Endothelial NO Synthase Is Increased in Regenerating Endothelium After Denuding Injury of the Rat Aorta

Veronica Poppa, Jody K. Miyashiro, Marshall A. Corson, Bradford C. Berk

Abstract—Endothelial nitric oxide synthase (eNOS) has been shown to be regulated both transcriptionally and posttranslationally in cultured endothelial cells, but eNOS regulatory mechanisms in vivo have not been elucidated. Because one of the strongest stimuli for eNOS expression in tissue culture is cell proliferation and because increased NO production would be beneficial in the setting of arterial injury, we hypothesized that eNOS expression should be increased in regenerating endothelium after a denuding injury. Rat aortas underwent partial endothelial denudation by passage of a deflated balloon catheter, and eNOS expression was studied 48 hours after injury. Immunohistochemistry with eNOS monoclonal antibody, NADPH diaphorase activity assay under conditions specific for eNOS, and mRNA hybridization were performed in situ on perfusion-fixed rat aortic segments. The vessels were studied en face to enhance visualization compared with cross sections. eNOS protein and mRNA expression were significantly increased in regenerating and migrating endothelial cells at the wound edge, with translocation of eNOS to the plasma membrane at the leading edge. Similar results were obtained when endothelial cells were studied in a tissue culture wound model. An important role for transforming growth factor (TGF)-β in regulating eNOS expression was suggested by the ability of a TGF-β₁-neutralizing antibody to limit induction of eNOS at the wound edge. Increased eNOS expression after wounding appears to be related to signal events associated with cell migration as well as proliferation, because eNOS expression in vivo increased in nonproliferating cells and TGF-β₁-neutralizing antibody inhibited eNOS expression but stimulated proliferation. The current study is the first to suggest an important role in vivo for increased eNOS, and perhaps NO production, in the process of endothelial regeneration and wound repair. (Arterioscler Thromb Vasc Biol. 1998;18:1312-1321.)

Key Words: endothelial nitric oxide synthase ■ endothelial regeneration ■ balloon injury ■ transforming growth factor-β₁

Alterations in endothelial function are likely to contribute to the pathogenesis of cardiovascular disease, including hypertension, atherosclerosis, and restenosis after coronary interventions. During the process of wound repair after arterial injury, which involves migration and proliferation of endothelial cells, increased production of NO appears to be beneficial because of its positive effects on vasodilation, prevention of platelet aggregation, and regulation of endothelial cell migration. However, the mechanisms that regulate endothelial nitric oxide synthase (eNOS) expression and activity, responsible for NO production in vessels, have not been studied in vivo during endothelial regeneration.

Both eNOS protein and mRNA have been shown to be dynamically regulated by multiple stimuli in tissue culture. Steady-state levels of eNOS mRNA and protein are increased in proliferating bovine aortic endothelial cells (BAECs) before cell growth is inhibited by cell-cell contact. eNOS expression and activity are increased in response to transforming growth factor-β₁ (TGF-β₁), and vascular endothelial growth factor (VEGF), and eNOS activity is inhibited by agonists that stimulate protein kinase C. eNOS function is likely regulated by changes in mRNA transcription, mRNA stabilization, protein synthesis, and enzyme activity (related to posttranslational modifications such as myristoylation and palmitoylation and subcellular localization). E₂/E₃ eNOS expression is increased in blood vessels exposed to high shear stress compared with low shear stress. However, little is known regarding changes in eNOS expression during endothelial cell regeneration in vivo after arterial injury. On the basis of in vitro studies that have demonstrated increased eNOS expression during cell proliferation, we hypothesized that eNOS expression should be increased in regenerating endothelium in vivo. Furthermore, because TGF-β₁ is highly expressed in injured arteries and is a known stimulus for eNOS expression in vitro, we hypothesized that TGF-β₁ would be a key mediator of changes in eNOS function.

To test our hypotheses, we performed a gentle partial denudation of the rat aorta and then studied eNOS expression by en face immunohistochemistry, NADPH diaphorase activity, and in situ mRNA hybridization. Our en face results demonstrate that regenerating endothelial cells express increased levels of eNOS mRNA and protein, that eNOS...
protein translocates from a perinuclear location (most likely the Golgi) to the plasma membrane, and that the increase in eNOS exceeds that expected due to cell proliferation, suggesting that other factors regulate eNOS in vivo. Additional studies in tissue culture suggest that TGF-β is an important factor for eNOS regulation during wound repair.

