Nitric oxide modulates murine yolk sac vasculogenesis and rescues glucose induced vasculopathy

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Accepted 4 February 2004

Development 131, 2485-2496
Published by The Company of Biologists 2004
doi:10.1242/dev.01131

Summary

Nitric oxide (NO) has been demonstrated to mediate events during ovulation, pregnancy, blastocyst invasion and preimplantation embryogenesis. However, less is known about the role of NO during postimplantation development. Therefore, in this study, we explored the effects of NO during vascular development of the murine yolk sac, which begins shortly after implantation. Establishment of the vitelline circulation is crucial for normal embryonic growth and development. Moreover, functional inactivation of the endodermal layer of the yolk sac by environmental insults or genetic manipulations during this period leads to embryonic defects/lethality, as this structure is vital for transport, metabolism and induction of vascular development. In this study, we describe the temporally/spatially regulated distribution of nitric oxide synthase (NOS) isoforms during the three stages of yolk sac vascular development (blood island formation, primary capillary plexus formation and vessel maturation/remodeling) and found NOS expression patterns were diametrically opposed. To pharmacologically manipulate vascular development, an established in vitro system of whole murine embryo culture was employed. During blood island formation, the endoderm produced NO and inhibition of NO (L-NMMA) at this stage resulted in developmental arrest at the primary plexus stage and vasculopathy. Furthermore, administration of a NO donor did not cause abnormal vascular development; however, exogenous NO correlated with increased eNOS and decreased iNOS protein levels. Additionally, a known environmental insult (high glucose) that produces reactive oxygen species (ROS) and induces vasculopathy also altered eNOS/iNOS distribution and induced NO production during yolk sac vascular development. However, administration of a NO donor rescued the high glucose induced vasculopathy, restored the eNOS/iNOS distribution and decreased ROS production. These data suggest that NO acts as an endoderm-derived factor that modulates normal yolk sac vascular development, and decreased NO bioavailability and NO-mediated sequela may underlie high glucose induced vasculopathy.

Key words: Nitric oxide, Vasculogenesis, Yolk sac, Hyperglycemia, Reactive oxygen species, Mouse

Introduction

Establishment of the extra-embryonic vascular system is vital for survival, growth and homeostasis in the vertebrate embryo. Vasculogenesis, the in situ development of blood vessels from angioblasts, begins in the extra-embryonic yolk sac prior to vasculogenesis in the embryo proper (Boucher and Pedersen, 1996). Vasculogenesis occurs during cardiovascular organogenesis, a period when the developing embryo undergoes profound biochemical and metabolic changes as landmark morphogenetic movements are occurring. Development of the vitelline circulation allows the embryo to shift from reliance on diffusion-dependent nutrient delivery to a system of vascular conduits (Jollie, 1990). Moreover, normal development of the cardiovascular system is particularly important as it is the first functional organ/system to develop during organogenesis in the vertebrate embryo; thus, heart/vascular defects will affect subsequent embryonic development.

Studies with knockout mice have revealed several essential molecules that participate in vertebrate vasculogenesis, including ephrins, TIE2, angiopoietin (ANG), platelet-derived endothelial cell adhesion molecule (PECAM), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Gerety and Anderson, 2002; Sato et al., 1995; Suri et al., 1996; Duncan et al., 1999; Gerber et al., 1999; Yasuda et al., 1992). Loss of many of these molecules leads to vascular dysfunction characterized by enlarged, hyperperfused capillaries and embryonic lethality due to cardiovascular defects. Despite the knowledge gained from these studies, the regulatory molecules and signaling events underlying vessel development and patterning remain largely unknown (Darland and D’Amore, 2001; Tallquist et al., 1999). One possible candidate is nitric oxide (NO), a small multifunctional gaseous molecule that acts as a vasoactive agent, signaling molecule and free radical in mammalian
systems (Bogdan, 2001; Papapetropoulos et al., 1999; Schmidt and Walker, 1994).

NO participates in numerous biological functions that are relevant to reproduction and embryogenesis, including gene expression, cell growth and matrix remodeling. All nucleated mammalian cells possess at least one of the three conserved NOS enzymes, neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), which generate NO by the oxidation of L-arginine. Gouge et al. have demonstrated that NO was produced by preimplantation murine embryos and that embryos (two-cell to blastocyst stage) cultured with a NOS inhibitor (L-NMMA) were developmentally delayed or nonviable (Gouge et al., 1998). The authors speculated that NO participates in implantation and demonstrated for the first time that NO is required for early embryonic development in mice. Accordingly, eNOS knockout mice display defects in ovulation and oocyte meiotic maturation, and produce fewer pups (Jablonska-Shariff and Olson, 1998).

