10–25% (3, 6, 26). Flow and diameter remain unchanged in the contralateral RC. As seen in Fig 1 *A*, ligation of left external carotid artery for 2 wk in mice homozygous for the eNOS locus (+/+) resulted in a significant reduction in lumen diameter of the LC compared with the RC ($n = 6$ mice from two separate experiments, $P < 0.05$), qualitatively similar to remodeling seen in the same model in rats and rabbits. In contrast, mice with homozygous disruption of the eNOS locus (−/−) did not exhibit lumenal remodeling of the LC ($n = 7$ mice from two separate experiments). The magnitude of the lumen diameter reduction of the LC (normalized to the contralateral RC as an internal control) in +/+ mice was significantly greater than that seen in −/− mice (10.0±2.9 and 1.3±1.8%, respectively; $P < 0.05$). There were no differences between LC and RC in age-matched, sham operated C57BL/6J mice (placement and removal of ligature on the external carotid artery, $n = 3$, data not shown). In addition, there was a nonstatistically significant trend towards a smaller RC in −/− mice compared with their +/+ littermates ($P = 0.34$). Thus, abnormalities in carotid arterial diameters were found in −/− mice only after a remodeling stimulus.

Histological examination of common carotids from +/+ mice demonstrated a normal endothelial monolayer enveloping three layers of smooth muscle cells in RC and an anatomically identical, but smaller remodeled vessel in LC (Fig. 1 *B*, *top*). In contrast, −/− mice revealed an increase in LC medial mass relative to the contralateral RC with more rounded nuclei (Fig. 1 *B*, *bottom*). Direct measurements of wall thickness showed a significant increase in LC wall thickness relative to RC of −/− mice with no differences between RC and LC of +/+ mice (Fig. 2 *A*) or between RC of +/+ and −/− mice. Thus, in the absence of NO, vessel wall thickness increased in response to a remodeling stimulus without reducing lumen diameter.

