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Co-expression of Endothelial and Neuronal Nitric Oxide Synthases in the Developing Vasculatures of the Human Fetal Eye

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Abstract

Background—Nitric oxide (NO) is a multifunctional gaseous molecule that regulates various physiological functions in both neuronal and non-neuronal cells. NO is synthesized by nitric oxide synthases (NOSs), of which three isoforms have been identified. Neuronal NOS (nNOS) and endothelial NOS (eNOS) constitutively produce low levels of NO as a cell-signaling molecule in response to an increase in intracellular calcium concentration. Recent data have revealed a predominant role of eNOS in both angiogenesis and vasculogenesis.

Methods—The immunohistochemical localization of nNOS and eNOS was investigated during embryonic and fetal ocular vascular development from 7 to 21 weeks gestation (WG) on sections of cryopreserved tissue.

Results—eNOS was confined to ECs of developing vessels at all ages studied. nNOS was prominent in nuclei of vascular endothelial and smooth muscle cells in the fetal vasculature of vitreous and choriocapillaris. nNOS was also prominent in the nuclei of CXCR4⁺ progenitors in the inner retina and inner neuroblastic layer.

Conclusion—These findings demonstrate co-expression of n- and eNOS isoforms in different compartments of vasoformative cells during development. Nuclear nNOS was present in vascular and nonvascular progenitors as well as endothelial cells and pericytes. This suggests that nNOS may play a role in the transcription regulatory systems in endothelial cells and pericytes during ocular hemo-vasculogenesis, vasculogenesis and angiogenesis.

Keywords

choroidal vasculature; fetal vasculature of vitreous; retinal vasculature; nitric oxide synthase; nucleus; progenitors

INTRODUCTION

In normal adult tissues, nitric oxide (NO) regulates neurotransmission, blood vessel tone, platelet aggregation, vascular permeability, elimination of certain free radicals and leukocyte-endothelial interactions [1, 2]. Nitric oxide is produced by nitric oxide synthase (NOS) of which there are three known isoforms: neuronal NOS (nNOS, also referred to as type 1 NOS); inducible NOS (iNOS, type 2); and endothelial NOS (eNOS, type 3). These three mammalian enzyme isoforms reflect the tissues of origin for the original protein and cDNA isolates: nNOS from neuronal tissue; iNOS from an immuno-activated macrophage

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line; and eNOS from vascular endothelium. Since then, all three isoforms have been identified in a myriad of tissues, cells and various subcellular compartments [1, 2].

Nitric oxide is involved in the control of basal blood flow in ocular tissues such as the choroid, optic nerve, and the retina. In the adult human retina, nNOS is expressed in photoreceptors, amacrine cells, ganglion cells and retinal pigment epithelium while eNOS is expressed in endothelial cells of retinal blood vessels [3, 4]. In choroid, nNOS is associated with perivascular nerve fibers, smooth muscle cells of arteries and in some scattered cells in stroma [3]. eNOS is localized to choriocapillaris endothelium, to a lesser extent in endothelial cells of large choroidal blood vessels, and individual cells in choroidal stroma. NO formed by constitutive eNOS, particularly in endothelial dysfunction, participates in ocular hemodynamic abnormalities that appear to trigger generation and development of various eye diseases including glaucoma and ischemic retinopathies as occurs in diabetes [5]. Additionally, we have previously shown that NOS isoforms are reduced in eyes with age-related macular degeneration [3]. While much is known regarding the expression and regulation of NOS isoforms in adult tissue, little information is available regarding their cellular localization during embryonic and fetal development.