The importance of NO as a regulator of early developmental events has been established by studies that demonstrated that the oocyte and preimplantation embryo are exposed to NO, and oocyte maturation, preimplantation embryogenesis and implantation require NO (Biswas et al., 1998; Gagiotti et al., 2000; Maul et al., 2003; Novaro et al., 1997; Purcell et al., 1999; Sengoku et al., 2001; Shukovsky and Tsafriri, 1995; Telfer et al., 1995). Moreover, in vitro and in vivo studies showed that administration of either NOS inhibitors or NO donors hinders development of preimplantation embryos (i.e. expansion to blastocyst stage), which revealed the importance of maintaining appropriate levels of NO during preimplantation embryogenesis (Barroso et al., 1998; Biswas et al., 1998; Gouge et al., 1998; Sengoku et al., 2001).

The complex effects of NO are highly dependent on micro-environment, with several groups reporting diametrically opposing effects of NO, which are probably due to differences in cell/tissue type, local microenvironment and redox state (Dulak and Jozkowicz, 2003; Kroncke et al., 1997; Shaul, 2002; Wink and Mitchell, 1998). The role of NO in the developmental milieu of vasculogenesis in the murine yolk sac has not been studied. During murine development, both embryonic and extra-embryonic mesoderm is produced giving rise to the embryonic and extra-embryonic vasculature, respectively. At E7.5, extra-embryonic mesodermal cells proliferate forming angioblastic cords (Pulis et al., 1995). At E8.0, blood islands fuse establishing the primary capillary network (E8.5), which is intimately associated with mural cells (Flämme et al., 1997; Risau and Flämme, 1995; Pinter et al., 1999). Subsequently, by E9.5, the capillary plexus has remodeled into a complex hierarchy of mature large and small vessels, and a functional vitelline circulation is established (Folkman and D’Amore, 1996).

The yolk sac serves an essential function at the maternal-fetal interface, and its capacity to tolerate environmental insults is vital for the normal progression of cardiovascular and embryonic organ development (Brent et al., 1971; Freeman et al., 1981; Jollie, 1990; Lerman et al., 1986; Waddell and Marlowe, 1981). Environmental insults during this developmental window lead to defects in the cardiovascular system, which subsequently affect the development of several embryonic organs. An in vitro whole embryo culture model permits study of this crucial developmental period and has shown that short exposure to hyperglycemia is teratogenic, causing dose dependent growth retardation, neural tube lesions and yolk sac failure (Cockroft and Coppola, 1977; Pinter et al., 1999; Reece et al., 1996; Rashbass and Ellington, 1988; Sadler, 1980a; Sadler, 1980b; Freinkel et al., 1986). Additionally, this model system mimics the morphological and biochemical vascular defects in the embryo and yolk sac observed in vivo (New et al., 1976; Mills et al., 1979; Sadler and Warner, 1984).

Though the underlying mechanisms are unknown, the yolk sac has been suggested as a target of the hyperglycemic insult via excess production of reactive oxygen species (ROS), and a link between yolk sac injury and multi-system embryopathy has been proposed (Eriksson and Borg, 1991; Eriksson and Borg, 1993; Hagay et al., 1995; Hunter and Sadler, 1992; Pinter et al., 1986; Reece et al., 1989; Reece et al., 1994; Wentzel and Eriksson, 1998; Zusman et al., 1987).

In this study, we determined the profile of expression/localization of NOS isofoms and NO production during the well-defined stages of yolk sac vascular development. Additionally, we used the in vitro whole conceptus culture system to describe the stage specific morphological defects induced by temporal disruption of NO/NOS expression and/or activity induced by NO inhibitors, NO donors and hyperglycemia. Furthermore, we determined the ability of a NO donor to rescue vascular defects induced by a known environmental insult: hyperglycemia. In this study, hyperglycemia was chosen as a pathophysiologically relevant condition, which has been extensively studied, known to induce ROS and cause vasculopathy (Eriksson and Borg, 1991; Eriksson and Borg, 1993; Pinter et al., 1986; Wentzel and Eriksson, 1998). Finally, we begin to explore the relationships between the hyperglycemia insult and NO inhibition through a pathway involving ROS.

Materials and methods

Mice

CD-1 mice were purchased from Charles River (Wilmington, MA) and maintained under standard conditions. Timed-matings were performed and detected by the presence of a vaginal plug [designated day 0.5 postconceptional (dpc)]. The Yale University Animal Care and Use Committee approved all animal protocols.

Conceptus culture

To study vasculogenesis (E7.5-9.5 in mice) an established in vitro whole embryo culture was employed (Chen and Hsu, 1982; New, 1978; New, 1991; Sadler, 1979). Cultures were performed as previously described (Pinter et al., 1999). Briefly, conceptuses were harvested at 7.5 dpc from timed-pregnant mice. After complete removal of the trophoblast and Reichert’s membrane, the developmental stage was determined using Downs and Davies’ morphological criteria for staging morphological landmarks of the primitive streak, neural plate and head fold (Downs and Davies, 1993). The conceptuses were cultured in pooled rat serum for 48 hours and examined under a dissecting microscope for structural and functional defects (neural tube closure, axial rotation completion, yolk sac circulation and heart beat). Subsequently, conceptuses were processed for histological analysis to evaluate complexity of branching, organization, presence of a hierarchical network of large and small vessels, and vessel diameter.