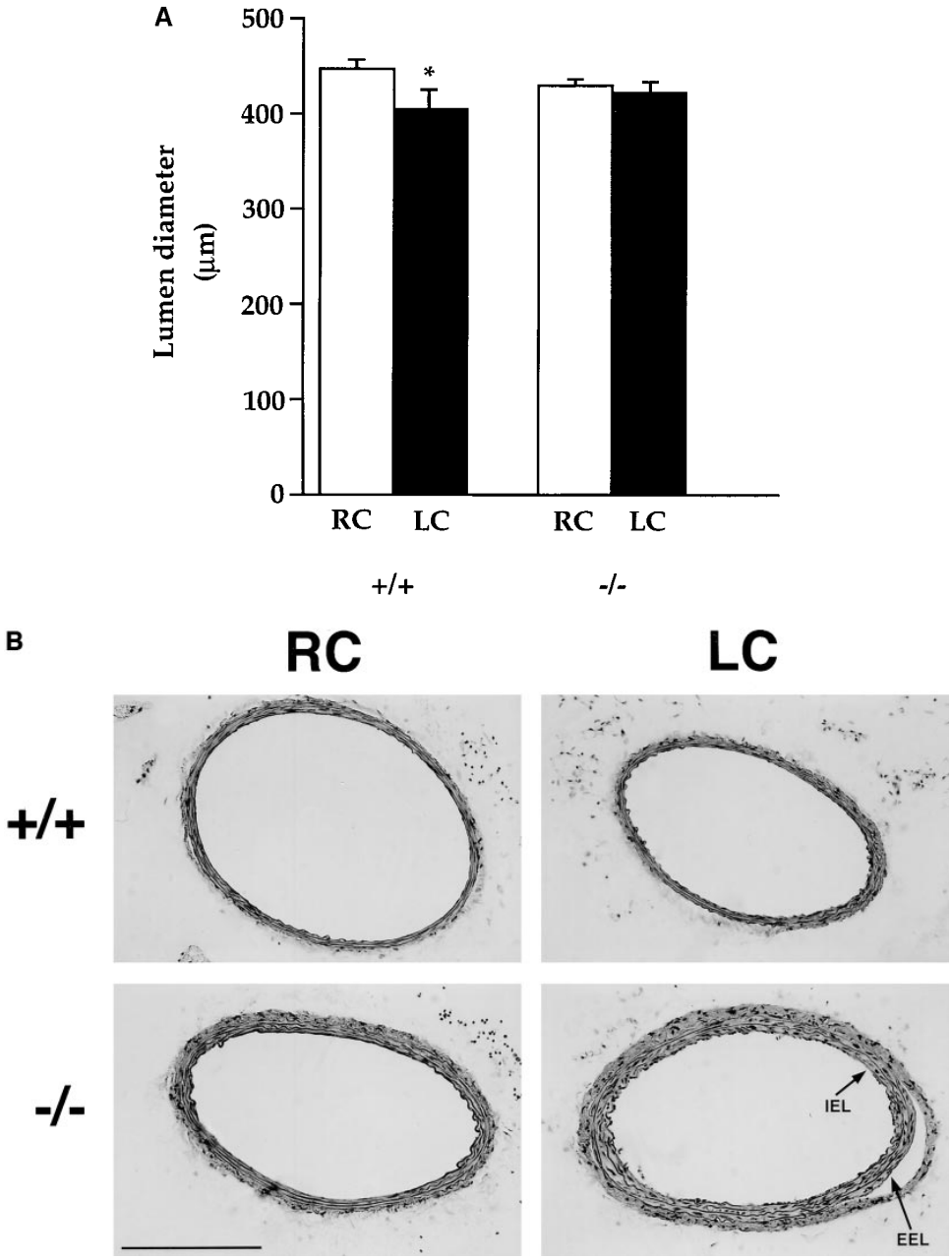

Figure 1. Impaired vascular remodeling in eNOS knockout mice. The left external carotid arteries of F2 generation littermate mice with wild-type (+/+) and homozygous mutant (−/−) eNOS genotypes were ligated for 2 wk after which the common carotids were perfusion fixed and analyzed as described (25). (*A*) Lumen diameter of LC in +/+ mice was reduced in response to a remodeling stimulus but was not in LC of −/− mice. In all mice (from various litters in two separate experiments) the magnitude of the reduction in lumen diameter of LC compared with RC in +/+ mice exceeded that of −/− mice. Values are mean±SEM; $*P < 0.05$ with a two-tailed, paired Student's *t* test; $n = 6$ and 7 animals from for +/+ and −/− mice, respectively. (*B*) Depicts representative hematoxylin and eosin stained cross sections of RC and LC from mice with +/+ and −/− eNOS genotypes. In the top two panels, the LC diameter and media are reduced proportionally compared with the contralateral RC of +/+ mice. In the bottom panel, lumen diameter is not changed in LC compared with RC of −/− mice and a visible increase in medial thickness is seen in LC. The lighter eosin stained layer on the outermost aspect of the vessel is the adventitia. Bar, 200 μm; *IEL*, internal elastic lamina; *EEL*, external elastic lamina.

This finding suggests that NO is required for coupling lumenal hemodynamic changes to underlying architectural reorganization of the vessel wall.

To examine if the increase in vessel wall thickness in −/− mice was due to smooth muscle hypertrophy or hyperplasia, total nuclei and BrdU-labeled nuclei in RC and LC from +/+ and −/− mice were determined. Total vessel wall nuclei were decreased by 18% in LC of +/+ mice relative to RC, consistent with a proportional reduction in lumen diameter and the number of vascular smooth muscle cells without changing wall thickness. In contrast, −/− mice showed a 50% increase in total nuclei in LC relative to RC which is indicative of cellular hyperplasia (Fig. 2 *B*). The morphometric assessments of wall thickness and total nuclei were confirmed by results assessing the incorporation of BrdU into carotid vessels of +/+ and −/− mice. The number of BrdU positive cells between RC and LC of +/+ did not differ whereas the LC of −/− mice had a marked increase in BrdU positive nuclei compared with RC (Fig. 2 *C*). Finally, the BrdU labeling index (percent BrdU labeled cells/total nuclei) was unchanged between RC and LC of +/+ mice (~ 1% for both RC and LC), however this was increased in LC from −/− mice (from 1 to 6% in RC versus LC). Fig. 3 depicts the increase in BrdU incorporation, wall thickness and nuclei found in abnormally remodeled LC from −/− mice compared with LC remodeled LC from +/+ mice. Collectively, these data indicate that the increase in wall thickness in the remodeled artery from mice lacking eNOS is due to a hyperplastic response, since both the total cell number and the number of proliferating cells were increased.

