Functional Reconstitution of Endothelial Nitric Oxide Synthase Reveals the Importance of Serine 1179 in Endothelium-Dependent Vasomotion


Abstract—Phosphorylation of endothelial nitric oxide synthase (eNOS) at serine 1179 can activate the enzyme, leading to NO release. Because eNOS is important in regulating vascular tone, we investigated whether phosphorylation of this residue is involved in vasomotion. Adenoviral transduction of endothelial cells (ECs) with the phosphomimetic S1179DeNOS markedly increased basal and vascular endothelial cell growth factor (VEGF)–stimulated NO release compared with cells transduced with wild-type virus. Conversely, adenoviral transduction of ECs with the non-phosphorylatable S1179AeNOS suppressed basal and stimulated NO release. Using a novel method for luminal delivery of adenovirus, transduction of the endothelium of carotid arteries from eNOS knockout mice with S1179DeNOS completely restored NO-mediated dilatation to acetylcholine (ACh), whereas vasomotor responses in arteries transduced with S1179AeNOS were significantly attenuated. Basal NO release was also significantly reduced in arteries transduced with S1179AeNOS, compared with S1179DeNOS. Thus, our data directly demonstrate that phosphorylation of eNOS at serine 1179 is an important regulator of basal and stimulated NO release in ECs and in intact blood vessels. (Circ Res. 2002;90:904-910.)

Key Words: adenovirus ■ endothelium ■ vascular endothelial growth factor ■ signal transduction ■ nitric oxide

The release of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) is important in regulation of cardiovascular homeostasis. Recently, several groups have shown that phosphorylation of eNOS by the serine/threonine protein kinase Akt (protein kinase B) at serine 1179 (bovine eNOS) or 1177 (human eNOS) can activate the enzyme, leading to NO release.\(^1\)\(^-\)\(^3\) In addition, other kinases such as AMP kinase\(^4\) and protein kinases A and G\(^5\) can also phosphorylate this residue, implying that serine 1179 integrates several signaling systems to eNOS activation and NO release. Mechanistically, phosphorylation of serine 1179 increases NO release by enhancing the rate of electron flux through the reductase domain of eNOS and by improving the calcium sensitivity of the enzyme.\(^6\)

There is evidence that phosphorylation of this residue may be important for NO release in intact blood vessels. Indeed, inhibitors of phosphatidylinositol 3-kinase (PI3K), the upstream activator of Akt, inhibit endothelium-dependent responses in the rat cerebral circulation in vivo.\(^7\) Further evidence of the importance of this pathway are experiments showing that dominant-negative Akt attenuates endothelium-dependent relaxation in rat\(^8\) and mouse aorta\(^9\) ex vivo and acetylcholine (ACh)-induced blood flow changes in rabbit femoral arteries in vivo.\(^9\) Therefore, these studies suggest that the PI3K/Akt pathway is involved in NO-mediated regulation of vascular tone, and hence, blood flow. However, both PI3K and Akt can influence a variety of cellular metabolic and survival pathways that may influence NO release.\(^10\) To directly address the importance of eNOS phosphorylation in intact blood vessels, we have developed adenoviruses that encode for the constitutively active form of eNOS,\(^1\) a phosphomimetic form of eNOS, by mutating serine 1179 to an aspartate residue (S1179DeNOS), or a non-phosphorylatable form of eNOS, by mutating serine 1179 to an alanine residue (S1179AeNOS). In the present study, we show that S1179DeNOS, but not S1179AeNOS, augments basal and vascular endothelial cell growth factor (VEGF)–stimulated NO production in endothelial cells (ECs) and completely restores endothelium-dependent vasodilatation in pressurized arteries from eNOS knockout mice. These data underscore the importance of this residue in regulation eNOS-dependent responses in vivo.

Materials and Methods

Adenoviral Vectors

Replication-deficient adenoviruses expressing the gene of interest, under the control of the cytomegalovirus (CMV) promoter, were...
generated using the pAdTrack-CMV vector and AdEasy System.\textsuperscript{11} We used 6 adenoviral vectors: eNOS (10\textsuperscript{10} pfu/mL),\textsuperscript{12} LacZ (10\textsuperscript{13} pfu/mL)-expressing nuclear-targeted β-galactosidase (β-gal), and 4 vectors that also express enhanced green fluorescent protein (EGFP): WTeneNOS (5×10\textsuperscript{10} pfu/mL), S1179DeNOS (3×10\textsuperscript{10} pfu/mL), S1179AeNOS (5×10\textsuperscript{10} pfu/mL), and GFP (10\textsuperscript{10} pfu/mL). Viruses were amplified in HEK293 cells, purified using CsCl, titered using a cytopathic effect assay,\textsuperscript{13} and stored in PBS containing 10% glycerol, 0.5 mmol/L MgCl\textsubscript{2}, and 0.5 mmol/L CaCl\textsubscript{2}.