The following pharmacological reagents were used: DETA NONOate (half-life of NO release is 20 hours at 37°C/pH 7.4), N°-Monomethyl-L-arginine (L-NMMA), N°-Monomethyl-D-arginine
Prepared and imaged.

Methylbutane cooled with liquid nitrogen. Slides were immediately washed and flash frozen in 2-

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ROS localization was performed using dihydroethidium (DHE), which reacts principally with superoxide anion. DHE enters the cytoplasm and becomes oxidized to ethidium, which intercalates into the DNA and produces red fluorescence. Conceptuses were incubated in the indicated conditions for 24 hours. DHE (10 μM) was added to the culture media for 30 minutes at 37°C. Subsequently, the samples were washed and flash frozen in 2-
methylbutane cooled with liquid nitrogen. Slides were immediately prepared and imaged.

**NO detection**

NO localization was performed using 4-aminomethyl ester hydrochloride (L-NAME) (Sigma-Aldrich, St Louis, MO). For the hyperglycemic condition 20 mM D-glucose was used (normoglycemic serum contains 5 mM). Control cultures contained vehicle (PBS), D-NMMA or D-NAME. All reagents were added at the beginning of culture.

**Immunohistochemistry and immunofluorescence microscopy**

Conceptuses were harvested from timed-pregnant mice or culture conditions at the indicated time points. The yolk sacs were separated from the embryos and fixed in 4% PFA. Polyclonal anti-murine PECAM-1 (Pinter et al., 1999) was used and detected with biotin-conjugated secondary antibody and the ABC kit (Vector, Burlingame, CA). The reaction was visualized with diaminobenzidine. For immunofluorescence, after fixation, the samples were flash frozen in 2-methylbutane cooled in liquid nitrogen. Frozen sections of 10 μm were prepared. Immunodetection was performed using Alexa Fluor 594 (Molecular Probes, Eugene, OR).

**Western blotting**

Conceptuses were harvested from timed-pregnant mice or culture conditions at the indicated time points. Samples of 10-20 were pooled and lysed RIPA Buffer (Upstate, Waltham, MA) supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktails (Calbiochem, San Diego, CA). The samples were homogenized and soluble extracts obtained by centrifugation. Protein concentrations were determined by BCA Assay (Bio-Rad, Hercules, CA). For western blotting, 40 μg of protein was loaded onto SDS-PAGE gels under reducing conditions. For immunoprecipitation, 400 μg of lysate was precleared with normal rabbit serum and precipitated with protein A/G Sepharose. The supernatant was incubated with the primary antibody, washed with decreased salt conditions and electroblotted. Primary antibodies used included eNOS, iNOS, Akt and phosho-Akt from Santa Cruz (Santa Cruz, CA), phospho-eNOS (Ser-1177) (Cell Signaling Technology, Beverly, MA) and phospho-eNOS (Ser-1177) (Zymed, San Francisco, CA). Luminescence was preformed using the Western Lightening Chemiluminescence Reagent (PerkinElmer, Boston, MA) and phospho-eNOS (Ser-1177) (Zymed, San Francisco, CA). The reaction was visualized with diaminobenzidine.

**Morphometrics**

Yolk sac vessel diameters were measured using the IP Lab Spectrum program. Micrographs of PECAM1-stained yolk sacs were generated using an Olympus IX71 inverted microscope (20× objective) and saved as Photoshop 5.0 TIFF files. These files were opened using IP Lab Spectrum and the vessel diameters of several randomly selected fields measured.

**Statistics**

The data were analyzed by t-test using StatView (SAS Institute). P values less than or equal to 0.05 were considered significant.

**Results**

**Temporal distribution of NOS protein during vascular development**

In CD-1 mice, vasculogenesis occurs in three defined stages: blood island formation stage (E7.0-8.0), primary capillary plexus stage (E8.5-9.5), and vessel maturation/remodeling stage (E8.5-9.5). To investigate the distribution of NOS enzymes during vascular development, yolk sac protein extracts were generated from conceptuses harvested at each developmental stage (7.5, 8.5 and 9.5 dpc). Protein levels were determined by western blotting.

An inverse relationship between eNOS and iNOS isoforms exists during vascular development in the yolk sac (Fig. 1). During the blood island formation stage, iNOS protein level is high, while eNOS protein level is barely expressed. However, a switch in expression levels of iNOS and eNOS isoforms began just prior to the primary capillary plexus stage (~8.0 dpc) and persisted during the primary plexus stage. At the vessel maturation stage, iNOS protein was absent while eNOS protein expression continued. nNOS was not detected in the yolk sac at any of the three developmental stages examined (data not shown).

