Published in final edited form as:

Microvasc Res. 2019 March ; 122: 34–40. doi:10.1016/j.mvr.2018.11.004.

Endothelial nitric oxide synthase protein distribution and nitric oxide production in endothelial cells along the coronary vascular tree

Cristine L. Heaps, Jeffrey F. Bray, Avery L. McIntosh, and Friedhelm Schroeder

Department of Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843

Abstract

Objective: Freshly isolated endothelial cells from both conduit arteries and microvasculature were used to test the hypothesis that eNOS protein content and nitric oxide production in coronary endothelial cells increases with vessel radius.

Methods: Porcine hearts were obtained from a local abattoir. Large and small arteries as well as arterioles were dissected free of myocardium and homogenized as whole vessels. Additionally, endothelial cells were isolated from both conduit arteries and left ventricular myocardium by tissue digestion with collagenase, followed by endothelial cell isolation using biotinylated-anti-CD31 and streptavidin-coated paramagnetic beads. Purity of isolated endothelial cells was confirmed by immunofluorescence and immunoblot.

Results: In whole vessel lysate, immunoblot analysis revealed that protein content for eNOS was greater in arterioles compared to small and large arteries. Nitric oxide metabolites (nitrite plus nitrate; NO_x) levels measured from whole vessel lysate decreased as vessel size increased, with both arterioles and small arteries displaying significantly greater NO_x content than conduit. Consistent with our hypothesis, both eNOS protein level and NO_x were significantly greater in endothelial cells isolated from conduit arteries compared with those from coronary microvasculature. Furthermore, confocal microscopy revealed that eNOS protein was present in all conduit and microvascular endothelial cells, although eNOS staining was less intense in microvascular cells than those of conduit artery.

Conclusions: These findings demonstrate increased eNOS protein and NO_x content in endothelial cells of conduit arteries compared with the microcirculation and underscore the importance of comparing endothelial-specific molecules in freshly isolated endothelial cells, rather than whole lysate of different sized vessels.

Corresponding author: Cristine L. Heaps, Ph.D., Dept of Physiology & Pharmacology, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX 77843-4466, Telephone (979) 458-0753, cheapsc@cvm.tamu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures

There are no potential conflicts of interest, financial or otherwise to disclose.

Keywords

Microcirculation; confocal microscopy; coronary hemodynamics; vascular heterogeneity; eNOS distribution

Introduction

Nitric oxide is an important regulator of blood pressure and vascular function. The vascular endothelial enzyme, eNOS, is the primary source of nitric oxide production in the vascular wall. The distribution of eNOS protein in different branch orders of the coronary arterial tree has been compared previously using immunoblot analysis¹. These studies were completed utilizing whole vessel lysate and revealed that the content of eNOS protein relative to total vessel protein was similar across multiple diameter ranges of arteries and arterioles; only arterioles smaller than 50 μm displayed less eNOS content than the large arteries. However, as acknowledged by the authors¹, these studies were completed in lysate from whole coronary arteries and arterioles and thus were influenced by a relative increase in the contribution of smooth muscle and connective tissue proteins as vessel diameter increased. Indeed, these investigators¹ examined the relationship between vessel diameter and the number of smooth muscle cells in the wall of porcine coronary arteries and arterioles. Histological analysis revealed that the smallest arterioles (<50 μm) contained only one layer of smooth muscle cells, whereas larger coronary arteries (>300 μm) had 10–15 smooth muscle cells per endothelial cell¹, indicating that eNOS protein would be diluted to a greater extent in lysate from large arteries compared with that from arterioles. Taken together, these findings suggest that endothelial cells of large arteries contain greater eNOS protein levels compared with arterioles.

The assertion that larger vessels contain more eNOS protein was also supported by past studies in cultured primary coronary microvascular endothelial cells, in which ~30% expressed eNOS, whereas ~100% of cultured primary aortic endothelial cells expressed eNOS². Importantly, these studies by Ando et al.² also revealed that eNOS levels in primary coronary microvascular endothelial cells decreased while in culture although aortic endothelial cell eNOS levels were not affected. These findings suggest that eNOS measures in cultured microvascular cells may not reflect levels in freshly isolated cells and that hemodynamic and other stimuli present in the *in vivo* setting of the microcirculation may be necessary to maintain eNOS levels.

For the present study, we freshly isolated endothelial cells from epicardial coronary arteries and the microcirculation to test the hypothesis that eNOS protein content and NO_x levels in coronary endothelial cells increases in parallel with vessel radius. This novel approach allowed us to determine eNOS protein levels and NO_x content without influence from a relative increase in the contribution of smooth muscle and connective tissue proteins as vessel diameter increased. Furthermore, isolation of fresh coronary endothelial cells provides an advantage over cultured cells by studying cells that have been exposed to the *in vivo* stimuli associated with blood flow and pressure within hours of being snap-frozen for subsequent analysis. Finally, using confocal microscopy, we also explored the presence of

eNOS protein at the cellular level in endothelial cells isolated from both the conduit artery and microvasculature.

Materials and Methods

Isolation of coronary arteries and arterioles.