Methods

Arterial Injury Model and Fetal Calf Vessel Preparation

All animal studies were approved by the Animal Care Committee of the University of Washington, Seattle. Male Sprague-Dawley rats, 3 to 4 months old (~400 g), were anesthetized by intraperitoneal injection of xylazine (2.2 mg/kg) and ketamine (50 mg/kg). The carotid artery was cannulated and the aorta was partially denuded by passing a deflated 2F balloon catheter along the vessel, which was removed a 2- to 3-mm-wide strip of endothelium along the long axis of the vessel. Bromodeoxyuridine (BrDU) tablets (Boehringer Mannheim) were placed subcutaneously 24 hours before the animals were euthanized. For identification of deendothelialized portions, intravenous injection of Evans blue (0.3 mL of a 5% solution in saline) was performed 30 minutes before death. The animals, after being anesthetized with a 50 mg/kg pentobarbital sodium (Abbott) intraperitoneal injection, were killed by perfusion fixation with phosphate-buffered 4% paraformaldehyde (pH 7.4) 48 hours after the initial denuding procedure. Aortas were prepared for en face immunohistochemistry or in situ hybridization by cutting them into 8 segments, which were opened longitudinally, and the tissue was pinned out flat, luminal side up, on polytetrafluoroethylene cards and stored in phosphate-buffered 4% paraformaldehyde (pH 7.4) at 4°C for up to 5 days.

Fetal calf aortas were obtained from a local abattoir, rinsed with PBS, and perfused with 4% paraformaldehyde after ligation of the intercostal arteries. The vessels were dissected, pinned out as described above, and immersion postfixed for 1 hour before in situ histochemistry.

Tissue Culture Injury Model

BAECs at low passage (3 and 4) were grown in medium 199 supplemented with 10% FCS on gelatin-coated glass slides or on Permanox chamber slides and allowed to reach confluence. A 3- to 5-mm strip of cells was removed with an incising loop, and regeneration of the monolayer was allowed to proceed for up to 48 hours. For histological analysis, the cells were immersed in fixation, phosphate-buffered 4% paraformaldehyde and stored in the fixative at 4°C for up to 3 days. For assessment of proliferation, BrdU (Amersham) was administered at 10 μg/mL.

Immunohistochemistry

Fixed samples were incubated in 80% methanol containing 0.6% H₂O₂ for 30 minutes to quench endogenous peroxidases and blocked with 5% normal horse serum for 30 minutes. All solutions were purchased from Vector unless specified otherwise, prepared in PBS with Ca²⁺- and Mg²⁺-containing 1% BSA, and applied at room temperature unless specified otherwise. There were at least three 5- to 10-minute washes between each solution application. All washes were done in PBS. Mouse monoclonal antibodies for eNOS (1:500 to 1:5000 dilution), inducible NOS (iNOS, 1:500 to 1:50 dilution), caveolin (1:1000; Transduction Laboratories), and mannosidase II (1:10 000; BAbCo) and rabbit polyclonal antibody for von Willebrand factor (1:1000; DAKO) were applied on whole mounts or tissue culture slides in pools, and the segments or slides were incubated overnight at 4°C in a humidifier box. Biotinylated secondary antibody (horseradish peroxide, 1:500 or goat anti-rabbit) was used for 1 hour, followed by a 30-minute incubation in ABC® and a 3- to 10-minute development in 0.5% diaminobenzidine (DAB) in 50 mmol/L Tris-buffered saline at pH 7.6. For a negative control, the primary antibody was substituted with normal mouse IgG at 1:2000 dilution. As a positive control for iNOS, rat aortic smooth muscle cells were stimulated as described in Geng et al. with tumor necrosis factor-α (500 U/mL), interleukin-1β (20 ng/mL), and γ-interferon (500 U/mL) for 24 hours.

For BrdU immunohistochemistry, a methanol-H₂O₂ incubation was followed by digestion with 0.5 mg/mL pepsin (Sigma) in 0.1N HCl at 37°C for 30 minutes. Tissue segments or tissue culture slides were then placed into 1.5N HCl for 15 minutes at 37°C and stabilized by washes in 0.1 mol/L Borax solution (pH 8.5). Mouse monoclonal BrdU antibody (DAKO) was applied at 1:200 dilution, and the tissues were incubated for 1 hour at 37°C in a humidifier box. After application of the biotinylated secondary antibody, the procedures were followed as described above. All tissues were counterstained with hematoxylin.