**Spatial expression pattern of NOS enzymes and NO during vascular development**

The cellular expression patterns of eNOS and iNOS in the yolk sac was established for each stage of vascular development by harvesting conceptuses at 8.0, 8.5 and 9.5 dpc and performing immunofluorescence microscopy. In blood islands, eNOS was expressed in the yolk sac vasculature. During the blood island formation stage, iNOS protein level is high, while eNOS protein level is barely expressed. However, a switch in expression levels of iNOS and eNOS isoforms exists during vascular development in the yolk sac (Fig. 1). During the blood island formation stage, iNOS protein level is high, while eNOS protein level is barely expressed. However, a switch in expression levels of iNOS and eNOS isoforms exists during vascular development in the yolk sac (Fig. 1).
weakly detected in the mesoderm (Fig. 2A), while iNOS was strongly present in the endoderm (Fig. 2D). At the primary capillary plexus stage, the yolk sac consists of the outer endodermal, inner endothelial and innermost mesothelial layers. At this stage, eNOS primarily localized to the endothelium, with faint staining present in some endodermal cells (Fig. 2B), while iNOS was modestly present in occasional endodermal cells (Fig. 2E). By the vessel maturation stage, eNOS expression increased in both layers with highest immunoreactivity in the vasculature, including the large vessels, and modest immunoreactivity in the endoderm (Fig. 2C). iNOS was not detected at this stage (Fig. 2F).

Next, the production of NO in 7.5 dpc conceptuses was evaluated using DAF-FM. During normal development, NO is produced by trophoblast giant cells, the ectoplacental cone and endoderm. By carefully removing the endoderm from the conceptus, NO localization was observed in the endoderm but not the underlying mesoderm (Fig. 3A-D). Owing to the short-lived nature of the DAF-FM reagent, NO staining is only suitable for whole-mount imaging of thin specimens. Therefore, this method could not be used to evaluate NO in the endothelium when the eNOS isoform is present. To evaluate the activity of eNOS, a mixture of phosphoserine-specific eNOS antibodies was employed. Using a large pool of conceptuses, we immunoprecipitated eNOS and demonstrated that a fraction of eNOS was phosphorylated and presumably activated at this developmental stage (Fig. 3E). As a possible mechanism for eNOS phosphorylation, Akt and phospho-Akt levels were determined by western blot in E8.5 conceptuses. Akt phosphorylation was demonstrated (Fig. 3F) consistent with previous reports in the murine yolk sac (Adini et al., 2003).

**NOS inhibition arrests vascular development at the primary plexus stage**

Conceptuses were harvested at 7.5 dpc and cultured with a broad-spectrum NOS inhibitor, L-NMMA, in order to assess the effects of NO depletion on vascular development in the yolk sac. Whole-mount immunohistochemistry was performed with PECAM1 to enable histological analysis of vascular morphology at 9.5 dpc. A dose-response curve was performed (5 μM, 50 μM, 250 μM, 500 μM, 1 mM, 2 mM and 5 mM) to determine the effective dose range of L-NMMA. Dose-dependent impairment of vascular development was observed at 250 μM and higher. Doses of 50 μM and less caused no defects (data not shown), and doses of 1-5 mM caused severe defects. Treatment with 2 mM L-NMMA resulted in complete arrest of vascular development at the primary capillary plexus stage, reminiscent of the vasculopathy phenotype of several different growth factor null murine strains (Fig. 4B,E).
The nonspecific and toxic effects of the pharmacological agent used in this study were assessed using following controls: (1) D-NMMA (a stereoisomer of L-NMMA) and (2) L-NMMA (competitive inhibitor) plus excess L-arginine (5 mM), a substrate of NO synthesis. As shown in Fig. 4A,C, both controls developed normal vasculature, ruling out the possibility of toxic effects and nonspecific drug interactions of L-NMMA with other molecules.

In L-NMMA-treated conceptuses the hierarchy of vessels was lost and capillaries were enlarged while normal vascular development occurred in the presence of D-NMMA and L-NMMA + L-Arginine (Fig. 4A-C). Histograms of the size distribution of vessels in the control versus L-NMMA conditions revealed a peak shift from 10-15 μm to 20-25 μm, respectively. The fraction of vessels in the 25-30 to 55-60 μm categories rose from <5% to 5-14% in control versus L-NMMA conditions, respectively (Fig. 4F). The median vessel diameter in L-NMMA treated conceptuses harvested at 9.5 dpc was 27.6 μm compared with 30.6 μm in control E8.5概念uses (capillary plexus stage), confirming arrest at this stage. Additionally, a functional circulation was not present and the vessels contained fewer blood cells.

In order to determine the developmental window of susceptibility to L-NMMA, streak, neural plate and headfold stage conceptuses were separately treated. L-NMMA induced stage-specific defects in streak and neural plate conceptuses, while headfold stage conceptuses were resistant to the insult. Primitive streak conceptuses displayed the most vulnerability to the insult (i.e. complete arrest at the capillary plexus stage); therefore this stage was used for subsequent studies. Areas of normal vasculature, enlarged vessels and areas devoid of vasculature (endothelial clusters present) were all observed in neural plate stage conceptuses (data not shown).

Fig. 4. L-NMMA inhibits yolk sac vascularization. The vascular morphology of L-NMMA-treated conceptuses was evaluated by PECAM1 staining at 9.5 dpc: (A) D-NMMA, (B) L-NMMA and (C) L-NMMA plus L-arginine. Hematoxylin and Eosin stained images of yolk sacs treated with D-NMMA (D) and L-NMMA (E). (F) Morphometric analysis of images from PECAM1-stained yolk sacs showing vessel diameter distribution. Experiments were repeated at least five times.