Characterization of eNOS Adenoviruses

Bovine aortic endothelial cells (BAECs) were cultured in 100-mm dishes (for basal NO measurement) or 6-well plates (for stimulated NO measurement) and infected with adenoviruses for 4 hours. Viruses were washed off and cells were incubated for 18 hours in complete medium. For basal NO release, medium was collected 48 hours after infection. For comparisons between viruses, equal eNOS protein levels were confirmed by Western blotting. For measurement of nitrite (NO\textsubscript{2}) production from ECs, adenoviruses were incubated for 4 hours. For comparisons between viruses, equal eNOS protein levels were confirmed by Western blotting. For measurement of nitrite (NO\textsubscript{2}) production from ECs, adenoviruses were incubated for 4 hours. For comparisons between viruses, equal eNOS protein levels were confirmed by Western blotting.

NO Release From ECs

After infection with adenovirus, media were processed for measurement of nitrite (NO\textsubscript{2}), the stable product of NO, by NO-specific chemiluminescence.\textsuperscript{14}

Adenoviral Gene Transfer Into the Endothelium of Mouse Carotid Arteries

Male C57Bl/6 or eNOS knockout mice\textsuperscript{15} (8- to 12-week old) were anesthetized with ketamine/xylazine and exsanguinated via transsection of the inferior vena cava followed by perfusion of saline through the left ventricle. The common carotid was cannulated, flushed with a small amount (~2 μL) of virus, and tied off proximally. Approximately 3 μL of virus was injected into the common carotid to fill and distend the vessel. The cannula was then removed, and the vessel was tied off distally. The virus-filled vessel was incubated in situ at 37°C for 2 hours and then dissected free from surrounding tissue and rinsed in saline before overnight (18 hours) incubation in complete DMEM at 37°C, 5% CO\textsubscript{2}. Control vessels were filled with viral storage buffer and treated in an identical manner.

Assessment of Gene Transfer

Efficiency of gene transfer was assessed by β-gal staining,\textsuperscript{16} visualizing GFP fluorescence in the vessel wall, or by Western blotting. After β-gal staining, vessels were either cut longitudinally and mounted onto glass cannulae for imaging en face using a laser scanning confocal microscope. Black and white confocal images were used to assess the area viral-infected, GFP-expressing cells per field using ScionImage software. White was assigned an arbitrary value of 1 and black as 250. Mean intensity was calculated from 4 vessels and each vessel was measured 5 times. Transgene expression was also determined by Western blot analysis. Infected carotid arteries (2 vessels/sample) were frozen in liquid N\textsubscript{2}, crushed, and homogenized. Proteins (11 μg/sample) and control samples of purified bovine eNOS were separated on 10% gel, transferred onto a nitrocellulose membrane, and probed with monoclonal antibody against eNOS (1:1000, Transduction Laboratories) and β-actin (1:5000, Sigma). Densitometry of each blot was used to calculate the relative expression of eNOS as a ratio of β-actin expression.

Ex Vivo Assessment of Gene Transfer on Vessel Reactivity

After overnight incubation, carotids were placed in cold (4°C) Krebs physiological saline solution (PSS) of the following composition (mmol/L): NaCl 119, KCl 4.7, CaCl\textsubscript{2} 1.8, MgSO\textsubscript{4} 1.4, NaHCO\textsubscript{3} 2.1, KH\textsubscript{2}PO\textsubscript{4} 1.2, glucose 11, ibuprofen 0.01, and gassed with 5% CO\textsubscript{2} in air. Vessels were cut into 2 rings for isometric tension studies or left intact and mounted onto glass cannulae for isobaric studies.