NADPH Diaphorase Activity Assay

A modified procedure based on O’Brien et al. was used. Rat aortic whole mounts, fetal calf aorta-to-intercostal transitional areas, and tissue culture BAEC slides were fixed in 4% parafomaldehyde for exactly 1 hour to deactivate all other NADPH diaphorases and rinsed in PBS, and a reaction solution of 0.1 mol/L PBS, 0.3% Triton X-100, 0.1 mg/mL nitroblue tetrazolium, and 1.0 mg/mL β-NADPH was applied to the segments or slides in pools. All reagents were acquired from Sigma Chemical Co. Tissues were incubated at 37°C in the dark for 1 hour, after which the reaction was stopped by rinsing the pinned vessel segments and slides in 70% ethanol. The nuclei were counterstained with nuclear fast red. As a negative control, NADP was substituted for NADPH at the same concentration. In addition, NADH was substituted for NADPH and showed a reticular cytoplasmic pattern of staining, which clearly differed from that observed with NADPH, thus further confirming the specificity of the protocol. Although eNOS expression in SF9 cells (V.P., unpublished data, 1998) and specific detection by this protocol showed the technique to be able to readily detect eNOS, we cannot exclude the possibility that some other diaphorases may still have been active.

In Situ Hybridization

Bovine cDNA for eNOS, ligated into Bluescript vector as described, was used to generate riboprobes. 35 S-UTP (Amersham) riboprobes for the radioactive in situ method and digoxigenin-labeled UTP (Boehringer Mannheim) riboprobes for the nonradioactive method were synthesized by T3 and T7 polymerases (Boehringer Mannheim) from a linearized ~400-bp-long template. Pinned vessel segments were treated with 1 μg/mL proteinase K (Boehringer Mannheim) at 37°C for 15 minutes, followed by a 2-hour prehybridization at 55°C in a buffer containing 0.3 mol/L NaCl, 20 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 1× Denhardt’s solution (Sigma), 10% dextran sulfate, 10 mmol/L DTT, and 50% deionized formamide. The riboprobes were diluted in the same buffer and applied to the tissue segments with 300 cpm/mL of the radiolabeled probes (both sense and antisense) and 12.5 ng/mL of the digoxigenin-labeled probes (both sense and antisense). Hybrization took place overnight at 72°C in a humidifier box, after which the specimens were washed with 2× SSC, treated with RNase A (Sigma) at 20 μg/mL dilution for 30 minutes at 37°C, and washed in increasing stringency buffers, with a final buffer of 0.1× SSC at 72°C for 2 hours. For the radioactive in situ method, the Haucutench procedure as described below was carried out after probe hybridization. The slides then were coated with autoradiographic emulsion (Kodak, NTB2), exposed for 1 week, and developed (Kodak, D-19). For digoxigenin-labeled probes, hybridization was followed by incubation in 1× blocking solution (Boehringer Mannheim) in maleic acid buffer (0.1 mol/L maleic acid, 0.15 mol/L NaCl, pH 7.5) for 1 hour and then with alkaline phosphatase–conjugated anti-digoxigenin (Boehringer Mannheim) in the same blocking solution overnight at 4°C. After extensive washing with maleic acid buffer, the specimens were allowed to equilibrate in detection buffer (0.1 mol/L Tris, 0.1 mol/L NaCl, and 50 mmol/L MgCl₂, pH 9.5) and placed upright into a color detection solution prepared by adding levamisole (0.25 mg/mL), 4.5 μL/mg nitroblue tetrazolium, and 3.5 μL/mL 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) solution (Boehringer Mannheim) to the detection buffer. The
color was allowed to develop for 5 hours at room temperature in the dark with no agitation, followed by nuclear fast red counterstaining.

Haeutchen Procedure for En Face Viewing of Endothelium

The Haeutchen procedure as described by Lindner and Reidy was carried out after immunohistochemistry or in situ hybridization was completed on pinned vessel segments. In brief, the tissues were dehydrated in an ethanol series, removed from polytetrafluoroethylene cards, and pressed luminal face down with a drop of 50% ether–50% ethanol–dissolved parlodion (Baxter) onto parlodion–coated slides, air dried, and soaked in 70% ethanol for 1 hour. All tissue except for the adherent endothelial cell layer was then peeled off, the parlodion film was taken off the glass slides and trimmed off around the endothelial cell sheets, and the sheets were individually pressed onto gelatin-coated slides luminal side up. The parlodion film was dissolved away from the face of the endothelial cell sheet in a 50% ether–50% ethanol solution overnight, and the specimens were processed for mounting or autoradiography.

TGF-β1–Blocking Antibody Procedures

TGF-β1–neutralizing antibody was purchased from Becton Dickenson Labware and applied at concentrations from 0.3 to 30 μg/mL to BAECs in tissue culture immediately before injury. Treated cells were fixed for 20 hours after injury and assayed for NADPH diaphorase activity, whereas BrdU immunohistochemistry was performed on the matching set of BAECs.