Exogenous NO treatment results in normal vascular development

Previous studies have shown that high concentrations of NO donor (0.1 and 1 mM DETA/NO) in vivo impaired embryo development while lower concentrations (1 and 10 μM) did not cause developmental abnormalities (Barroso et al., 1998; Sengoku et al., 2001). Therefore, we supplemented low doses of a slow, steady release NO donor (NOC-18) to the culture media and assessed the tolerance of the yolk sac to exogenous NO (20 μM) during vasculogenesis. Whole-mount immunohistochemistry was performed for PECAM at 9.5 dpc to assess vascular morphology. Exogenous NO treatment resulted in the formation of a normal hierarchy of large and small vessels (Fig. 5A,B) and a functional circulation. The size distribution of vessels in the NOC-18 versus control conditions produced overlapping histograms (Fig. 5C). Thus, a dose of 20 μM NOC-18 is not toxic to the vasculature and does not cause vascular dysfunction. Additionally, the ability of exogenous NO to regulate the expression of NOS isoforms was evaluated by western blot. Conceptuses were cultured with 20 μM NOC-18 and harvested at 8.5 dpc, when the switch in NOS isoforms occurs. Exogenous NO treatment correlated with increased baseline expression of eNOS and decreased baseline expression of iNOS normally observed at 8.5 dpc (Fig. 5D).

Hyperglycemia alters the developmental distribution of NOS protein and increases NO production

To evaluate the effect of a known environmental insult on NOS protein distribution in the developing yolk sac, 20 mM glucose was added to the culture media. Hyperglycemia was chosen as a pathophysiologically relevant condition in order to study a insult-mediated induction of vasculopathy. In addition, hyperglycemia has been demonstrated to arrest vascular development at the primary capillary plexus stage, resulting in vasculopathy that is similar to the L-NMMA phenotype (Fig. 6A, Fig. 4B) (Pinter et al., 1999). Histograms of the size distribution of vessels in the hyperglycemic condition revealed a peak of 25-30 μm, compared with 10-15 μm in the control (Fig. 6C). The fraction of vessels in the 35-40 to 55-60 μm categories rose from <5% to 5-12% in control versus hyperglycemic conditions, respectively.

In addition to the morphological defects, the endogenous expression pattern of NOS isoforms was dramatically altered by the hyperglycemic insult (Fig. 6D,E). The inverse expression pattern of iNOS and eNOS was lost because of iNOS upregulation and inappropriate maintenance. Though the expression pattern of eNOS throughout the developmental stages remained unchanged, its levels were decreased compared with controls at all time points. Thus the eNOS/iNOS isoform switch that occurs prior the primary...
plexus stage (~8.0 dpc) was no longer present. The activity of iNOS during the hyperglycemic insult was determined in 7.5 dpc conceptuses by evaluating the production of NO using DAF-FM. Hyperglycemic treatment for 3 hours induced increased NO production from the ectoplacental cone and endoderm (Fig. 6F-H).

NO donor rescues the vasculopathy induced by hyperglycemia
To investigate the possibility that NO bioavailability is a mediator of hyperglycemia-induced vasculopathy, conceptuses were cultured in hyperglycemic media supplemented with a NO donor (NOC-18). The addition of exogenous NO resulted in rescue of the vasculopathy induced by hyperglycemia (Fig. 6B). Morphologically, normal primary plexus and large vessels formed, establishing a functional circulation and allowing embryo development to progress. NOC-18 appeared to restore normal large vessel morphology and branching morphogenesis. Additionally, vessels were not dilated or enlarged. The size distribution of vessels in the NOC-18 supplemented hyperglycemic condition resulted in a near overlapping histogram with the control (Fig. 6C). Furthermore the fraction of vessels in the 35-40 to 55-60 μm categories decreased from 5-12% to <7% in the hyperglycemic versus hyperglycemic plus NOC-18 conditions, respectively.

The ability of exogenous NO to restore normal NOS isoform distribution during the hyperglycemic insult was evaluated at 8.5 dpc, when the NOS isoform switch normally occurs. Supplementation of NOC-18 to the hyperglycemic media restored eNOS levels and downregulated iNOS (Fig. 6I).

NO depletion increases ROS production: a common pathway in vasculopathy
Studies using low doses of NO donors demonstrated that NO directly acts as an antioxidant (Chang et al., 1996; Joshi et al., 1999; Kim et al., 1995; Wink et al., 1993). Therefore, we hypothesized that the vasculopathy induced by L-NMMA may be due to increased ROS, suggesting a common pathway with hyperglycemia. In order to evaluate the potential role of ROS in L-NMMA induced vasculopathy in the yolk sac, ROS production was evaluated by DHE at 8.0 dpc. This time point, just prior to the primary plexus stage, was chosen because it represents the point at which the NOS isoforms switch in distribution (Fig. 1). Furthermore, NO inhibition seems to cause a defect in primary plexus formation and arrest at this subsequent stage (8.5 dpc). L-NMMA induced a dramatic increase in ROS production (Fig. 7B). A gradient of ROS was present from the endoderm to the mesodermal/endothelial layer, with the highest ROS levels present in the endoderm. Additionally, cells within the blood islands and endothelial cells within the vessels expressed ROS. To determine if uncoupling NOSs contributes to superoxide anion (SO) generation, L-NAME was used. L-NAME, unlike L-NMMA, inhibits SO production by NOSs. L-NAME treatment induced similar ROS formation as L-NMMA treatment, suggesting that uncoupling of NOSs does not significantly contribute to ROS production (Fig. 7C).