Recent studies have suggested a role for NOSs in vasculogenesis, angiogenesis and neovascularization in vivo [6, 7] and in vitro [8, 9]. In endothelial cells, NO has been shown to signal the actions of vascular endothelial growth factor (VEGF), in increasing cell proliferation, migration and permeability, suggesting a role in vascular development. The fetal human ocular vasculatures form by several different processes. The fetal vasculature of vitreous and the choriocapillaris develop initially by hemo-vasculogenesis, whereby both blood vessel cells (endothelium and pericytes) and blood cells (hematopoietic) develop from a common progenitor, the hemangioblast [10, 11]. Angioblasts emerge from a CXCR4⁺ progenitor pool within the inner neuroblastic layer of retina and differentiate and aggregate to form the primordial vessels [12, 13]. In this study, we examined the localization of nNOS and eNOS during embryonic and fetal development of the ocular vasculatures (7–21 weeks gestation). We demonstrate that during development, the two constitutive isoforms of NOS (nNOS & eNOS) are co-expressed by developing endothelial cells and adventitial cells in different cellular compartments.

MATERIALS AND METHODS

Human fetal eyes, ranging in age from 7 to 21 WG, were obtained from Advanced Bioscience Resources, Inc (Alameda, CA). Utilization of this human tissue was in accordance with the Declaration of Helsinki with approval of the JCCI at Johns Hopkins University School of Medicine. The gestational age of each embryo or fetus was determined by the fetal foot size or sonography.

Streptavidin alkaline phosphatase (APase) immunohistochemistry was performed on cryopreserved tissue sections using a nitroblue tetrazolium (NBT) system developed by Bhutto et al [3]. The sections of human fetal eyes from 7 to 21 WG were incubated overnight at 4°C with either of the following primary antibodies: mouse anti-nNOS (1:500; BD Biosciences) or rabbit anti-eNOS (1:500; Assay Designed). Mouse anti-CD31 (1:500; Dako) was used as a hemangioblast and an endothelial cell marker. We have shown previously that CD31 is co-expressed with epsilon hemoglobin in endothelial progenitors in blood island-like formations in embryonic choroid (up to 10 WG). The same is true for fetal vasculature of vitreous (unpublished results McLeod 2011). After washing in TBS, sections were incubated for 30 min at room temperature with the appropriate biotinylated secondary antibodies diluted 1:500 (Kirkegaard and Perry, Gaithersburg, MD). Finally, sections were incubated with streptavidin APase (1:500; Kirkegaard and Perry), and APase activity was

developed with a 5-bromo-4-chloro-3-indoyl phosphate (BCIP)-NBT kit with levamisole (Vector Laboratories, Inc.), yielding a blue reaction product.

Immunofluorescence and confocal microscopy were used for double labeling on cryopreserved tissue sections to further confirm the nNOS nuclear localization in vascular and nonvascular cells (10, 12, 13). Cryosections were permeabilized with absolute methanol at -20°C and blocked with 2% normal goat serum in Tris-buffered saline (TBS; pH 7.4 with 1% BSA). After they were washed in TBS, the sections were incubated for 2 hr at room temperature with a mixture of two primary antibodies as follow: mouse anti-nNOS (1:100; BD Biosciences) and rabbit anti-aSMA (1:400; Abcam), mouse anti-nNOS (1:100; BD Biosciences) and rabbit anti-vWf (1:2500; Dako). After they were washed in TBS, sections were incubated for 30 min at room temperature with the mixed secondary antibodies: goat anti-mouse conjugated with Cy3 diluted 1:500 and goat anti-rabbit conjugated with Cy5 diluted 1:500 (Jackson Immuno Research). Finally, sections were mounted in DAPI counterstaining medium (Vector Laboratories, Inc.). Sections were then observed with a Zeiss Meta 510 confocal microscope (Carl Zeiss).

RESULTS

Fetal Vasculature of Vitreous

In the 7–9 WG fetal vasculature of vitreous, blood island-like structures composed of CD31^{+} hemangioblast aggregates were present without discernible lumen. These cells in aggregates were also positive for epsilon hemoglobin, which is present in hemangioblasts and erythroblasts [10](unpublished data). eNOS and nNOS were only weakly expressed in these primordial vessels (Figure 1). By 12–14 WG, when vascular lumen had formed and cellular components of the vessel walls began to differentiate, both NOS immunoreactivities had increased with eNOS being associated with endothelial cell cytoplasm (Figure 1–2) and nNOS associated with both the endothelial and smooth muscle cell nuclei (Figure 2–3). This was best demonstrated in double labeled sections where eNOS and nNOS were co-expressed in different compartments of the endothelial cells as well as in pericytes (Figure 4).