Image Analysis

Aortic segments assayed for NADPH diaphorase were photographed with a 40× objective in a systematic fashion (at the wound edge and 3 sites distant from the edge, at 25, 50, and 75 rows of cells away from the wound). Images (6 or 7 for each condition) were scanned and digitzed with Adobe Photoshop, and positive staining was counted with a 600-point grid on the computer screen. The intensity of the staining was intentionally disregarded; thus, a point with very dark staining at the wound edge was assigned the same value as a very faintly stained point away from the wound. To evaluate subcellular localization of eNOS, the ratio of points overlying nonperinuclear formazan precipitate to the total points overlying formazan precipitate was calculated. Statistical analysis was performed with Kruskal-Wallis and Scheffe’s tests by using StatView 4.01 for Macintosh.

The proliferation index was calculated as the ratio of the number of BrdU-stained nuclei to the total number of cells overlain by a systematically applied reticle (15 fields per each thoracic and abdominal aortic segment per rat or slide) positioned perpendicular to the edge of the wound.

Figure 1.1, Greater eNOS expression in regenerating endothelium: eNOS immunohistochemistry. eNOS-immunoreactive protein was detected with monoclonal eNOS antibody at 1:1000 dilution, as shown by brown DAB precipitate. Nuclei were stained with hematoxylin. Denuded area, which defines the wound edge, is marked with an asterisk. Blood flow is parallel to the wound edge in all figures. A, At the wound edge, regenerating endothelium, with its characteristic elongated and condensed nuclei that appear more darkly stained by hematoxylin, shows increased immunoreactivity compared with the undisturbed area. B, Wound edge stained with preimmune mouse IgG as a control. C, Higher magnification of wound edge stained for eNOS shows high level of eNOS staining both in perinuclear region (open arrow) and associated with plasma membrane (solid arrow). D, Higher magnification of undisturbed endothelium stained for eNOS shows low-level expression confined to perinuclear region (open arrows) and barely detectable levels in association with plasma membrane (solid arrow). Bar corresponds to 30 μm in A and B and to 5 μm in C and D. 1.2, Greater eNOS expression in regenerating endothelium: NADPH diaphorase activity. eNOS was detected by NADPH diaphorase activity as evidenced by blue formazan precipitate. Nuclei were stained with nuclear fast red. Denuded area is marked with an asterisk. A, Wound edge shows high levels of NADPH diaphorase activity. B, Wound edge stained with NADP rather than NADPH as a negative control. C, Higher magnification of wound edge assayed by NADPH diaphorase shows perinuclear localization (open arrow) and striking enhancement of plasma membrane (solid arrow) localization. D, Higher magnification of undisturbed endothelium shows low-level NADPH diaphorase activity, which is more evenly distributed between perinuclear (open arrows) and plasma membrane (solid arrow) sites. Bar corresponds to 30 μm in A and B and to 5 μm in C and D. 1.3, Mannosidase II and caveolin show no change in regenerating endothelium: immunohistochemistry. Mannosidase II and caveolin were detected with monoclonal antibodies as shown by brown DAB precipitate. Nuclei were stained with hematoxylin. A, Wound edge shows mannoseidase II present in perinuclear regions only (open arrow). B, Undisturbed endothelium shows similar localization, with no plasma membrane association. C, Wound edge shows caveolin present in perinuclear regions (open arrow) and associated with plasma membrane (solid arrow). D, Undisturbed endothelium shows caveolin present in perinuclear region (open arrow) and associated with plasma membrane (solid arrow). Bar corresponds to 10 μm.

To analyze the effects of TGF-β, blocking on eNOS expression as measured by NADPH diaphorase assay, the stained monolayers were photographed at the same exposure with a transmitted light microscope in a systematic fashion using a 40× objective. Ten photomicrographs per well were scanned and digitized with Adobe Photoshop, background was subtracted, and quantitative densitometry was performed with NIH Image 1.60 software. To yield pixel intensity per cell, pixel intensity of an image was divided by the number of cells in that image to yield pixel intensity per cell. The data were analyzed for statistical significance by ANOVA and Bonferroni pairwise comparison using SYSTAT for Macintosh, version 5.2.

Results

Increased eNOS at the Wound Edge: Protein Expression

Local variations in eNOS expression are likely to regulate vessel responses to mechanical, hormonal, and hemodynamic stimuli. To investigate changes in eNOS expression in regenerating endothelium, we performed a gentle denuding injury with a deflated balloon catheter in rat aorta. eNOS expression and activity were then assayed in situ 48 hours after injury. In all injured segments, there was an area of morphologically distinct endothelial cells at the wound edge, which were readily characterized by 3 important features. Compared with cells away from the edge, these cells were more elongated (Figure 1.1A and 1.1B), more darkly stained with hematoxylin (Figure 1.1B), and showed greater eNOS expression by immunohistochemistry (Figure 1.1A). Increased immunohistochemical staining was evident in segments without counterstain as well; therefore, this increase cannot be explained as a result of the generally darker counterstained appearance of the cells along the wound edge (Figure 1.1B). No DAB staining was evident in segments in which the primary antibody had been substituted with preimmune mouse IgG at the same dilution (Figure 1.1B) or in smooth muscle cells (not shown).