The pattern of ROS expression in L-NMMA and L-NAME treated conceptuses mirrored that of hyperglycemic treated conceptuses (Fig. 7B-D). To investigate the hypothesis that NO may have antioxidant properties in our system, the ability of NOC-18 to restore normal ROS levels was evaluated in hyperglycemia exposed conceptuses. Addition of 20 μM NOC-18 to the hyperglycemic media resulted in decreased hyperglycemia-induced ROS (Fig. 7E).

Discussion
We have established that there is an inverse relationship between eNOS and iNOS during yolk sac vasculogenesis. At the beginning of vasculogenesis when blood islands are forming, the endoderm strongly expresses iNOS and produces NO. Around 8.0 dpc, when blood islands are fusing into the primary plexus, eNOS localized to the mesoderm. eNOS expression increased in the endothelium and begun in the endoderm during the primary capillary plexus stage, while endodermal iNOS expression diminished. The eNOS expression pattern increased during maturation of the
vasculature, while iNOS was absent. This distinct pattern of NOSs may represent a developmental stage-specific need for appropriate NO production/bioavailability.

We next demonstrated that NO depletion arrested vascular development at the primary plexus stage and induced vasculopathy. The presence of blood islands suggests that endothelial specification, which begins during gastrulation (E6.0), and egress of mesodermal cells had occurred. However, as L-NMMA was added at the primitive streak stage, a time when mesodermal invagination occurs and mesodermal cell migration is still occurring, these events may be affected by NO depletion/bioavailability. The subsequent angioblastic cords were capable of forming a polygonal pattern of capillaries, but the capillaries were enlarged, typical of the vasculopathy induced in several diverse knockout mice (Gerety and Anderson, 2002; Sato et al., 1995; Suri et al., 1996). These defects have been associated with hyperfusion of blood islands, which suggests a role for NO in proper blood island formation. It could also be argued that L-NMMA treatment results in an immature or arrested vascular network, suggesting a defect in vascular remodeling processes and a possible role for NO in vessel size determination and vascular organization. In this

Fig. 6. Effects of hyperglycemia on iNOS/eNOS distribution and NO production, and rescue by NO donor. The ability of NOC18 to restore normal vascular morphology of hyperglycemic treated conceptuses was evaluated by PECAM1 staining at 9.5 dpc: (A) hyperglycemia and (B) hyperglycemia plus NOC18. Morphometric analysis of images from PECAM1-stained yolk sacs showing vessel diameter distribution (C). Graphs of ERK2 normalized data for iNOS (D) and eNOS (E) protein expression in pooled 7.5, 8.5 and 9.5 dpc conceptuses. The broken lines represent the hyperglycemic condition, whereas the unbroken black lines represent the control (n=4, *P<0.05, **P<0.01). The data (mean±s.e.m.) is relative to the control 7.5 dpc levels. Above each graph is a representative western blot of iNOS and eNOS at 8.5 dpc. (F) Phase-contrast image of the endoderm of a 7.5 dpc conceptus showing the intact endodermal surface. (G) DAF-FM fluorescence of the corresponding area of a conceptus cultured in normoglycemic conditions. (H) DAF-FM fluorescence of a similar area of a conceptus cultured in hyperglycemic conditions. (I) Representative western blot of the effect of a NO donor on NOS protein distribution of glucose treated conceptuses at 8.5 dpc and graph of ERK2 normalized averaged data for eNOS and iNOS protein expression (white columns represent eNOS and black columns represent iNOS; averages of two experiments). Nml, normoglycemic; HG, hyperglycaemic; HG + NOD, hyperglycemic + NOC-18.
During this stage, blood island formation occurs and iNOS localized to the visceral endoderm, the major secretory layer that sends inductive signals to the mesoderm (Antin et al., 1994; Jollie, 1990; Wilt, 1965). The endoderm has been demonstrated to be required for normal blood island formation, affecting endothelial cell differentiation (Miura and Wilt, 1969; Bielinska et al., 1996). Furthermore, endoderm-derived inductive signals were determined to direct endothelial differentiation during a specific developmental period: gastrulation (Belaoussoff et al., 1998). Molecules such as winged-helix genes, Indian hedgehog and VEGFA have been discovered as endoderm-derived factors that act locally on the mesoderm and are required for yolk sac vasculogenesis (Farrington et al., 1997; Dyer et al., 2001; Damert et al., 2002). Given the effects of NO depletion during late gastrulation/early organogenesis on vascular formation, we speculate that iNOS derived NO is one of the endoderm-derived molecules that acts as a paracrine regulator of endothelium development via direct or indirect effects (NO-mediated production of essential factors).