Isometric Studies

Rings of carotid artery were mounted in a 5-mL vessel myograph (Kent Scientific) on 2 tungsten wires (25-μm diameter), and basal tension was set at 0.5 g. After 45 minutes equilibration, rings were contracted with 125 mmol/L KCl substituted for NaCl in PSS (KPSS). Responses to KPSS were repeated until the contraction was reproducible. Concentration-response curves were constructed to prostanoid P\textsubscript{2} (PGE\textsubscript{2}, 10\textsuperscript{-7} to 3×10\textsuperscript{-6} mol/L) to assess endothelial function. Vessels were precontracted with a submaximal (~80%) concentration of PGE\textsubscript{2} (0.5 to 1×10\textsuperscript{-5} mol/L) before application of endothelium-dependent dilator acetylcholine (ACh, 10\textsuperscript{-10} to 3×10\textsuperscript{-6} mol/L). Basal NO production was measured by increasing tone by ~50%, using PGE\textsubscript{2} (3 to 5×10\textsuperscript{-6} mol/L), and applying L-NAME (3×10\textsuperscript{-4} mol/L, 20 minutes). Sensitivity to NO was tested by measuring relaxation to the NO donor sodium nitroprusside (SNP, 10\textsuperscript{-6} to 10\textsuperscript{-8} mol/L).

Isobaric Studies

Carotid arteries were mounted in a perfusion myograph (Living Systems), pressurized to 100 mm Hg, and superfused at 10 mL/min. Intraluminal pressure was maintained using a pressure servo system, and internal diameter was monitored using a dimension analyzer. Vessels were equilibrated for 45 minutes at an intraluminal flow rate of 0.13 mL/min and then primed with 10\textsuperscript{-6} mol/L phenylephrine (PE). After a 30-minute washout period, vessels were constricted with 10\textsuperscript{-7} to 10\textsuperscript{-6} mol/L PE to reduce internal diameter by ~15% and ACh (10\textsuperscript{-10} to 10\textsuperscript{-4} mol) was applied directly into the organ bath (5 mL). Basal NO production was measured by applying L-NAME (3×10\textsuperscript{-4} mol/L, 30 minutes) after preconstriction with PE. Dilatation to NO donor was tested by applying SNP (10\textsuperscript{-6} mol/L). Passive diameter was determined by superfusing with Ca\textsuperscript{2+}-free PSS containing 2 mmol/L EGTA at the end of each experiment. Passive diameter of S1179DeNOS-infected vessels was 503±17.8 μm (n=6) and S1179AeNOS was 509±31.1 μm (n=11). The passive diameter of wild-type vessels was significantly (P<0.001) greater than knockout vessels (590±9.3 μm, n=5).

Statistical Analysis

Results are expressed as mean±SEM. Responses to dilators were calculated as percentage reversal of induced tone or constriction. Contraction to L-NAME (isometric) was calculated as percentage increase above basal contraction to PGE\textsubscript{2}. Constriction to L-NAME (isobaric) was measured as the additional decrease in diameter above constrictor response to PE. Comparisons between groups were made using ANOVA followed by Bonferroni’s multiple comparison test. Statistical significance was considered when P<0.05.

Results

Characterization of eNOS Adenoviruses

Adenoviruses expressing wild type (WT), S1179DeNOS, or S1179AeNOS were generated using the pAdTrack-CMV vector allowing for coexpression of eNOS with GFP. As a control, a virus expressing GFP alone was used. BAECs were infected with 50 MOI of each eNOS virus or with GFP. At 8 hours post-infection, GFP expression was quantified as 250. Mean intensity was calculated from 4 vessels and each vessel was measured 5 times. Transgene expression was also determined by Western blot analysis. Infected carotid arteries (2 vessels/sample) were frozen in liquid N\textsubscript{2}, crushed, and homogenized. Proteins (11 μg/sample) and control samples of purified bovine eNOS were separated on 10% gel, transferred onto a nitrocellulose membrane, and probed with monoclonal antibody against eNOS (1:1000, Transduction Laboratories) and β-actin (1:5000, Sigma). Densitometry of each blot was used to calculate the relative expression of eNOS as a ratio of β-actin expression.