At the subcellular level, eNOS protein was evident both in the perinuclear region and associated with the plasma membrane in cells at the wound edge (Figure 1.1C, open and closed arrows, respectively). In contrast, in cells distant from the wound edge, there was only perinuclear expression of eNOS (Figure 1.1D). In addition, the pattern of perinuclear
staining in regenerating endothelium was different from that of the undisturbed endothelium. In undisturbed areas, eNOS appeared as grainy, perinuclear aggregates (Figure 1.1D), whereas in regenerating areas, eNOS had a more diffuse pattern that extended into the cell cytoplasm (Figure 1.1C). In contrast to an increase in eNOS levels at the wound edge, no change in von Willebrand factor immunoreactivity of adjacent aortic segments was observed. Thus, eNOS immunohistochemistry demonstrated an increase in protein expression at the leading edge of the regenerating endothelium and increased association of eNOS with the plasma membrane.

To investigate whether iNOS was present in the regenerating endothelium, iNOS immunohistochemistry was performed 48 hours after injury. No iNOS was detected in endothelial cells at the wound edge or elsewhere after gentle injury (not shown). The absence of iNOS suggests that in partially denuded aortas at early times after injury (<48 hours), eNOS plays the dominant role in cellular events involving NO production.

Increased eNOS Expression at the Wound Edge: NADPH Diaphorase Activity
To confirm the results of eNOS immunohistochemistry in rat aortic endothelium, an NADPH diaphorase activity assay was performed under conditions specific for eNOS. NADPH diaphorase activity assay also showed increased enzyme activity at the leading edge of the regenerating endothelium (Figure 1.2A). There was no formazan precipitate in tissue in which NADPH was replaced with NADP, which cannot be metabolized by eNOS (Figure 1.2B). The blue reaction product representing NADPH diaphorase activity was evident primarily in association with the plasma membrane at the leading edge, with some formazan precipitate in perinuclear regions (Figure 1.2C). In uninjured areas >200 μm away from the wound, the stain occurred primarily in association with the nuclei, although some membrane association was evident as well (Figure 1.2D). Similar results were observed in aortas from sham, uninjured animals. Quantitative image analysis confirmed the differences in subcellular localization of the enzyme between the wound edge and undisturbed endothelium. At the wound edge, 90.6±1.4% (n=7 segments from 4 animals) of the formazan precipitate was not associated with the nucleus, whereas in the undisturbed area (75 rows of cells away from the wound), 64.3±4.9% (n=6 segments from 4 animals) of the precipitate was not associated with the nucleus. The difference was statistically significant (P=0.0011). There was also a statistically significant difference between the wound edge and all other areas tested (25, 50, and 75 rows of cells away from the wound), whereas no differences were detected among these areas themselves. These results demonstrate an increase in eNOS levels and translocation of eNOS from a perinuclear to a plasma membrane location at the leading edge, as measured by NADPH diaphorase activity.

eNOS Colocalization With a Golgi Marker and Caveolin: Immunohistochemistry
To relate eNOS immunohistochemical results with specific subcellular compartments, immunohistochemistry for mannosidase II (a Golgi marker) and caveolin (a Golgi and caveola marker) was performed in aortic segments adjacent to those stained for eNOS (Figure 1.3). Mannosidase II staining showed primarily a perinuclear distribution (Figure 1.3A and 1.3B). Caveolin was detected both in the perinuclear regions and associated with the plasma membrane (Figure 1.3C and 1.3D). No changes in overall staining intensity or localization for either of these marker proteins were detected at the wound edge, nor were there significant differences between the wound edge (Figure 1.3A and 1.3C) and undisturbed endothelium (Figure 1.3B and 1.3D). Based on these markers it appears that eNOS localization is similar to that of the Golgi and caveolae in vivo.