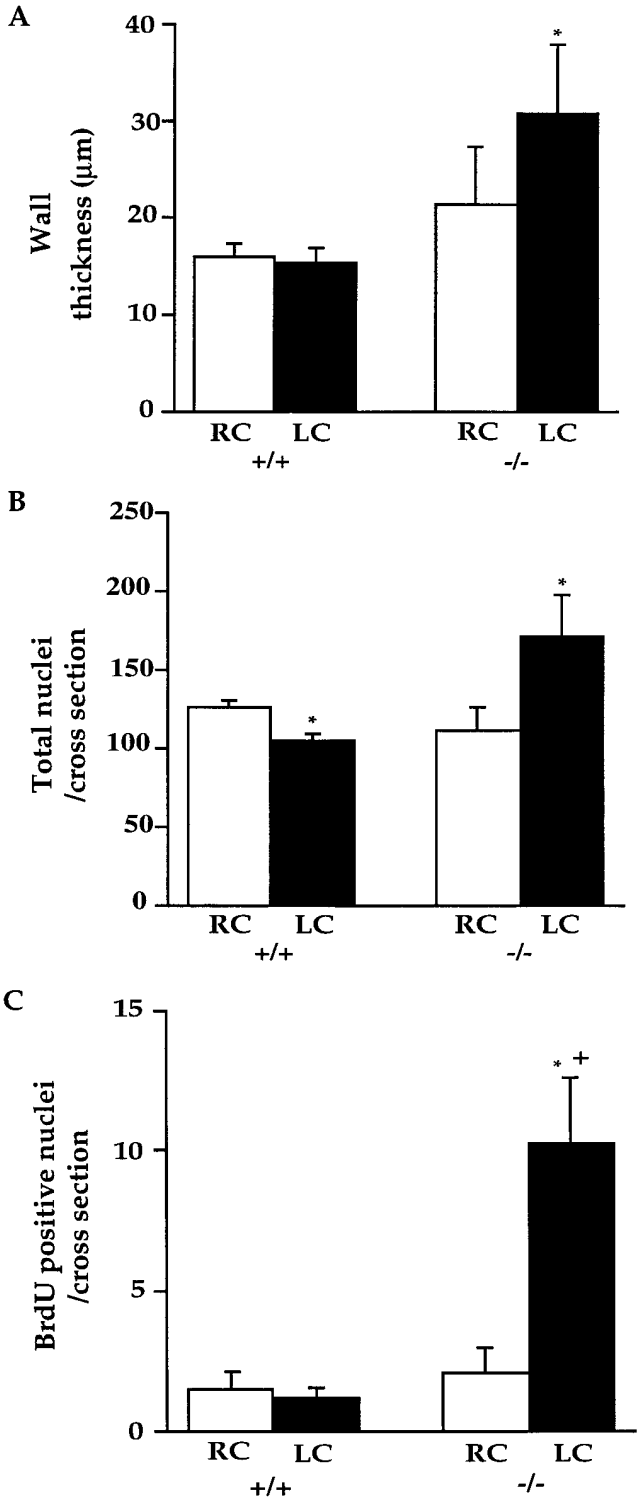

Figure 2. The remodeled carotid artery of eNOS knockout mice exhibits medial hyperplasia. (*A*) Medial wall thickness was directly measured from cross sections of RC and LC as described (17). Medial thickness increased in LC of −/− mice compared with the contralateral RC whereas wall thickness did not change in +/+ mice; changes in the +/+ mice are consistent with proportional remodeling of the lumen and medial smooth muscle. (*B*) Total vessel nuclei were counted from cross sections of RC and LC. Expressed relative to RC, the number of nuclei decreased in the LC of +/+ mice and increased in −/− mice. (*C*) The number of BrdU labeled cells were counted in RC and LC as described (21). BrdU incorporation in medial cells markedly increased in the LC of −/− mice. In all panels, values are mean±SEM; $^{*}P < 0.05$ comparing LC to RC of the given genotype; $^{+}P < 0.05$ compared with carotids and from +/+ mice; $n = 6$ and 7 animals for +/+ and −/− mice, respectively.