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Previously, we1 and others2,3 have shown that Akt-dependent phosphorylation of eNOS on serine 1179 is necessary for VEGF-induced NO release. As seen in Figure 1C, VEGF induces a 2-fold increase in NO\textsuperscript{2-} release from control and GFP-transduced cells. Infection of BAECs with WT-eNOS augmented VEGF-stimulated NO production, whereas infection with S1179DeNOS further increased basal and VEGF-stimulated NO production during the 30-minute collection period. Interestingly, transduction with S1179AeNOS resulted in less basal NO release than seen in WTeNOS- or S1179DeNOS-transduced cells but more than control or GFP-infected cells.

**Luminal Delivery of eNOS Adenovirus to eNOS Knockout Vessels Restores Endothelium-Dependent Relaxations**

Previous approaches to infect isolated blood vessels with adenoviruses have relied on incubation of vascular rings with virus in organ culture (for example see Lake-Bruse et al\textsuperscript{17}). Although efficient, one cannot selectively deliver the gene of interest to the endothelium or adventitia because both layers are bathed in the virus. In order to circumvent this issue, we developed a technique for luminal delivery of viruses to selectively infect the endothelium. As seen in Figures 3A and 3B, in situ cannulation and luminal transduction of the endothelium of the mouse carotid with a virus expressing β-gal resulted in ample expression of the virus in the endothelial layer. The transduction efficiency using a viral titer of 10\textsuperscript{11} pfu/mL, was 40% to 60% as assessed by en face imaging of the EC surface. Viral infection with LacZ had no significant effect on endothelial function as assessed by relaxation to ACh (see Figure 2C). Similarly, infection with adenovirus had no significant effect on contractile responses to PGF\textsubscript{2α} (n=4); EC\textsubscript{50} and maximum responses were 5.4±1.21 μmol/L, 0.4±0.04 g, and 3.9±0.06 μmol/L, 0.3±0.1 g without and after viral infection, respectively. To test the function of luminal gene transfer, carotid arteries from eNOS knockout mice were infected with WTeNOS virus and ACh-induced relaxations of mouse carotid arterial rings examined. The carotid artery was used because in our preliminary experiments, ACh responses in knockout vessels were absent and abolished by L-NAME in WT vessels (n=4).

As seen in Figure 2D, luminal transduction of mouse carotid arteries with WTeNOS virus completely reconstituted ACh-induced relaxations in eNOS knockout vessels, an effect abolished by L-NAME. EC\textsubscript{50} values and maximum responses were 46±13.9 mmol/L, 92±3.0% (n=4) and 66±15.5 mmol/L, 82±8.1% (n=5) in WT and infected knockout vessels, respectively. Furthermore L-NAME, which had no constrictor effect in control uninfected vessels, produced an increase in isometric contraction of 65.3±26.4% (n=7) of vessels transduced with WTeNOS. Therefore, using endothelium-selective, adenoviral-mediated gene transfer of eNOS, we were able to restore both endothelium-dependent and basal NO release in eNOS knockout arteries. Interestingly, despite differences in basal NO production, contractile responses to either PGF\textsubscript{2α} or KPSS were unaffected by eNOS gene transfer. Maximum responses to PGF\textsubscript{2α}, were 0.3±0.06 g (n=9) and 0.3±0.06 g (n=9), and to KPSS were 0.1±0.02 g (n=9) and 0.1±0.02 g (n=9) with and without viral infection, respectively. These results support previous findings by Lamp-
vessels, respectively (n=4 in both), representing approximately 50% of the endothelium virally infected. Relative protein expression of eNOS/β-actin was 1.1±0.1 (n=4) for vessels transduced with S1179DeNOS and 0.9±0.4 (n=4) for S1179AeNOS. Thus, using 2 independent quantitative measurements, the proteins were equally expressed. We then examined functional responses of the transduced vessels in a perfusion myograph system. We opted to use pressurized vessels because it is a more physiologically relevant preparation (ie, with constant transmural pressure throughout the vessel segment) and it permits evaluation of the time course of vasodilation. Carotid arteries from eNOS knockout mice infected with a virus encoding for β-gal did not exhibit significant changes in diameter to ACh (n=4; Figure 3D, open circles). Transduction of eNOS knockout arteries with either S1179DeNOS (n=6) or S1179AeNOS (n=11) restored dilatation to ACh to ~50% of responses seen in normal WT vessels infected with adenovirus encoding for β-gal, responses that were abolished after pretreatment with L-NAME (not shown). Maximum responses to ACh were 43±3.5% (n=6) and 38±9.8% (n=11) in the presence of S1179DeNOS and S1179AeNOS, respectively, whereas responses in β-gal–infected WT vessels were 91±4.3% (n=5). However, as seen in Figure 3D, transduction with S1179AeNOS significantly (P<0.05) reduced the dilator responses to doses of ACh compared with S1179DeNOS-transduced vessels. This effect was greater at lower doses (10^-3 mol/L) of ACh, suggesting that the sensitivity to ACh was modulated.