Pig hearts were obtained from a local abattoir and transported to the laboratory in iced Krebs bicarbonate buffer (0–4 °C). Animals were approximately 5 months old and 85–100 kg. With the aid of a dissection microscope, coronary epicardial arteries (2–5 mm diameter) were isolated from the heart, cleaned of myocardium, and trimmed of fat and connective tissue. Similarly, small arteries (150–350 µm) and arterioles (75–125 µm) were isolated from the subepicardium of the left ventricle for use in experimental protocols described below. Whole vessel homogenates from these vessel segments were compared in immunoblot analysis.

Preparation of coronary arteries for endothelial cell isolation.

Additional epicardial coronary arteries were dissected from the heart and endothelial cells isolated by opening the vessel and pinning lumen side up and then digesting in DMEM:F12 (1:1) media containing 2 mg/ml collagenase II (Worthington #4176; 210 U/mg; 90 min at 37 °C). Following digestion, the tissue was triturated with a 5-ml pipet and diluted 2× with DMEM:F12 media, then centrifuged at 1,000 rpm for 5 min. Supernatant was removed, the cell pellet was washed and then resuspended with DMEM:F12 media plus 5% FBS for subsequent isolation of endothelial cells, as described below.

Preparation of coronary microvasculature for endothelial cell isolation.

A myocardial section (~1.0–1.3 grams) from the left ventricle near the apical region of the heart was isolated and minced into small pieces (<2 mm squares) with scissors. The tissue was digested in 5 ml DMEM:F12 media containing 2 mg/ml collagenase II (Worthington #4176; 210 U/mg; 45 min at 37 °C). After addition of DNase I (10 µl; Thermo #90083), the sample was repeatedly pipetted to help disperse larger tissue pieces. The sample was digested an additional 45–60 min. Following digestion, the tissue was triturated with a 5-ml pipet and diluted 2× with DMEM:F12 (1:1). Digest was filtered through a 100-µm nylon mesh then centrifuged at 1,000 rpm for 5 min. Supernatant was removed, the cell pellet was washed with DMEM:F12 media, centrifuged again, and the cell pellet was resuspended in DMEM:F12 plus 5% FBS for subsequent isolation of endothelial cells, as described below.

Endothelial cell isolation from coronary artery and microvascular lysate.

Anti-CD31 (BD Biosciences no. 550300) was biotinylated using EZ-Link NHS-Biotin (Thermo Fisher Scientific no. 20217) per manufacturer's instructions. Biotinylated-anti-CD31 (7.5 µl) was added to DMEM:F12-resuspended cell pellets; final concentration of the biotinylated antibody was ~7.5 µg/ml. Following addition of biotinylated-antibody, the cell solution was rotated for 30–60 min at 4 °C after which streptavidin magnetic beads (10 µl; New England BioLabs no. S1420S) were added and mixed for an additional 30–60 min. Bead/cell complexes were collected using a magnet. Complexes were washed 3× with DMEM:F12 plus 5% FBS, using a magnet to recover cells after each wash.

Histology.

To assess the diameters of blood vessels from which microvascular endothelial cells were isolated, an analogous myocardial section from a separate set of hearts was isolated near the apical region of the left ventricular free wall and frozen by slow immersion (20–30 sec) into liquid nitrogen-cooled 2-methylbutane. The myocardium was sectioned (~6 µm thick) in a chilled cryostat (–20 °C) and stained with Masson's trichrome stain for assessment of blood vessel diameters in the myocardial segment. Vessel diameters were measured from bright-field images with the NIS Elements software (Nikon) distance feature; calibration was confirmed with a micrometer slide. We pursued these studies in frozen rather than fixed tissue to avoid tissue shrinkage and thus, change in blood vessel diameter that occurs with fixation.

Immunoblots.

Lysates were prepared from whole arteries and arterioles and isolated endothelial cells for immunoblot analysis. Coronary main epicardial arteries (2–5 mm diameter; 1 cm length), small coronary arteries (150–350 µm; ~10 mm total length), and arterioles (75–125 µm diameter; ~15–20 mm total length) were isolated, quick-frozen in liquid N₂ and stored at –80 °C for later immunoblot analysis. Alternatively, endothelial cells were isolated from coronary arteries and myocardial microvasculature as described above, quick-frozen and stored at –80 °C. Lysate (20 µg total protein) was subjected to SDS-polyacrylamide gel electrophoresis (4–20% gradient gel), transferred to PVDF membrane, blocked with 5% non-fat dry milk, then probed for 1 hour or overnight with primary antibody. Primary antibodies for immunoblot included eNOS (BD Biosciences no. 610297, 1:500), smooth muscle α-actin (Abcam no. ab21027, 1:1000), troponin-I (Advanced Immunochemical no. 19C7; 1:2,000), and GAPDH (Advanced Immunochemical no. RGM2–200; 1:5,000). After washing, membranes were incubated with the appropriate horse radish peroxidase-conjugated species-specific anti-IgG. Scanning densitometry was used to quantify signal intensities which were normalized with GAPDH or SYPRO ruby protein blot stain (Lonza no. 50565).