Increased eNOS Expression at the Wound Edge: In Situ mRNA Hybridization
To investigate whether changes in mRNA expression were involved in the increased eNOS expression at the leading edge of the regenerating endothelium, in situ mRNA hybridization for eNOS was performed. In situ hybridization demonstrated a strong signal in every cell along the wound edge (Figure 2A and 2E). The area of the positive in situ hybridization signal along the wound edge colocalized with the area positively stained for eNOS protein by histochemistry and immunohistochemistry. No signal was evident in segments hybridized to sense probe (Figure 2C) or in smooth muscle cells exposed to antisense probe. To obtain improved cellular localization of eNOS mRNA expression, nonradioactive in situ hybridization with digoxigenin-labeled probes was performed. The same pattern of mRNA expression was observed; there was a strong eNOS mRNA signal in every cell at the edge of the wound but not in cells farther away from the wound (not shown).

Correlation Between eNOS Expression and Cell Proliferation
To determine whether there was a correlation between endothelial cell proliferation and eNOS expression, BrdU tablets were administered to the animals subcutaneously 24 hours before they were killed. The greatest BrdU incorporation was observed at the edge of the wound, with a relatively narrow band of cells demonstrating stained nuclei (not shown). To quantify the number of endothelial cells that had entered the S phase of the cell cycle, a proliferation index was calculated as the ratio of the number of BrdU-stained nuclei to the total number of cells overlain by a systematically applied reticle positioned at the edge of the wound. The BrdU proliferation index was 21% (n=2 vessels). No BrdU incorporation occurred in the areas away from the wound. No staining was evident in segments in which antibody for BrdU had been substituted with preimmune mouse IgG at the same dilution. The relatively low ratio of BrdU-stained cells to eNOS-expressing cells suggests that factors other than cell proliferation also regulate eNOS expression in regenerating endothelium.

eNOS Expression in Regenerating Endothelial Cells in Tissue Culture
To investigate whether a similar change in eNOS expression occurs in an in vitro model of regenerating endothelium, a 3- to 5-mm scratch was made in a confluent BAEC monolayer.
Little difference in eNOS immunoreactivity and NADPH diaphorase activity was observed 6 hours after injury. After 24 hours, however, cells at the edge of the denuded area had increased levels of eNOS by eNOS immunohistochemistry (Figure 3A) and by NADPH diaphorase (Figure 3B). After 48 hours, the monolayer was totally restored. In preparations 24 hours after injury, an increase in NADPH diaphorase activity was obvious in perinuclear regions and in the cytoplasm of cells on the wound edge but not in association with the plasma membrane (not shown), in contrast to the results observed in vivo. The increased staining at the wound edge was not due to an edge effect, because no increase in formazan precipitate in NADPH diaphorase activity preparations or in DAB precipitate in immunohistochemical preparations was observed at the edge created by the inoculating loop after the cells were paraformaldehyde fixed. Average pixel intensity per cell in NADPH diaphorase-stained samples was 0.71 ± 0.04 at the wound edge (n = 3 independent experiments of 2 to 5 slides each) compared with 0.32 ± 0.03 in undisturbed areas (n = 3, P < 0.05), comprising a 123 ± 4% (n = 3, P < 0.001) increase in eNOS expression. The BrdU index was 4% (n = 2) at the wound edge compared with no BrdU staining in undisturbed regions. The magnitude of eNOS induction at the

Figure 2. Greater eNOS expression at leading edge of regenerating endothelium: in situ hybridization. eNOS mRNA is demonstrated by presence of silver grains, appearing as white dots in the dark-field photomicrographs. In light photomicrographs, cell position and wound edge are shown by hematoxylin-stained nuclei. Denuded areas are marked with an asterisk. A, Dark-field photomicrograph in which wounded endothelium was hybridized to antisense cDNA shows strong signal and greater association of silver grains with regenerating endothelium compared with undisturbed endothelium. B, Light photomicrograph of same field as in A. C, Dark-field photomicrograph of wound edge hybridized with sense cDNA shows very little signal. D, Light photomicrograph of same field in C. E, Higher-magnification dark-field photomicrograph of cells at edge shows very close association of silver grains with nuclei. F, Light photomicrograph of same field in E. Bar corresponds to 100 μm in A through D and to 20 μm in E and F.
wound edge measured by immunohistochemistry and NADPH diaphorase activity assay was smaller in tissue culture than in the rat aorta. Nevertheless, the similarities in NADPH activity and eNOS protein expression in tissue culture compared with the in vivo conditions indicate that the regulatory signals stimulated in endothelial cells by regeneration are similar in vivo and in vitro.