Our studies in infected BAECs show that S1179AeNOS releases considerably less basal NO than S1179DeNOS. Similarly, measurement of basal NO-dependent tone in pressurized carotid arteries (Figure 3E), assessed by constriction to L-NAME, was significantly (P<0.05) less in vessels transduced with S1179AeNOS (47±11 μm, n=11) than with S1179DeNOS (68±2.1 μm, n=6). Moreover, consistent with lower basal NO release, responses to NO donor SNP were significantly (P<0.05) greater in the presence of S1179AeNOS (81±3.2%, n=11) than S1179DeNOS (70±2.6%, n=6). However, this difference in basal NO release did not affect constrictor reactivity; application of 10^-5 mol/L PE resulted in decrease in diameter of 16.2±0.8% (n=6) and 16.3±1.4% (n=11) in S1179DeNOS- and S1179AeNOS-infected arteries, respectively.

To test whether the time course of eNOS activation was different between the two mutant forms of eNOS, we tested the duration of vasodilatation in response to doses of ACh given in pressurized vessels. Despite a trend toward shorter duration of endothelium-dependent responses to ACh in vessels transduced with S1179AeNOS, there was no significant difference between the two mutant forms of eNOS (Figure 3F).

**Discussion**

The major emphasis of this study was to examine the functional significance of serine 1179 in eNOS by transducing cultured endothelial cells and intact blood vessels with adenoviruses. Presently, we show that adenoviral knock-in of endothelial cells with the phosphomimetic form of eNOS

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**Figure 2.** Endothelium-selective expression of adenovirus in carotid arteries. A, En face image of carotid artery demonstrating efficient gene transfer of β-gal, and (B) cross-section of artery demonstrating β-gal expression in the endothelium but not in the media or adventitia. C, Transduction with β-gal (●) does not affect endothelium-dependent relaxation of rings of carotid artery to ACh in WT arteries (●) (n=4 from 4 animals). D, Transduction with WT.eNOS (●) restores endothelium-dependent relaxation in eNOS KO (○) arteries (n=5 from 5 animals). Relaxation to ACh in eNOS-transduced arteries was inhibited by L-NAME (300 μmol/L) (●). Data are shown as mean±SEM.
Vegetative propagation of plants is a significant means of reproduction. In the context of cellular biology, the term "vegetative propagation" typically refers to the process by which plant cells or tissues grow and differentiate without undergoing sexual reproduction. This method involves the transfer of vegetative parts of plants, which can include stems, leaves, or roots, to new environments where they can develop into whole plants. Vegetative propagation is often used in horticulture and agriculture to propagate plant varieties that reproduce asexually. It is an essential technique for the propagation of many ornamental and crop plants, offering advantages such as disease resistance, uniformity of the plant, and a rapid propagation rate compared to sexual reproduction. 