Choroid

In the embryonic choriocapillaris, the blood island-like CD31^{+} hemangioblast aggregates with no discernable lumen expressed low levels of eNOS and nNOS (Figure 5a, d, g&j). Like the fetal vasculature of vitreous, the eNOS appeared diffuse and cytoplasmic while the nNOS had a decidedly nuclear appearance. Additionally, nNOS immunostaining was observed within the mesenchymal cells of choroidal stroma and in scattered retinal pigment epithelial cells (arrowhead in j). Nuclear nNOS was also present in adult RPE cells [4]. At 14 WG when choriocapillaris is more mature and intermediate choroidal vessels are forming (Figure 5b,e, h&k), eNOS immunoreactivity increased in blood vessels and nNOS continued to be expressed in vascular cell nuclei and progenitors in choroidal stroma. By 21 WG (Figure 5c, f, i&l), when 3 layers of blood vessels are present, eNOS immunostaining was prominent throughout the choroidal vasculature while nNOS expression was decreased in vascular and non-vascular cells. Choroidal endothelial and smooth muscle cell nuclear localization of nNOS was confirmed in sections of a 17 WG fetal eye using double labeling with von Willebrand factor (Figure 6) and alpha smooth muscle actin (Figure 7).

Retina

In the 12 WG fetus, the retina is avascular and consists of an undifferentiated pool of progenitors in the innermost portion of neuroblastic layer (Figure 8a). The inwardly migrating cells consist of neuroblasts and angioblasts, which will ultimately aggregate and differentiate to form the primordial vessels within the nerve fiber layer [12, 13]. Because

there were no blood vessels, CD31 and eNOS immunostaining was absent (Figure 8d&g), however, nNOS expression was observed in the progenitors of the putative ganglion cell layer and inner neuroblastic layer (Figure 8j). At 17 WG, the retina consisted of a nerve fiber layer, ganglion cell layer, inner plexiform layer and neuroblastic layer (Figure 8b). Blood vessels had begun to form in the nerve fiber layer and were CD31⁺ and eNOS⁺ (Figure 8e&h). The cells of these primordial vessels expressed nNOS in their nuclei with APase staining (Figure 8k) which was confirmed with double labeling and confocal microscopy (Figure 9). At 21 WG, the retina still consisted of a nerve fiber layer, ganglion cell layer, inner plexiform layer and neuroblastic layer; however, blood vessels had formed throughout much of the inner retina (Figure 8c&f). CD31 clearly labeled the retinal vascular endothelium (Figure 8f), while eNOS and nNOS immunostaining was observed in blood vessels and progenitors in the ganglion cell layer. In APase stained sections, eNOS appeared diffuse and cytoplasmic (Figure 8i), however, nNOS was prominent in cell nuclei (Figure 8L). Nuclear nNOS staining of endothelial cells was confirmed in sections double-labeled with von Willebrand factor using confocal microscopy (Figure 9).

DISCUSSION

In the normal adult human retina, nNOS expression is confined to ganglion cells, presumed amacrine cells in the inner nuclear layer and photoreceptors of the outer retina [3, 4], while eNOS is expressed exclusively in vascular endothelial cells [3]. In normal adult choroid, nNOS expression is associated with RPE nuclei, perivascular nerve fibers and smooth muscle cells of arteries and eNOS is confined to vascular endothelial cells [3]. Therefore, NOS expression in the adult retina and choroid fits principally with the conventionally accepted notion that NO generation by endothelial eNOS regulates basal microvascular tone and NO synthesized by neuronal nNOS has neuromodulator functions. However, in this study, we clearly show that eNOS and nNOS are co-expressed in developing vasculatures of the embryonic and fetal human eye. Coexpression of nNOS and eNOS has been observed previously in endothelial cells [14].