Inhibition of eNOS Induction by TGF-β1 Block In Vitro
To investigate the role of TGF-β1 in eNOS induction at the wound edge, a TGF-β1-neutralizing antibody was added to wounded BAEC monolayers. Compared with the cultures treated with the same concentrations of preimmune chicken

Figure 3. Greater eNOS expression at leading edge of regenerating BAEC monolayer in vitro: eNOS immunohistochemistry and NADPH diaphorase activity. BAEC monolayers were wounded and allowed to regenerate for 24 hours. Denuded areas are marked with an asterisk. A, eNOS immunohistochemistry shows increase in DAB precipitate at edge of regenerating monolayer. No counterstain was used. B, NADPH diaphorase activity assay shows increase in formazan precipitate at wound edge. No counterstain was used. Notice similarity in appearance of eNOS expression in the 2 assays. Bar corresponds to 30 μm.

Figure 4. TGF-β1 blockade diminishes eNOS induction at wound edge: NADPH diaphorase activity in BAECs. BAECs were grown, 3- to 5-mm wound was created as described in Methods, and cultures were studied 24 hours later. TGF-β1 antibody was applied to cells immediately before injury at concentrations of 0.3 to 30 μg/mL. Preimmune chicken serum (10 μg/mL) was used as negative control. BrdU (10 μg/mL) was added to a matching set of TGF-β1 antibody–treated cells. A, Wound edge of control culture that received chicken serum. B, Wound edge of TGF-β1–neutralizing antibody (10 μg/mL)–treated cultures. Bar corresponds to 5 μm. C, Histogram of pixel intensity per cell (arbitrary light units) shows dose-dependent decrease in eNOS NADPH diaphorase activity at wound edge in TGF-β1 antibody–treated cells.

Figure 3.

Figure 4.
serum as a control (Figure 4A), the intensity of NADPH staining at the wound edge was reduced in a concentration-dependent manner (0.3 to 30 µg/mL) by TGF-β1 antibody (Figure 4B and 4C). eNOS activity measured by densitometry showed a significant dose-dependent decrease, which resulted in a 64±3% inhibition (n=4 independent experiments of 2 wells each, P<0.05) in eNOS activity at the wound edge in the presence of TGF-β1 antibody concentrations $\leq$1 µg/mL (Figure 4C). Some cell death occurred in monolayers treated with doses of TGF-β1 antibody $\geq$10 µg/mL. Neutralization of the biological activity of TGF-β1 was confirmed by an increase in the number of BrdU-stained nuclei, from 4% in untreated cultures to 12% (n=2) in cultures treated with TGF-β1 antibody, consistent with the antiproliferative effect of TGF-β1 on endothelial cells.

Dynamic Regulation of eNOS In Vivo by Shear Stress

Regenerating endothelium in vivo occurs under conditions of varying fluid shear stress. Because shear stress is an important regulator of eNOS expression,22,13 we extended our in vivo analyses of eNOS expression to sites of varying hemodynamics; specifically, flow dividers, which include regions of increased, decreased, and turbulent flow. We analyzed the levels of eNOS protein and mRNA expression in areas around intercostal openings in the rat thoracic aorta. Both NADPH diaphorase activity (Figure 5A) and eNOS immunohistochemistry (not shown) showed greater eNOS expression in a “crescent” of cells at the downstream region of the intercostal branches. In situ hybridization demonstrated greater deposition of silver grains in the same areas as well (not shown). To verify that increased expression was not due to an edge effect, the aorta-to-intercostal transitional area in fetal calf vessels was pinned out flat (as shown in Figure 5B), and NADPH diaphorase activity was assayed in the tissue. As in the rat, the same crescent of cells with high NADPH diaphorase activity was present at the flow dividers without the possible artifactual folding of the endothelium (Figures 5C through 5E). Based on these findings, increased levels of eNOS mRNA and protein are present in localized areas of higher shear stress in vivo.

Discussion

The major finding of this study is that regenerating endothelial cells in vivo and in vitro show increased eNOS protein and mRNA expression, which is localized to cells at the wound edge. In addition, we show similar highly localized changes in eNOS expression at arterial branch points in a manner consistent with regulation by fluid shear stress. This study is the first to characterize localized eNOS expression in vivo in response to physiological stimuli. Our ability to demonstrate this localized change in eNOS expression was a consequence of our use of en face histochemical and immu-
The lack of VEGF, which is produced by vascular smooth wound edge, accounting for expression and activity. Thus, it is possible that increased regulators of eNOS expression in deendothelialized vessels.