Measurement of NOx levels.

Nitrite plus nitrate (NO_x), the stable oxidation products of nitric oxide, were measured in vessel extracts as indicators of nitric oxide synthesis using the Griess assay modified as described previously³. For these studies, frozen epicardial arteries (2–5 mm diameter, 2–3 mm length, n=1 artery/sample), small arteries (150–350 µm diameter, 2–3 mm length, n=3–5 arteries/sample), and arterioles (75–125 µm diameter, 2–3 mm length, n=30–40 arterioles/sample) were ground to a fine powder, extracted with phosphate buffered saline (PBS; 55 µl for small arteries and arterioles and 125 µl for conduit) for 20 min at room temperature, and centrifuged at 12,000 g for 5 min. For isolated endothelial cell samples, PBS (55 µl) was added and extracts were obtained by three freeze/thaw/vortex cycles, then centrifuged at 12,000 g for 5 min. Supernatant from vessel or endothelial cell extracts was used in the Griess assay for measurement of combined nitrite and nitrate. Duplicate samples were adjusted to 100 µl volume with PBS to which equal volume of Griess reagent was added. Griess reagent consisted of (in mg/ml): 2.5 vanadium chloride, 1 sulfanilamide, 0.05 N-(1-

Naphthyl)ethylenediamine dihydrochloride in 0.6 N HCl. Potassium nitrate was used to generate a standard curve (10 μ m to 0.15 μ m using 2 $\times$ serial dilutions). A sodium nitrite curve was also run at the same concentrations to determine nitrate reduction efficiency. Plates were incubated for 16 hours in the dark at room temperature, to optimize conversion of nitrate to nitrite as indicated previously,³ and then read at A540. Nitrate plus nitrite concentrations were calculated based on the standard curve and these values were normalized to total protein.

Immunolabeling of freshly isolated endothelial cells.

Resuspended cells were seeded onto glass slides and fixed with paraformaldehyde diluted to 2% (Thermo Fisher Scientific no. 157–8; electron microscopy grade) in PBS (pH 7.4; 10 min at room temp). Slides were rinsed in PBS, incubated in glycine (100 mM for 10 min), followed by PBS washes (3 $\times$ 3 min). Primary antibodies were diluted in 0.1% Triton X-100, 2% BSA (Sigma no. A7030), and SSC buffer (150 mM NaCl, 15 mM NaCitrate, pH 7.4) and included eNOS (Thermo Fisher Scientific no. PA5–16887; 1:200), CD31 (BD Biosciences no. 550300; 1:100), and CD105 (Thermo Fisher Scientific no. MA1–19408; 1:200). Fixed cells were incubated with primary antibody overnight, 4 $^{\circ}$ C, followed by washes in PBS (4 $\times$). Secondary antibodies (anti-mouse Alexa 488 and anti-rabbit Alexa 594 at 1:500 in PBST) and Hoechst 33342 (0.5 μ g/ml) were added to cells and incubated (1 hr at room temp). After washes in PBS (5 $\times$), cells were covered with ProLong anti-fade solution (Thermo Fisher Scientific Molecular Probes no. P36934) and a cover slip. Optimal dilutions for antibodies were based on experiments where staining was performed with a range of dilutions. Control experiments were also performed with normal rabbit and mouse sera, omitting the primary antibodies and using secondary antibodies only. These procedures assured optimization of antibody concentration and hence minimized non-specific staining.

Epifluorescence microscopy.

Endothelial cells were excited with a 175-W xenon arc lamp. Hoechst and Alexa 488 were excited at wavelengths of 350 nm and 495 nm and fluorescence emission captured at 450 nm and 515 nm, respectively. Excitation wavelengths were generated by a Lambda-DG4 controller (Sutter). Images were collected using a Nikon Eclipse Ti microscope equipped with an x10 Plan Fluor oil immersion DIC objective, numerical aperture of 0.3 coupled with a Quantem 512SC camera. Fluorescence images were acquired using NIS Elements (Nikon) software.

Laser scanning confocal microscopy.

A MRC-1024 point scanning laser confocal microscopy system (Bio-Rad) equipped with a Zeiss Axiovert 135 inverted microscope, fitted with x63, 1.4 numerical aperture oil immersion lens. Alexa 488 and Alexa 594 were excited with the 488-nm and 564-nm laser lines of an Ar⁺/Kr⁺ laser, respectively, while Hoechst was excited with a 408-nm laser line from a 408-nm diode laser. Alexa 488, Alexa 594, and Hoechst were detected through a HQ530/40, HQ615/30, and HQ470/20M filters, respectively. Confocal images were acquired with Laser Sharp Software (Bio-Rad) and analyzed with Laser Sharp, Metamorph (Molecular Devices), and Adobe Photoshop.

Data Analysis.

Statistical analyses were completed using parametric or non-parametric tests as appropriate for the data analyzed, followed by post-hoc tests for multiple comparisons when a main effect was identified. Specific statistical tests used for each data set are provided in the figure legends. For all analyses, a P value ≤ 0.05 was considered significant. Data are presented as mean $\pm$ S.E.M., and n values in parentheses reflect the number of animals studied.