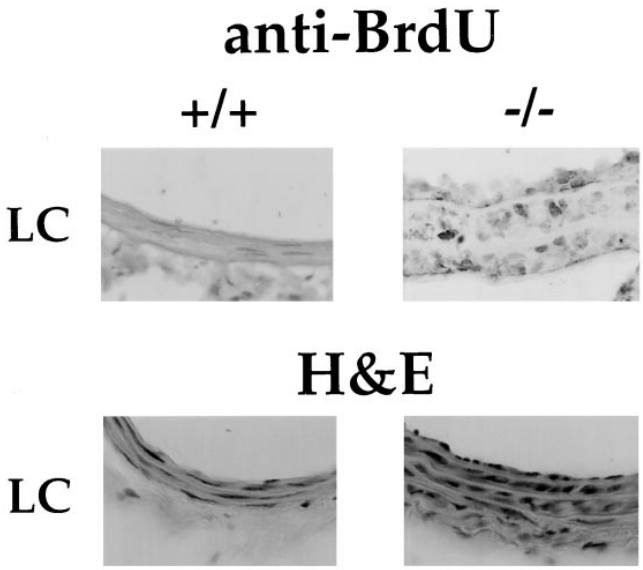

Figure 3. Abnormal vessel architecture in remodeled vessels from eNOS knockout mice. In the top panel, the number of BrdU positive cells (21) are markedly increased (dark cells in intima and medial smooth muscle) in −/− mice. Note the lack of staining in +/+ mice. This section of LC was lightly hematoxylin stained to show nuclei. In the bottom panel, hematoxylin and eosin stained remodeled vessels from −/− mice were markedly thicker with more rounded nuclei compared with remodeled vessels from +/+ mice.

Discussion

This study demonstrates that NO derived from eNOS is a major regulator of vessel reorganization in response to a remodeling stimulus. More importantly, in the absence of eNOS, not only is lumenal remodeling impaired, but vessel wall thickness doubles due to proliferation of vascular smooth muscle cells. Thus, these data directly support NO as a major determinant of vessel architecture in response to a hemodynamic stimulus and fits the criteria of an endothelium-derived mediator responsible for vessel remodeling (6).

Another salient feature of this paper is the development of a remodeling model in mice. Previous studies evaluating vascular abnormalities in mice were dependent on genes causing embryonic lethality through vascular defects (27, 28) or the use of an injurious stimulus to evoke a response-to-injury (29, 30). Here we show the feasibility of using a previously described method (6) of noninjurious remodeling via external carotid arterial ligation in mice. The magnitude of the reduction in LC diameter (10%) after a 2-wk ligation of the external carotid artery was similar to that seen in rats exposed to chronic carotid arterial stenosis (10%) (26) but less than that observed in rabbits exposed to complete ligation of the external carotid artery (20%) (3, 6). In our study, a consistent reduction in LC diameter relative to the RC was seen in every wild-type mouse stud-

ied. Possible differences that can account for the smaller reduction in diameter seen in rodents may be age, species, or the duration of the ligation. It is clear that vascular remodeling in response to increased or decreased flow is highly dependent on age, i.e., older animals exhibit less remodeling (3, 31). Another potential caveat of the mouse model described in this study is the inability to measure the flow reduction initiated by left external carotid ligation. Accurate measurements of absolute blood flow by velocimetry in conduit vessels is difficult in mice and indirect measurements by Doppler flowmetry are compromised by relative measurements of flow and the difficulty in standardizing flows between vessels and animals. Irrespective of flow measurements, in this study, the decrease in LC diameter after external carotid artery ligation and the decrease in medial nuclei number is consistent with flow-dependent remodeling toward a smaller vessel.