endothelial cells, and to stimulate endothelial cell production of VEGF has been shown to be increased in balloon-injured arteries,14 suggesting that in proliferating BAEC cultures, there is no increase in eNOS gene transcription on the basis of nuclear runoff experiments. In addition, these investigators found decreased expression of an eNOS mRNA–destabilizing protein in proliferating endothelial cells. The current study suggests that stimuli in addition to cell proliferation are important in increased eNOS expression in regenerating endothelium. Specifically, all cells on the wound edge exhibited increased eNOS expression, but only 21% of them had undergone the S phase within 24 hours before the animals were killed, based on BrdU uptake. Therefore, most of the cells on the wound edge are migrating cells that have not undergone recent cell mitosis. These findings suggest that in regenerating endothelium, ENOS induction may be related to signal events associated with either cell migration or switching from a locomotor to an adherent phenotype, as well as cell cycle progression. This conclusion is supported by the findings in tissue culture that endothelial cells treated with TGF-β1 to the membrane is functionally important, because mutations that in proliferating BAEC cultures also suggest that TGF-β1 is locally regulated, similar to eNOS. The results of our TGF-β1–blocking experiments with BAECs also suggest that TGF-β1, which is produced by vascular smooth muscle cells, may explain the smaller induction of eNOS expression at the wound edge in tissue culture compared with in vivo. Because VEGF induction in balloon-injured vessels is stimulated by TGF-β1, it is clear that TGF-β1 is one of the key regulators of ENOS expression in deendothelialized vessels. However, it is possible that VEGF is the primary, direct stimulus for eNOS upregulation. Interestingly, recent data show that NO can downregulate VEGF expression in reendothelializing vessels,21 suggesting an efficient negative-feedback mechanism.

Components of the plasminogen activator system, including tissue plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and its receptor (UPAR), as well as plasminogen activator inhibitor-1, are increased after arterial wounding.24 These molecules are involved in cell-matrix interactions, and UPAR expression is associated with increased migration of endothelial cells.25 Interestingly, plasmin (produced by the action of tPA and uPA) has been shown to activate extracellular latent TGF-β1, secreted by cells,26 suggesting that the plasminogen activator system exerts both direct effects (uPA-UPAR) and indirect effects (TGF-β1 activation) on eNOS expression. Finally, increased shear stress or shear rate may contribute to eNOS expression. Cells at the edge of a wound are subjected to the same flow velocity as cells of the undisturbed endothelium; however, they may experience increased shear stress or shear rate because of a change in shape. It has long been noted that endothelial cells at wound edges have elongated shapes,17 and this appearance has been termed the locomotor phenotype. It is possible that locomotor phenotype cells are not only elongated but thickened as well, on the basis of their more intense staining with counterstains (compare Figure 1.1C with 1.1D). If this is true, their greater protrusion into the vessel lumen compared with flatter, nonmigrating cells may be sufficient to cause increased shear rate and shear stress,27 a stimulus for eNOS induction. Further studies with scanning and transmission electron microscopy will be necessary to quantify cell morphological changes at the wound edge.

An exciting finding of the en face studies performed here was the apparent translocation of eNOS to the plasma membrane in cells at the wound edge. Translocation of eNOS to the membrane is functionally important, because mutations of carboxyl amino acids that define the myristoylation or palmitoylation sites10 prevent association with the plasma membrane and result in decreased production of NO. In addition, Shaul et al2 and Liu et al2 have suggested that localization of eNOS to caveolae is necessary for maximal production of NO. eNOS has been shown to be targeted to the plasma membrane and to caveolae7,11 by several mechanisms, including myristoylation, palmitoylation, and binding to caveolin. Although there was a small increase in immunoreactive caveolin at the plasma membrane in cells at the wound edge, caveolin in the endothelial cells at the wound edge, the mechanisms responsible for eNOS translocation in regenerating endothelium remain undefined.

Several important physiological consequences may result from increased eNOS expression and, by extrapolation, increased NO production at the wound edge. First, NO may regulate endothelial cell migration,20–22 which is orchestrated in an orderly fashion in regenerating endothelium. Appropriate regeneration requires signals first to initiate cell migration and then to inhibit migration (once cells have repopulated the denuded area). Migration-initiating signals may include increased expression of osteopontin and its receptors (eg, α,β,γ,δ),
integrin), as well as uPA/UPAR, which have been shown to be greatly increased on the leading edge of regenerating endothelium and to stimulate endothelial cell migration in vitro.6,32 In contrast, a recent report based on tissue culture studies31 indicated that NO inhibits endothelial cell migration. Thus, a balance between promigratory actions of osteopontin and UPAR and the antimigratory action of NO31 may orchestrate endothelial cell regeneration. Second, NO may regulate the switch from the endothelial cell locomotor phenotype to the vasoactive phenotype, as suggested by neuronal cell differentiation in response to NO.36 Finally, NO has been well documented to inhibit platelet adhesion,34 smooth muscle cell proliferation,35 and smooth muscle cell migration,36 suggesting a role for NO in the prevention of neointima formation after injury.

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References

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Veronica Poppa, Jody K. Miyashiro, Marshall A. Corson and Bradford C. Berk

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