Results

We examined the protein levels of eNOS in lysate of isolated arterioles (A; 75–125 μ m), small arteries (SA; 150–350 μ m), and large conduit arteries (LA; 2–5 mm). HUVEC (H) were used as positive control for eNOS and GAPDH. Protein levels of eNOS were normalized to both GAPDH and SYPRO ruby protein gel stain; staining for eNOS, GAPDH, and SYPRO were completed on the same blot. These studies demonstrated that eNOS protein levels were significantly increased in arteriole lysate compared with that of large arteries when eNOS was normalized to GAPDH or SYPRO (Figure 1). GAPDH levels were not different across vessel sizes. Furthermore, although eNOS protein levels in small arteries were somewhat less than that observed in arterioles and slightly more than that of large arteries, these differences did not achieve statistical significance.

To specifically ascertain eNOS protein content in endothelium without contamination of other vascular wall cells types, we optimized a protocol for the fresh isolation of conduit and microvascular endothelial cells. In these studies, microvascular endothelial cells were isolated from a myocardial section near the apical region of the heart. Histological analysis of sections from four pigs revealed that the single largest vessel through the section taken from each pig averaged $266 \pm 29 \mu$ m (range 181–304 μ m). All other vessels in the microvascular myocardial sections were less than 150 μ m, as represented in Figure 2A. For these experiments, conduit artery segments and myocardial sections were digested enzymatically and endothelial cells isolated using biotinylated-anti-CD31 and streptavidin-coated paramagnetic beads. Traditional fluorescence microscopy of isolated cells labeled with the DNA stain, Hoechst (Figure 2B), and the endothelium-specific marker, CD31 (Figure 2C) was used to determine purity of our endothelial cell isolation. Hoechst-positive and CD31-positive images were colocalized (Figure 2D) and a pixel fluorogram of labeled cells in the image constructed (Figure 2E) as described previously⁴. Correlation coefficients (Figure 2E) revealed that in this sample, 100% (Red=1) of Hoechst-positive cells were also CD31-positive, as determined by the ratio of the sum of intensities from colocalized pixels (CD31- and Hoechst-positive cells) to the sum of intensities of all red pixels (Hoechst-positive). CD31-positive pixels that were Hoechst-negative (non-nucleated; Green=0.47) were likely endothelial cellular debris because the low resolution conditions (traditional fluorescence microscopy and low magnification) likely would not discern intact cell plasma membrane CD-31 from nuclear Hoechst. Overall, the effectiveness of our method revealed that microvascular endothelial cells comprised $97 \pm 2\%$ of nucleated cells in all myocardial samples examined ($n=3$ pigs). As further confirmation, immunoblot analysis of cell lysate demonstrated preservation of the endothelial marker, eNOS protein, while α -smooth muscle actin was markedly reduced compared with that found in small artery or arteriole lysate

(Figure 2F). In addition, cardiac troponin I was not detectable in cell lysate compared with myocardial lysate as positive control (Figure 2G). Hoechst-positive and CD31-positive images were similarly colocalized and pixel fluorograms constructed for conduit artery endothelial cell isolation and revealed 94 ± 2 % sample purity (n=4 pigs).

We next compared total eNOS protein levels in lysate of endothelial cells isolated from coronary conduit arteries or the microcirculation, as well as whole arteriole and conduit segments (Figure 3). Immunoblot images for eNOS and GAPDH as loading control are shown in Figure 3A. These studies revealed that eNOS protein level in endothelial cells isolated from conduit arteries was significantly greater than that observed in all other sample preparations (Figure 3B). Importantly, eNOS protein level in endothelial cells isolated from conduit arteries was eight-fold greater than that observed in the coronary microvascular endothelial cells. Interestingly, eNOS protein levels in whole arteriole lysate was very similar to that observed in isolated microvascular endothelial cells (Figure 3B). Comparable to that observed in Figure 1, eNOS protein content in whole arterial lysate was less than half of that detected in arteriolar lysate. Furthermore, GAPDH levels were not different across samples.

To begin to determine whether the lower eNOS content in endothelial cells isolated from the microcirculation compared with that from conduit artery is a reflection of an absence of eNOS protein in some microvascular endothelial cells or just an apparent overall lower level of eNOS, we evaluated isolated endothelial cells using laser scanning confocal microscopy. Cells were doubly immunolabeled for eNOS and CD105 (endothelial marker) and also labeled with the DNA stain, Hoechst (Figure 3C–H). Findings from these studies revealed that eNOS protein was present in all endothelial cells of large coronary artery as well as that of the microvasculature. However, comparison of confocal images of isolated endothelial cells from thirteen conduit and nineteen microvascular samples that were processed concurrently revealed that eNOS staining consistently appeared less intense in microvascular endothelial cells than those of the large artery as represented in Figures 3D and 3G. Indeed, contrast and gain of the confocal microscope had to be increased substantially to visualize eNOS in endothelial cells isolated from the microvasculature relative to endothelial cells of the large artery. As apparent in Figure 3G, small punctate areas of intense eNOS staining were also typical of microvascular endothelial cells, found in seventeen of nineteen samples, whereas the intensity of staining was more uniform in conduit endothelial cells.