The importance of NO in remodeling has been previously suggested based on the known functions of NO. In addition, diseases associated with an impairment of vascular remodeling such as hypertension, stenotic atherosclerotic lesions, vascular graft failure and diabetes are associated with endothelial dysfunction and compromised endothelium-dependent relaxations to endothelium-dependent vasodilators (1). Previous studies using NOS inhibitors have shown that blockade of whole body NOS blunts high flow-dependent remodeling in rabbits (23) and causes coronary microvascular remodeling and fibrosis in rats (32). Our study using eNOS knockout mice confirms and extends these previous reports. Using the external carotid arterial ligation model, we find that the LC of −/− mice exhibit abnormal changes in vascular morphology (increased wall thickness and cellularity with no change in lumen diameter) only after ligation of the external carotid artery whereas +/+ mice exhibit the expected decrease in lumen diameter and total nuclei number. The precise mechanism for the focal impairment of remodeling is not known. Since the eNOS −/− mice have modest hypertension (24), it is possible that elevated arterial pressure and the attendant circumferential wall tension is amplified during remodeling in the absence of endothelium-derived NO. However, the increase in arterial pressure, per se, most likely did not directly cause abnormal remodeling since the right and left carotid arteries were exposed to the same systemic pressures yet the abnormal response was only seen in the left carotid artery. This focal response of the vessel to a remodeling stimulus indicates that NO in normal mice, but not in eNOS −/− mice, either may stimulate an inhibitor of vascular smooth muscle proliferation such as transforming growth factor-β (TGF-β) or counteract the actions of known smooth muscle mitogens such as platelet-derived growth factor (PDGF) and/or fibroblast growth factor during normal vessel remodeling. In support of PDGF as a mitogen responsible for remodeling lumen diameter after flow reduction is a recent report demonstrating increased expression of PDGF in vessels exposed to a reduction in flow (33). Indeed, NO delays G1-S progression (34), and blocks growth-factor induced proliferation and migration of cultured smooth muscle cells in culture, suggesting that low levels of endothelial-derived NO keep the underlying smooth muscle dormant and likely regulate the proportional turnover of vascular smooth muscle cells during remodeling of the vessel lumen.

Interestingly, and somewhat unexpected was the lack of a decrease in lumen diameter in remodeled carotid arteries of −/− mice based on the known vasodilatory role of NO. It is believed that in response to rapid decreases in blood flow, the shear rate that is sensed by a vessel will be normalized by reducing lumen diameter via vasoconstriction (5). Conversely, in response to increases in flow the shear rate will be normalized by increasing vessel diameter via vasodilation. Over time, vessel structure reorganizes commensurate to the stimulus imposed upon it (i.e., chronic flow reduction and the accompanying vasoconstriction remodels the structural and cellular elements towards a smaller vessel lumen), implying that chronic vascular adaptation and remodeling reflect the summation of many short term vasomotor events (1, 25). Based on this theory, the removal of NO should constrict vessels and reduce resting lumen diameter and wall thickness. Further, the imposition of a flow-reducing stimulus should proportionally reduce vessel lumen size and wall thickness even further in the absence of the endogenous vasodilator, endothelial-derived NO. The eNOS −/− mice displayed the opposite response to a remodeling stimulus, i.e., no change in lumen diameter with an increase in medial wall thickness. This suggests that endothelial-derived NO, in addition to its role as a vasodilator, may have a profound, unappreciated role in controlling aspects of vessel wall geometry. Alternatively, it is possible that in the absence of eNOS, the compensatory expression of iNOS in endothelial or vascular smooth muscle cells may be the source of large quantities of NO that dilate the vessel undergoing active remodeling and impair the ability of the lumen to decrease in response to the hemodynamic change (35, 36). However, it is unlikely that iNOS induction in smooth muscle would participate in the hyperplastic response seen in the remodeled eNOS −/− vessels as NO from this source typically negatively regulates smooth muscle proliferation and neointima formation after injury (37).

We suggest that in response to a remodeling stimulus, eNOS localized in endothelial caveolae (38) may act as a mechanosensor to couple NO release to long term hemodynamic changes that regulate aspects of extracellular matrix turnover, endothelial and smooth muscle cell proliferation, migration, organization, and responsiveness to growths factors; events that are ultimately responsible for vessel remodeling. In conclusion, eNOS is necessary for vascular remodeling, and its loss of function results in an abnormal vascular phenotype that may synergize with additional etiologic factors observed in cardiovascular diseases associated with endothelial dysfunction such as hypertension, diabetes, and atherosclerosis.

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