To evaluate the effect of excess NO on vascular development, we applied a low dose of NO donor to the culture media and found that NOC18 did not cause vascular defects. Furthermore, NOC18 treatment correlated with increased eNOS and decreased iNOS during primary capillary plexus formation. At this time we can not confirm that the induction of eNOS is a direct or indirect effect of NO. Potentially, feedback loops exist in which NO regulates endodermal growth factors that subsequently affect NOS expression. For example, NO is known to induce VEGF, which has been demonstrated to increase eNOS (Cha et al., 2001; Dulak and Jozkowicz, 2003). Alternatively, the conceptus may be capable of NO sensing and modulation of NOS isoforms by yet unidentified mechanisms, in order to maintain the appropriate concentration of NO during organogenesis. This switch between NOS isoforms just prior to the primary plexus stage seems to represent an important event in the progression of normal vascular development. Its significance is further demonstrated by the hyperglycemic experiments.

Hyperglycemia resulted in the persistence of the iNOS isoform and depression of the eNOS isoform. Potentially, in order for normal primary plexus formation to occur, NO-dependent signaling mechanisms are required in the endothelium. Therefore, the switch from endodermal NO production to endothelial NO production and autocrine NO-mediated events must occur. We speculate that the abrupt decline in iNOS represents a phase of normal development when a sustained increase in NO production via iNOS may alter further vascularization.

Though hyperglycemia increased endodermal NO production at 7.5 dpc, we observed that NO donor treatment rescued the hyperglycemia-induced vasculopathy and restored the eNOS/iNOS distribution. This seemingly conflicting result may be explained by decreased bioavailability of NO leading to a requirement for additional NO. Excessive NO produced by the high NO output NOS isoform, iNOS, is upregulated in vascular diseases and known to cause cytotoxicity and vascular dysfunction (Hibbs et al., 1989; Moncada et al., 1991; Zhang et al., 2003). However, despite increased production of NO in vascular diseases, there is impaired vasodilation, suggesting decreased NO bioavailability (Minor et al., 1990).

It has been demonstrated that glucose scavenges NO in a dose-dependent manner by the formation of a glucose-NO adduct, providing evidence for a direct action of glucose on NO bioavailability (Brødskjær et al., 2001). Additionally, hyperglycemia increases the production of ROS, of which SO

**Fig. 7.** Induction of ROS: a common pathway in vasculopathy. ROS production and localization in the yolk sac was evaluated by DHE (red) in 8.0 dpc conceptuses treated with D-NMMA (A), L-NMMA (B), L-NAME (C), glucose (D) and glucose plus NOC-18 (E). Images were captured at 20× magnification and merged with DAPI images (n=4).
is known to combine with NO and deplete it from the system, thus inhibiting NO-mediated intracellular signaling and downstream cellular responses (Mügge et al., 1991; Urbich et al., 2002; Zhang et al., 2001). The reaction between SO and NO is rapid, occurring at a rate of $6.7 \times 10^6 \text{M}^{-1} \text{s}^{-1}$, a near diffusion-limited rate (Huie and Padmajas, 1993). This reaction occurs about six times faster than the dismutase of SO by superoxide dismutase (SOD), thus NO is an efficient scavenger of SO (Koppenol, 1998). By increasing the concentration of NO, once saturation of SO has occurred, the excess NO is available to act in the endoderm. Furthermore, NO is membrane permeable, unlike SO, and capable of diffusing over 100 µm in a few seconds at 37°C (Meulemans, 1994; Wise and Houghton, 1969; Malinski et al., 1993a; Malinski et al., 1993b). NO has been demonstrated to diffuse through tissues without consumption, establishing its role as an intracellular messenger (Lancaster, 1994; Wood and Garthwaite, 1994). Therefore, it is possible for NO released by the donor to diffuse through the single layer of endodermal cells and reach the mesoderm, where SO concentrations are lower.

The diffusion capabilities of the NO donor and its ability to restore the normal eNOS/iNOS distribution in the hyperglycemic condition may provide the required local concentrations of NO for vascular development to progress normally. As eNOS produces less concentration of NO than does the iNOS isoform, we expect the physiological concentration of NO during normal development in the endothelium to be low. In other tissues, such as the cerebrum, the concentration of NO was measured at 10 nM during physiological conditions (Malinski et al., 1993a; Malinski et al., 1993b). Furthermore, only 5 nM of NO is needed to induce relaxation of vessels and is the minimum concentration to activate guanylate cyclase. Thus, low concentrations of NO are sufficient for NO-mediated actions in physiological conditions and, potentially, developmental states. We demonstrated that, under normal conditions, a small fraction of eNOS was phosphorylated; thus, we speculate that eNOS produces a low [NO] which is required for optimal vascular development. The survival factor, Akt, is known to activate eNOS (Fulton et al., 1999; Michell et al., 1999). Furthermore, Akt plays a role in endothelial cell survival as blood vessels assemble during vascular development through a mechanism involving RhoB (Adini et al., 2003). As phospho-Akt was detected in E8.5 yolk sacs, this pathway may serve as a mechanism to activate eNOS and also promote endothelial cell survival.