In Figure 4, we used equivalent data acquisition parameters (laser power, aperture, and photomultiplier voltages) for direct comparison of fluorescence intensity of labeled eNOS in endothelial cells isolated from conduit coronary artery and coronary microcirculation with equivalent data acquisition parameters. Representative confocal images of endothelial cells from conduit artery and the microvasculature are shown in Figures 4A and 4B, respectively. Due to the low intensity of eNOS in the microvasculature (Figure 4B), 8-bit grayscale images were pseudo-colored for visualization using a lookup table and a grayscale ramp was added. Quantitative analysis revealed that the average intensity of the eNOS fluorescence was 5.6 ± 0.5 -fold lower in microvascular compared with conduit endothelial cells.

NOx levels.

We examined NOx levels under basal conditions in lysate of isolated arterioles (75–125 μ m), small arteries (150–350 μ m), and large conduit arteries (2–5 mm). NOx levels were normalized to total extracted protein levels. These studies demonstrated that NOx levels were significantly decreased in large arteries compared with that of arteriole and small artery lysates (Figure 5A). We next evaluated NOx levels in lysate of endothelial cells isolated from both the microvasculature (MEC) and conduit arteries (CEC). These studies revealed NOx levels in endothelial cells isolated from the microvasculature was significantly reduced compared with that observed in conduit arteries (Figure 5B). Importantly, NOx levels in endothelial cells isolated from conduit arteries was more than four-fold greater than that observed in the coronary microvascular endothelial cells.

Discussion

These studies are the first to examine eNOS protein content and NOx levels in endothelial cells freshly isolated along the coronary vascular tree. Using this novel approach, we were able to compare eNOS protein levels and nitric production in endothelial cells from both epicardial conduit arteries and the coronary microcirculation. Findings from these studies reveal that conduit artery endothelial cells contain significantly greater eNOS protein and NOx content than endothelial cells from the microcirculation. Laser scanning confocal microscopy of isolated endothelial cells stained with the DNA-specific stain Hoechst, the endothelial-specific marker anti-CD105, and anti-eNOS indicated that eNOS protein was detectable in every endothelial cell examined from both large coronary artery and the microcirculation.

Contrary to our findings in isolated endothelial cells, comparison of eNOS protein content in whole vessel lysate demonstrated that eNOS protein in arterioles was significantly greater compared with large arteries. These data are supported by a previous finding in five sequential conduit arteries (range 1–8 mm luminal diameter) providing blood flow to the hind limb of pig, where eNOS protein content increased as vessel diameter decreased⁵. We anticipated that this would be the outcome of our studies because in whole vessel lysate, endothelial-specific proteins are influenced by variable amounts of non-endothelial cell types, such as smooth muscle and connective tissue proteins, in the lysate as vessel diameter increases. Laughlin and colleagues have previously documented using histological analyses that the number of smooth muscle layers increases as vessel diameter increases¹, providing evidence that endothelial-specific proteins would be diluted to a greater extent in lysate from large arteries compared with that from arterioles.

It is important to note that our findings regarding eNOS protein content in freshly isolated endothelial cells vary from those in primary culture. Ando et al. previously isolated and subsequently cultured porcine aortic and coronary microvascular endothelial cells². These studies revealed that eNOS protein levels decreased during culture of coronary microvascular endothelial cells while aortic endothelial cell eNOS levels were not affected. Indeed in primary culture, ~30% of microvascular endothelial cells contained eNOS protein whereas nearly 100% of primary aortic endothelial cells were eNOS positive². With subsequent passages, microvascular endothelial cells containing eNOS protein dropped off

even further, whereas aortic endothelial cell eNOS levels remained unchanged. In contrast to Ando et al., findings from our studies using freshly isolated endothelial cells suggest that eNOS protein is present in all endothelial cells of both large coronary artery and microvasculature. However, eNOS staining was consistently less intense in microvascular endothelial cells than that of the large artery. Taken together with our immunoblot data comparing eNOS protein content in isolated endothelial cells of large artery and the microvasculature, these data suggest that eNOS is present in all freshly isolated endothelial cells but appears to be less abundant in cells of the microcirculation.