The image contains a figure (Figure 3) illustrating the phosphorylation of S1179 in eNOS. The figure shows the expression of eNOS protein as detected by Western blot analysis in eNOS KO carotid arteries infected with (A) S1179DeNOS and (B) S1179AeNOS. C, Expression of eNOS protein as detected by Western blot analysis in eNOS KO carotid arteries infected with β-gal, S1179DeNOS, or S1179AeNOS. D, Endothelium-dependent dilations to ACh are attenuated in S1179AeNOS-transduced (●) vessels compared with S1179DeNOS-transduced (▲) arteries. Transduction of eNOS KO vessels with a β-gal-expressing virus did not influence vasomotion (○). E, Duration of ACh response in S1179AeNOS-transduced (●) vessels compared with S1179DeNOS-transduced (▲) arteries. F, Constriction to L-NAME is greater in vessels infected with S1179DeNOS (▲) than S1179AeNOS (●). Data are mean±SEM; *P<0.05 by 2-way ANOVA.
serine 1179. As stated previously, other kinases can indeed phosphorylate this site and other sites too. In order to directly study the importance of phosphorylation of serine 1179 in isolated blood vessels, we used luminal delivery to selectively infect endothelium of arteries with adenoviruses encoding for S1179DeNOS or S1179AcE-NOS. We did not compare responses of eNOS mutants to WT enzyme because the phosphorylation state of S1179 in WTeNOS cannot be controlled in our experiments or any experiments. By comparing S1179DeNOS with S1179AcE-NOS, we directly assessed the effects of phosphorylation on this particular residue on endothelial function. Infection with either S1179DeNOS or S1179AcE-NOS restored the ability of ACh to elicit dilatation, indicating that both eNOS mutants are capable of releasing sufficient amounts of NO to mediate these responses. However, the sensitivity and magnitude of vasodilatation to receptor-mediated stimulation was reduced with S1179AcE-NOS compared with that of S1179DeNOS. The negative charge at serine 1179, due to phosphorylation or the presence of aspartate, improves the rate of electron flux and decreases the dissociation of eNOS/calmodulin complex at low Ca$^{2+}$ concentrations. Hence, the apparent increase in sensitivity of S1179DeNOS to ACh may be due to enhanced stability of eNOS/calmodulin complex at low-dose stimulation. However, we cannot rule in or out the importance of threonine 495 dephosphorylation synergizing with serine 1179.$^{23,26}$ A recent report has shown that dephosphorylation of threonine 495 increases the ability of calmodulin (CaM) to interact with eNOS and enhances eNOS activity in vitro.$^{26}$ The dephosphorylation is transient in nature and may relate to the initial binding of CaM to the enzyme. In this context, threonine 495 may initiate eNOS activation, an effect sustained through serine 1179 phosphorylation. Consistent with an important role for eNOS phosphorylation on serine 1179 in regulating basal NO release, the ability of L-NAME to further enhance vessel tone, as an index of basal NO release,$^{27,28}$ was also less in S1179AcE-NOS-transduced vessels compared with S1179DeNOS-transduced vessels. Concomitantly with the greater basal release of NO, we observed a decrease in sensitivity of S1179DeNOS-transduced vessels to the NO donor SNP. This downregulation is typically seen in vessels exposed to NO$^{29}$ or in eNOS transgenic mice.$^{30}$ Because neither S1179AcE-NOS nor S1179DeNOS can be phosphorylated/dephosphorylated at S1179, we anticipated that the duration of the ACh response may be regulated by cycles of phosphorylation/dephosphorylation; however, to our surprise, there was no difference in the temporal kinetics of ACh-mediated dilatation of vessels transduced with either virus. This implies that other mechanisms, most likely related to CaM binding and release from eNOS, are more important than S1179. Thus, in summary, phosphorylation of eNOS on S1179 influences endothelium-dependent responsiveness of mouse carotid arteries. Previous studies have documented that gene therapy with eNOS is an attractive strategy for treatment of several cardiovascular diseases, including atherosclerosis,$^{31}$ diabetes,$^{32}$ and restenosis.$^{33}$ Expression of the phosphomimetic S1179DeNOS in endothelium modulates VEGF-induced NO synthesis in vitro, and expression in arterial endothelium results in greater basal NO release and enhanced endothelium-dependent responsiveness. Therefore, S1179DeNOS may be more beneficial than conventional WTeNOS for use in gene therapy in disease states associated with reduced NO release and impaired angiogenesis. In particular, because Akt activity is inhibited by atherogenic stimuli,$^{34,35}$ perhaps the phosphomimetic S1179DeNOS may be a novel treatment for endothelial dysfunction associated with hypercholesterolemia and atherosclerosis.

**Acknowledgments**

This work is supported by grants from the National Institutes of Health (RO1 HL57665, HL61371, and HL64793 to W.C.S. and T32HL10183 to D.F.). R.S.S. is funded by a Wellcome Trust Traveling Research Fellowship and J.P.G. by a fellowship from the Canadian Institutes of Health Research. W.C.S. is an Established Investigator of the American Heart Association. The authors wish to thank Dr Zvonimir Katucis for WTeNOS virus used in preliminary studies, Dr Kevin Claffey for assistance with imaging GFP in the vessel wall, and Dr Ken Walsh with help and advice for propagation of adenoviruses.

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Circ Res. 2002;90:904-910; originally published online March 28, 2002; doi: 10.1161/01.RES.0000016506.04193.96
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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