The protective effect of upregulation of the low NO output NOS isoform, eNOS, by the NO donor during the hyperglycemic insult is supported by several studies in eNOS knockout mice, which revealed that this isoform serves cardioprotective and vasoprotective functions (Connelly et al., 2003; Gewaltig and Kojda, 2002; Jablonka-Shariff and Olson, 1998). These functions include modulation of vasoactivity and blood pressure, and inhibition of platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation (Limbourg et al., 2002; Jugdutt, 2002; Papapetropoulos et al., 1999). Additionally, several studies have demonstrated that NO acts as an antioxidant (Chang et al., 1996; Herrmann et al., 1997; Joshi et al., 1999; Kim et al., 1995; Paxinou et al., 2002; Zhang et al., 2001). The reaction between SO and NO, once saturation of SO has occurred, the excess NO acts as an antioxidant (Chang et al., 1996; Hermann et al., 1997; Joshi et al., 1999; Kim et al., 1995; Paxinou et al., 2002; Zhang et al., 2001). The reaction between SO and NO, once saturation of SO has occurred, the excess NO acts as an antioxidant (Chang et al., 1996; Hermann et al., 1997; Joshi et al., 1999; Kim et al., 1995; Paxinou et al., 2002; Zhang et al., 2001). The reaction between SO and NO, once saturation of SO has occurred, the excess NO acts as an antioxidant (Chang et al., 1996; Hermann et al., 1997; Joshi et al., 1999; Kim et al., 1995; Paxinou et al., 2002; Zhang et al., 2001).
2001; Wink et al., 1993). Accordingly, we showed that NO supplementation to hyperglycemic media reduces hyperglycemia-induced ROS. Furthermore, we demonstrated that L-NMMA treatment induced ROS in the endoderm and mesoderm/endothelium. These results suggest that endogenous NO has antioxidant functions during blood island formation.

The expression pattern of ROS in L-NMMA treated conceptuses was similar to that induced by hyperglycemia, indicating that the induced vasculopathies may share a common pathway of ROS induced vascular defects. High concentrations of ROS injure tissues and cause vascular dysfunction (Channon and Guzik, 2002). Accordingly, mice deficient in copper-zinc superoxide dismutase, the cytosol/nuclear SOD, display increased SO, decreased relaxation response to NO, and vascular dysfunction (Didion et al., 2002). Furthermore, administration of superoxide scavengers protects against vascular dysfunction in experimental animal models (Iadecola et al., 1999; Mayhan, 1997).

Under normal conditions in vivo, ROS and NO levels are balanced and low, and thus their combination is limited by their ability to diffuse and interact with each other. In this situation, both ROS and NO are essentially free to initiate their respective signaling cascades. NO has been demonstrated to interact with several intracellular signaling cascades, including mitogen-activated protein kinase (MAPK), janus kinase (JAK) and Jun N-terminal kinase (JNK) (Lander, 1997; Kim et al., 1997; So et al., 1998). Additionally, transcription of several gene classes, including cytokines, matrix proteins and hormones, are modulated by NO regulation of nuclear factor κB (NFκB), hypoxia inducible factor 1 (HIF1) and zinc-finger transcription factors (Huang et al., 1999b; Kroncke and Carlberg, 2000; Matthews et al., 1996; Tabuchi et al., 1996; Torres and Forman, 2000). ROS, however, regulates transcription of protein kinases, growth factors and transcription factors, and plays a role in signaling in the vasculature (Burdon, 1996; Giaccia and Kastan, 1998; Maulik, 2002; Meyer et al., 1994; Nose, 2000). Additionally, ROS inhibits low molecular weight (LMW) phosphatases, which have been shown to downregulate proliferation by attenuating growth factor receptor signaling (Chiarugi, 2001; Huang et al., 1999a). It is essential for ROS and NO to carry out these respective signaling cascades because, if they interact, as they do at high concentrations, vasculopathy occurs.

Taken together, the results of this study suggest that tight regulation of the stage appropriate levels of NO and NO-mediated events are required for normal vascular development. The downstream NO-mediated signaling events and molecules are yet to be determined, as is the crosstalk between NO and other endoderm-derived factors (Fig. 8). However, the observed antioxidant effect of NO during early vasculogenesis broadens the protective activities of the yolk sac endodermal layer. Future studies using this model are needed to examine the role of NO in antioxidant events during vasculogenesis, particularly the regulation of SODs and other protective mechanisms such as peroxisome proliferator activated receptor γ, PPARγ. Moreover, the relationship between high glucose, NO bioavailability and downstream antioxidant events needs to be further explored.

This work was supported by an NIH Institutional Training grant to A.K.N., an American Heart Association grant AHA 01151194T to E.P., USPHS grant R37-HL28373 to J.A.M. and a Yale Diabetes Endocrine Research Center grant NIH 5P30-DK45735 to J.A.M. and E.P.

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