Mechanisms that underlie increased eNOS protein content in conduit coronary arterial endothelial cells compared with that of the microcirculation is unclear. The pulsatile nature of blood pressure and flow creates hemodynamic forces in the vasculature that are especially complex in the coronary circulation as a result of extensive cyclical variations in coronary blood flow during the cardiac cycle. Hemodynamic forces, including oscillatory shear stress, cyclic strain, and unidirectional shear stress act on the vascular endothelium to produce vasoactive responses as well as influence gene expression. Oscillatory shear stress, which stems from antegrade and retrograde components of blood flow, has been shown to decrease eNOS mRNA expression^{6, 7} and increase reactive oxygen species⁸ in cultured endothelial cells potentially scavenging nitric oxide, further reducing bioavailability. Cyclic strain results from circumferential stress created by the pulsatile pattern of arterial blood pressure and has shown mixed results regarding eNOS expression in cultured cell models^{6, 9, 10}. Cyclic stretch did not alter eNOS mRNA expression above that found with shear stress alone in endothelial cells grown inside an elastic tube^{6, 10}, although others have shown increased eNOS expression in response to cyclic strain in standard cell culture⁹. Alternatively, shear stress exerted by the unidirectional flow of blood consistently has been shown to increase eNOS expression^{6, 11–13}. Review of the literature suggests that wall shear stress is inversely related to the diameter of the artery^{5, 14, 15}. Hence, the magnitude of shear stress is suggested to be greater in the microcirculation compared with the conduit arteries in humans and large animal models. It is thus difficult to reconcile the significantly greater levels of eNOS protein in the large coronary artery when shear stresses are reported to be lower in the conduit coronary artery. Despite findings that hemodynamic parameters alter eNOS expression, evidence also exists that molecular heterogeneity may exist between endothelial cells of the vascular tree as a result of genetic inclination rather than hemodynamics^{16, 17}. This concept suggests that significantly greater eNOS levels in the conduit artery may be at least in part independent of the mechanics of blood flow. Finally, our findings support previous assertions that mechanisms responsible for vasomotor responsiveness across the vascular tree are highly varied. The increased eNOS protein content that we observed in large coronary arteries compared with the microcirculation is consistent with an apparent greater dependence on nitric oxide-mediated dilation in larger arteries compared to the small arterioles¹⁸.

Study Limitations

Using immunofluorescence and *en face* confocal microscopy of the rat heart, previous studies suggested that coronary venous and capillary endothelium contain less eNOS than arteriolar endothelial cells¹⁹. Similarly, acute exposure (90 min) of excised human

saphenous vein exposed to arterial flow conditions significantly increased eNOS protein levels²⁰, suggesting that the different hemodynamic profile of the venous and potentially capillary beds of the vasculature relative to that of the arterioles likely contribute to lesser eNOS protein levels in endothelial cells of these vascular beds. Furthermore, arteriolar endothelial cells in our microvasculature samples may represent only approximately one-third of the isolated endothelial cells with remainder coming from venules and capillaries. These findings are especially pertinent since our data of protein levels in microvascular endothelial cells revealed that eNOS protein levels were significantly lower compared to isolated endothelial cells from conduit artery (Figures 3 and 4). Thereby, one limitation of our studies is that eNOS protein content from the microcirculation reflects eNOS from arterioles, capillaries, and venules. Therefore, based on studies by Andries et al.¹⁹, microvascular eNOS protein may have been diluted by lesser eNOS levels in endothelial cells of the venules and capillaries and thus not fully reflective of arteriolar eNOS. However, whether eNOS protein levels in venous and capillary endothelial cells are reduced compared to that of arteriolar cells specifically in the coronary vasculature is unknown.

In summary, we have pursued a novel approach using freshly isolated coronary endothelial cells to demonstrate that eNOS protein content is significantly increased in cells of epicardial conduit artery compared with that found in the microvasculature. Our images obtained with confocal microscopy, revealed that eNOS protein was present in all endothelial cells of large coronary artery as well as that of the microvasculature. This finding differs from that observed in primary coronary microvascular endothelial cells where eNOS protein levels decreased while in culture although aortic endothelial cell eNOS levels were not affected². Furthermore, we demonstrate that eNOS protein of endothelial cells isolated from conduit coronary artery is greater and more diffuse throughout the cell, whereas endothelial cells from the microcirculation appear to have a more punctate distribution. The basis for the phenotypic variation in eNOS protein levels observed across the vasculature is unclear. Our findings underscore the significance of comparing endothelial-specific proteins in freshly isolated endothelial cells to more clearly discern protein content, rather than examination in whole vessel lysate or isolated cells that have undergone culture.

Perspectives

Comparison of eNOS protein levels and NOx content in coronary endothelial cells freshly isolated across the vascular tree reveals that eNOS protein and NOx are significantly increased in cells of epicardial conduit artery compared with that of the microcirculation. Furthermore, images obtained with confocal microscopy revealed that eNOS protein was present in all endothelial cells of the microvasculature as well as that of large coronary artery; a finding that differs from that observed in primary coronary microvascular endothelial cells where eNOS protein levels decreased while in culture. Despite previous evidence of an apparent greater dependence on nitric oxide-mediated dilation in larger arteries compared to small arterioles, our data provide new information regarding eNOS distribution and highlight the significance of comparing endothelial-specific molecules in freshly isolated endothelial cells to more clearly discern molecular content, rather than examination in whole lysate from different sized vessels or isolated cells that have undergone culture.