Recent studies have shown that a myriad of non-neuronal cell types express nNOS including endothelial cells where it may play a vasculoprotective role [15]. Of interest is a previous report where retinal blood vessels develop normally in eNOS knockout mice (-/-), suggesting increases in vascular-associated nNOS activity compensate for the eNOS deficiency [16]. Similarly, nNOS compensated for the loss of eNOS in cerebral ischemia in eNOS -/- mice [17]. Wei et al also reported that transgenic mice deficient in either endothelial or neuronal isoforms of NOS produced NO in brain that was ~50% of wild-type mice NO production. Several studies have reported that the NO generation from one of the two isoforms can dilate blood vessels by compensatory mechanisms following the disruptions of the other isoform [18]. Our results showing both eNOS and nNOS isoforms expressed in different compartments of angioblasts and differentiating endothelial cells during fetal development suggest that these cells have the capacity to augment NO synthesis via one of the two isoforms.

NO has been shown not only to promote vasculogenesis and angiogenesis by itself, but also mediate the functions of angiogenic factors. Evidence indicates that NO plays an integral role in vascular endothelial growth factor (VEGF) signaling [19]. Vascular endothelial growth factor (VEGF) is an important cytokine that regulates vasculogenesis during development and angiogenesis in many physiologic and pathologic conditions. VEGF promotes NO production and also induces eNOS and iNOS expression in vascular endothelial cells in vitro [20]. We have recently observed the anti-angiogenic VEGF_{165b} in the nucleus of the vascular progenitors that have nNOS in their nuclei and the angiogenic splice variant VEGF₁₆₅ was in the cytoplasm of the same cells [21]. Furthermore, inhibition

of in vivo NO production results in reduced angiogenesis and vascular permeability induced by VEGF. NO at an appropriate concentration induces VEGF synthesis through an HIF-1 mediated pathway, and VEGF enhances NO production by eNOS. Therefore, NO may have a pivotal role in normal vessel development, because it is both an upstream and downstream mediator of VEGF.

Perhaps the most interesting and novel observation from the current study was the co-localization of nuclear nNOS and cytoplasmic eNOS within angioblasts and endothelial cells of developing ocular vasculatures. This is not the case in adult human retinal or choroidal vasculatures [3]. Emerging evidence lends credence to the validity of nuclear NOS expression. In fact, all three isoforms have now been reported to be in the cell nucleus [22–25]. In cultured cerebral astrocytes, nNOS has been observed to change localization from cytosol to nucleus and back to cytosol [1]. In neural and glial cells, recent studies have reported nNOS localization exclusively in nucleus [26]. Additionally, eNOS has been localized in nuclei of rat hepatocytes and in subcellular nuclear fractions of the same origin [23]. In that study, the authors provided clear evidence for involvement of nuclear NOS and derived NO in the control of signaling cascades leading to gene expression. Several routes of transportation to nucleus have been elaborated in recent years. Feng et al reported that eNOS transport to nucleus was stimulated by VEGF and the translocation was accomplished via the caveolae system [27]. Transport of nNOS to different subcellular locations including nucleus is probable because nNOS has a PDZ domain, which can interact with many proteins including CAPON and Caveolin-3 [26, 28]. It is known that nNOS regulates hematopoiesis in vivo and in vitro and NO is critical for hemangioblast activity in vascular development [29–31]. How the compartmentalization of cytosolic eNOS and nuclear nNOS functions in angioblasts and endothelial cells during ocular vasculogenesis in human retina [12, 13] and hemangioblasts during hemo-vasculogenesis in human choroid [10, 11] and fetal vasculature of vitreous (unpublished data) is intriguing and clearly requires additional study. It is interesting that the fetal vasculature of vitreous, which regresses in human at 12–14 WG [32–34], had similar nNOS and eNOS localizations as retinal and choroidal blood vessels, which are just forming during the ages studied. This suggests that these NOSs are not involved in regression of the fetal vasculature because the localization remained even at 14 WG when regression is occurring [32–34].

In summary, we examined the localization of eNOS and nNOS during embryonic and fetal ocular vascular development. These two NOS isoforms were co-expressed in different cellular compartments of vasoformative cells during vasculogenesis, hemo-vasculogenesis, and angiogenesis. These results strongly suggest a role for NO in human ocular vascular development and indicate a likelihood that nuclear nNOS may function in the transcription regulatory system in vascular and nonvascular progenitors and endothelial and smooth muscle cells as well as pericytes during differentiation and growth of the vasculature.