Acknowledgements

We gratefully acknowledge Mr. Kent Fisher of K & C Meats, Navasota, TX for supplying porcine hearts used for these studies. We also appreciate direction provided by Dr. Louise Abbott, Texas A&M University, regarding histology experiments. Our studies were supported by research funds from the National Institutes of Health R56-HL122612 and R01-HL139903. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Grant Funding: NIH R56-HL122612 and R01-HL139903

Abbreviations

eNOS	endothelial nitric oxide synthase
PVDF	polyvinylidene difluoride
CEC	conduit endothelial cells
MEC	microvascular endothelial cells

References

- Laughlin MH, Turk JR, Schrage WG, Woodman CR and Price EM. Influence of coronary artery diameter on eNOS protein content. *Am J Physiol Heart Circ Physiol.* 2003;284:H1307–H1312. [PubMed: 12595288]
- Ando H, Kubin T, Schaper W and Schaper J. Cardiac microvascular endothelial cells express a-smooth muscle actin and show low NOS III activity. *Am J Physiol Heart Circ Physiol.* 1999;276:H1755–H1768.
- Patton CJ and Kryskalla JR. Colorimetric determination of nitrate plus nitrite in water by enzymatic reduction, automated discrete analyzer methods. *US Geological Survey Techniques and Methods, Book 5, Chapter B8*; 2011.
- Atshaves BP, Starodub O, McIntosh A, Petrescu A, Roths JB, Kier AB and Schroeder F. Sterol carrier protein-2 alters high density lipoprotein-mediated cholesterol efflux. *J Biol Chem.* 2000;275:36852–36861. [PubMed: 10954705]
- Guo X and Kassab GS. Role of shear stress on nitrite and NOS protein content in different size conduit arteries of swine. *Acta Physiologica.* 2009;197:99–106. [PubMed: 19432590]
- Ziegler T, Silacci P, Harrison VJ and Hayoz D. Nitric oxide synthase expression in endothelial cells exposed to mechanical forces. *Hypertension.* 1998;32:351–355. [PubMed: 9719066]
- Gambillara V, Chambaz C, Montorzi G, Roy S, Stergiopulos N and Silacci P. Plaque-prone hemodynamics impair endothelial function in pig carotid arteries. *Am J Physiol Heart Circ Physiol.* 2006;290:H2320–H2328. [PubMed: 16415081]
- Hwang J, Ing MH, Salazar A, Lassègue B, Griendling K, Navab M, Sevanian A and Hsiai TK. Pulsatile versus oscillatory shear stress regulates NADPH oxidase subunit expression: implication for native LDL oxidation. *Circ Res.* 2003;93:1225–1232. [PubMed: 14593003]
- Awolesi MA, Sessa WC and Sumpio BE. Cyclic strain upregulates nitric oxide synthase in cultured bovine aortic endothelial cells. *J Clin Invest.* 1995;96:1449–1454. [PubMed: 7544806]
- Ziegler T, Bouzourène K, Harrison VJ, Brunner HR and Hayoz D. Influence of oscillatory and unidirectional flow environments on the expression of endothelin and nitric oxide synthase in cultured endothelial cells. *Arterioscler Thromb Vasc Biol.* 1998;18:686–692. [PubMed: 9598825]
- Woodman CR, Muller JM, Rush JWE, Laughlin MH and Price EM. Flow regulation of eNOS and Cu/Zn SOD mRNA expression in porcine coronary arterioles. *Am J Physiol Heart Circ Physiol.* 1999;276:H1058–H1063.
- Woodman CR, Price EM and Laughlin MH. Shear stress induces eNOS mRNA expression and improves endothelium-dependent dilation in senescent soleus muscle feed arteries. *J Appl Physiol.* 2005;98:940–946. [PubMed: 15531569]

13. Uematsu M, Ohara Y, Navas JP, Nishida K, Murphy TJ, Alexander RW, Nerem RM and Harrison DG. Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *Am J Physiol.* 1995;269:C1371–C1378. [PubMed: 8572165]
14. Stepp DW, Nishikawa Y and Chilian WM. Regulation of shear stress in the canine coronary microcirculation. *Circulation.* 1999;100:1555–1561. [PubMed: 10510060]
15. Reneman RS and Hoeks APG. Wall shear stress as measured in vivo: consequences for the design of the arterial system. *Med Biol Eng Comp.* 2008;46:499–507.
16. Aird WC. Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circ Res.* 2007;100:174–190. [PubMed: 17272819]
17. Torres-Vázquez J, Kamei M and Weinstein BM. Molecular distinction between arteries and veins. *Cell Tissue Res.* 2003;314:43–59. [PubMed: 14505031]
18. Jones CJH, Kuo L, Davis MJ and Chilian WM. Regulation of coronary blood flow: coordination of heterogeneous control mechanisms in vascular microdomains. *Cardiovasc Res.* 1995;29:585–596. [PubMed: 7606744]
19. Andries LJ, Brutsaert DL and Sys SU. Nonuniformity of endothelial constitutive nitric oxide synthase distribution in cardiac endothelium. *Circ Res.* 1998;82:195–203. [PubMed: 9468190]
20. Golledge J, Turner RJ, Harley SL, Springall DR and Powell JT. Circumferential deformation and shear stress induce differential responses in saphenous vein endothelium exposed to arterial flow. *J Clin Invest.* 1997;99:2719–2726. [PubMed: 9169502]

Highlights

Greater eNOS protein levels in coronary endothelial cells of conduit vs. microvessel

Greater nitrite/nitrate in coronary endothelial cells of conduit vs. microvessel

eNOS protein present in all coronary conduit and microvascular endothelial cells

eNOS staining less intense in microvascular cells than those of conduit artery

Figure 1. Total eNOS protein levels in whole coronary arterioles and small and large coronary arteries.

Protein levels for eNOS in lysate from whole arterioles (75–125 μ m), small arteries (150–350 μ m), and large arteries (2–5 mm) were quantified by densitometry analysis, and normalized to GAPDH and SYPRO. A main effect was identified by one-way ANOVA; Bonferroni post-hoc tests revealed that eNOS protein normalized to either GAPDH or SYPRO was significantly decreased in conduit arteries compared with arterioles. A, arteriole; SA, small artery; LA, large artery; H, HUVEC. Values are means $\pm$ S.E.M. Number of animals studied is provided in parentheses. *P 0.05 vs. large artery normalized to GAPDH; †P 0.05 vs. large artery normalized to SYPRO.

Figure 2. Isolation of coronary microvascular endothelial cells (MEC) with limited contamination from other cell types.

Myocardial sections were digested and MEC isolated using biotinylated-anti-CD31 and streptavidin-coated paramagnetic beads. (A) Histological evaluation using Masson trichrome stain revealed that no vessels in the myocardial sections used exceeded ~300 µm luminal diameter and all but one vessel in each myocardial sample were under 150 µm diameter. Effectiveness of the methodology was determined by immunofluorescence and immunoblot to evaluate purity of the isolated MEC. (B-E) Hoechst-positive and CD31-positive images from MEC using epifluorescence microscopy were colocalized and a pixel fluorogram constructed. (F-G) Protein levels of eNOS, smooth muscle α -actin (SMA), and cardiac troponin I (cTnI) were compared by immunoblot analyses in lysates from endothelial cells (EC), small arteries (SA), arterioles (Art), and myocardium (M). EC1 & EC2 are microvascular endothelial cell preps from two different pig hearts. These data demonstrate that 97% of cells isolated from the myocardial sample preparation were endothelial cells. Similar analyses in endothelial cells isolated from conduit arteries revealed comparable purity.

Figure 3. Total eNOS protein levels in endothelial cells isolated from each coronary microcirculation (MEC) and conduit coronary artery (CEC) and whole arteriole and conduit artery lysate.

(A-B) Protein levels for eNOS in isolated endothelial cell as well as whole arteriole and artery lysate were quantified by densitometry analysis, and normalized to GAPDH. HUVEC serves as positive control. Statistical analysis identified a main effect by Kruskal–Wallis; Student-Newman-Keuls post-hoc tests revealed that eNOS protein levels were significantly increased in CEC compared with MEC, arterioles, and conduit arteries. Furthermore, eNOS protein was significantly decreased in conduit arteries compared with MEC and arterioles. (C-H) Representative laser scanning confocal microscopy images stained for CD105, eNOS, Hoechst (blue) revealed that all conduit and microvascular endothelial cells contain eNOS protein. (G). Microvascular endothelial cell image was acquired using 2-fold higher laser power (568 nm excitation) and increased photomultiplier voltage level (i.e. increased gain) as compared to conduit endothelial cells (D) for visualization and colocalization purposes. Values are means $\pm$ S.E.M. Number of animals studied is provided in parentheses. *P < 0.05 vs. CEC; †P < 0.05 vs. MEC and arterioles.

Figure 4. Comparison of fluorescence intensity of labeled eNOS in endothelial cells isolated from conduit coronary artery (CEC) and coronary microcirculation (MEC) with equivalent data acquisition parameters.

Representative confocal images of fluorescently labeled eNOS in (A) CEC and (B) MEC. Data acquisition parameters for both images (i.e., laser power, aperture, and photomultiplier voltages were kept constant). Due to the low intensity of eNOS in the MEC (B), the 8-bit grayscale images were pseudo-colored for visualization using a lookup table and a greyscale ramp was added. The average intensity of the eNOS labeling in the MEC vs CEC for n=6 cells was 5.6 ± 0.5 fold lower.

Figure 5. Nitrite plus nitrate (NOx) levels in lysate from whole vessel segments and isolated endothelial cells along the coronary vascular tree.

(A) NOx levels determined in lysate from whole arterioles (75–125 μm), small arteries (150–350 μm), and large arteries (2–5 mm) were quantified using a modified Griess assay and normalized to total protein. NOx content was significantly increased in lysate from whole arterioles and small arteries compared with large arteries, as identified by one-way ANOVA followed by Bonferroni post-hoc tests. (B) NOx levels determined in lysates of endothelial cell isolated from the microvasculature and conduit arteries. Statistical analysis by Mann-Whitney indicated that NOx levels were significantly increased in CEC compared with MEC. A, arteriole; SA, small artery; LA, large artery. Values are means ± S.E.M. Number of animals studied is provided in parentheses. *P 0.05 vs. large artery; †P 0.05 vs. CEC.