Acknowledgments

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Figure 1.

APase immunostaining for eNOS and nNOS in fetal vasculature of vitreous in human fetal eyes from 9 WG to 14 WG. H&E staining (a-c) shows morphological features of the vasculature in vitreous, which is intensely labeled for CD31 (d-f). Note that eNOS is prominently localized diffusely in endothelial cells (g-i), whereas nNOS is present in endothelial cell (EC) nuclei of the vasculature (j-l). Arrow indicates a blood vessel in each set of micrographs. Scale bar in "a" = 10 μ m

Figure 2.

Fetal vasculature of vitreous at 12 WG double-labeled with vWf (red fluorescence) and nNOS (green fluorescence). Endothelial cells (ECs) are positive for vWf (c). nNOS immunofluorescence is seen in EC nuclei (paired arrow) (d). Nuclei were counter stained with DAPI (blue fluorescence)(a). The merged images (a-b) demonstrate nNOS within endothelial cell nuclei Scale bar "a" = 10 μ m

Figure 3. Fetal vasculature of vitreous at 12 WG double-labeled for SMA (red)(c) and nNOS (green) (d) shows nuclear nNOS expression in SMA+ cells (paired arrow)(merged in a-b). Nuclei were counter stained with DAPI (blue fluorescence)(a). Scale bar “a” = 10 μ m

Figure 4. Fetal vasculature of vitreous at 12 WG double-labeled for eNOS (red)(c) and nNOS (green) (d) shows eNOS in the cytoplasm and nNOS in nuclei of both endothelial cells (paired arrows) and pericytes (arrow)(merged images a-b). Scale bar “a” = 10 μ m

Figure 5.

APase immunostaining for eNOS and nNOS in choroid of human fetal eyes from 7 WG to 21 WG. H&E staining (a-c) shows morphological features of developing choroid. Choriocapillaris (arrow in all) and deeper choroidal vessels (arrowhead in 14 & 21 WG) are labeled for CD31 (d-f). eNOS is prominently localized to the ECs of all choroidal vessels, whereas nNOS is present in mesenchymal precursor cells of stroma (bottom), in RPE nuclei and EC's of blood vessels. Scale bar in "a" = 10 μ m

Figure 6. Choroid at 17 WG double-labeled for vWf (red fluorescence)(c) and nNOS (green fluorescence)(d). Nuclear nNOS expression is seen in RPE (arrowhead), ECs of CC (arrow), and large choroidal vessels (paired arrow)(merged a-b). Nuclei were counter stained with DAPI (blue fluorescence)(a). Scale bar in “a” = 10 μ m

Figure 7. Choroid at 17 WG double-labeled for SMA (red)(c) and nNOS (green)(d) shows nuclear nNOS expression in α SMA⁺ cells in large choroidal vessel (paired arrow)(merged a-b). Nuclei were counter stained with DAPI (blue fluorescence)(a). Scale bar in “a” = 10 μ m

Figure 8.

APase immunostaining for eNOS and nNOS in developing retina of human fetal eyes from 12 WG to 21 WG. H&E staining shows morphological features of developing retina (a-c). Developing retinal blood vessels (present after 12 WG), are intensely labeled for CD31 (e-f). eNOS is prominently localized to the ECs of retinal developing vessels (arrow h-i), whereas nNOS is present in progenitors (arrowhead in "j") in neuroblastic layer, migrating angioblasts in nerve fiber and ganglion cell layer, as well as ECs of developing retinal blood vessels. Arrow indicates developing retinal blood vessels. Scale bar in "a" = 10 μ m

Figure 9. Seventeen WG retina double-labeled for vWf (red fluorescence)(c) and nNOS (green fluorescence)(d). Nuclear nNOS expression is seen in retinal vessel (arrow)(merged a-b). Nuclei were counter stained with DAPI (blue fluorescence)(a). Scale bar in “a” = 10 μ M